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Covalent and non-covalent strategies for surface modification of different textile materials with antimicrobial properties

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“The best way to predict the future is to create it.”
Peter Drucke

Dedicatory

To all those whom might use this knowledge to take a step forward towards something

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Resumo alargado

Os têxteis são substratos propícios ao crescimento e proliferação microbiana sob as condições apropriadas de humidade, nutrientes e temperatura. Em ambiente hospitalar, podem ser uma fonte perigosa de bactérias e fungos que contaminam pacientes e profissionais de saúde. As bactérias e fungos, patogénicos ou não, são normalmente encontrados na pele humana, na cavidade nasal e na área genital. A libertação de microrganismos do nosso corpo contribui para a contaminação e proliferação sobre o vestuário e têxteis. Vários estudos sustentam que a contaminação de têxteis usados em ambiente hospitalar pode contribuir para a libertação de agentes patogénicos no ar, contaminando o ambiente e sendo, presumivelmente, das causas mais prováveis de infeções hospitalares.

A maioria das infecções nosocomiais são causadas por microorganismos oportunistas, i.e., raramente causam doenças num sistema imunológico saudável, mas conseguem explorar o estado debilitado de pacientes imunocomprometidos, como vítimas de queimaduras, pacientes oncológicos ou acamados com feridas abertas, para causar infecções. Estas estirpes têm a capacidade de crescer em qualquer ambiente, apresentam importantes fatores de virulência e têm resistência a uma grande variedade de antibióticos.

Milhões de pessoas são infetadas com bactérias que causam infecções hospitalares, sendo uma das principais causas de mortalidade em hospitais, causando anualmente a morte de cerca de 700 mil pessoas a nível mundial.

O uso de têxteis antimicrobianos, especialmente em contato próximo com pacientes, mas também no ambiente circundante, pode reduzir significativamente o risco de infecções. Contudo, vários requisitos se impõem: os têxteis bioativos devem possuir propriedades biocidas de largo espectro, ser seguros para o utilizador e altamente eficazes no combate aos microrganismos resistentes a antibióticos, incluindo os que são usados no tratamento de infeções hospitalares, e não devem permitir o desenvolvimento de microrganismos resistentes ao composto ativo usado nem ser a causa de irritação da pele.

Vários agentes antimicrobianos têm sido testados em têxteis. Os compostos de amónio quaternário, a prata, o polihexametileno de biguanidina e o triclosano são usados como agentes antimicrobianos para têxteis, com sucesso, já que possuem uma atividade bactericida poderosa, como indicado pelo MIC (concentração mínima inibitória). No entanto, a maioria dos agentes antimicrobianos tem um espectro reduzido de inibição microbiana e pode causar irritação da pele, ecotoxicidade e resistência bacteriana. Além disso, a sua incorporação nos têxteis reduz substancialmente a atividade e limita a disponibilidade dos grupos bioativos, obrigando à utilização de concentrações elevadas nos processos de funcionalização. O biocida pode também perder gradualmente a sua atividade durante o uso e lavagens dos materiais têxteis. Assim, grandes quantidades são aplicadas aos têxteis para controlar o crescimento bacteriano e manter a sua durabilidade.

Na tentativa de superar estas desvantagens, vários compostos como o quitosano, ácido hialurónico e extractos de plantas têm sido avaliados como potenciais agentes antimicrobianos para têxteis. A par disso, a exigência dos consumidores por vestuário higiénico e o mercado crescente dos têxteis médicos e bioativos estimularam a investigação nesta área.

Baseada numa revisão cuidada da literatura, a nossa proposta prevê uma estratégia totalmente nova: o uso de aminoácidos L-cisteína (L-Cys), bacteriofágos e péptidos antimicrobianos (AMPs) como agentes bioativos em têxteis.

Assim sendo, numa primeira abordagem realizámos estudos no sentido de conferir propriedades antimicrobianas a superfícies têxteis e poliméricas de forma a que atraissem, ligassem e eliminassem os microorganismos, de forma irreversível, originando uma barreira protectora dinâmica. Para este efeito, foram utilizados o aminoácido L-Cys e os AMPs Magainina I, LL-37, e Cys-LC-LL-37, de modo a conferir propriedades antimicrobianas a materiais fibrosos de algodão. A seleção da L-Cys deveu-se ao facto das suas reconhecidas propriedades antimicrobianas conferidas pelo seu grupo tiol e que, para além disso, provou ter capacidades antioxidantes, pelo reagente 2,2-difenil-1-picrilidrazil (DPPH).

Estratégias de imobilização covalentes e não covalentes foram testadas em diferentes materiais fibrosos e submetidos a ciclos de lavagem intensos, tais como algodão, seda, policaprolactona, e polipropileno, com o objectivo de imobilizar a L-Cys de forma durável. Para uma melhor compreensão das interacções material-L-Cys-bactéria, substratos têxteis de algodão foram quimicamente modificados com N,N-carbonyldiimidazole (CDI) e posteriormente funcionalizados com diferentes concentrações de L-Cys. Estes estudos revelaram que havia uma quantidade específica de ativador CDI (4%) que seria a ideal para ligar com mais eficiência L-Cys (5%). Estes resultados revelaram uma maior eficiência antimicrobiana em comparação com outro estudo, no qual o substrato de algodão foi imobilizado não covalentemente com Magainina I e LL-37. O algodão-L-Cys provocou maior morte microbiana, após ciclos de lavagem, devido exclusivamente à sua ligação covalente que conseguiu imobilizar a L-Cys, de forma mais permanente. Para corroborar esta hipótese levantada, modificou-se um polímero difícil de ser modificado – polipropileno – com L-Cys, o que fortaleceu a sua estrutura em nanofibras e dotou-o com grupos tiol que permitiram ligar o péptido Cys-LC-LL-37 por ligação dissulfureto (covalente). Verificou-se que esta Cys-LC-LL-37 resistiu aos sucessivos ciclos de lavagem, e a flexibilidade deste péptido foi ímpar para a eliminação dos microorganismos.

Posteriormente, os conhecimentos adquiridos aquando da utilização do algodão e polipropileno foram transferidos para seda e policaprolactona, de forma a testar a aplicabilidade do conceito desenvolvido a outras estruturas fibrosas potencialmente usadas como têxteis antimicrobianos. Foram imobilizadas, por diferente química de superfície, diferentes percentagens de L-Cys em amostras destes polímeros com potencial biomédico, e

realizados também ensaios de espectroscopia de raios X (EDS), espectroscopia de infravermelho da transformada de Fourier (FT-IR), calorimetria exploratória diferencial (DSC), reagente de Ellman, e ângulo de contacto para verificar químicamente a imobilização de L-Cys, bem como ensaios antimicrobianos e de citotoxicidade, para assegurar que as aplicações não seriam tóxicas para o ser humano. Também com seda e policaprolactona, as amostras ligadas covalentemente por 1 e 5% de L-Cys, respetivamente, eliminaram os microorganismos de forma bastante boa. Para além disto, estas amostras retiveram L-Cys durante diversos ciclos de lavagem.

O trabalho desenvolvido e o conhecimento adquirido nesta fase permitiu evoluir para uma nova estratégia de imobilização de bacteriofagos em materiais fibrosos. Testou-se, assim, a ligação covalente da cápside do fago vB-Pae-Kakheti25 à superfície de nanofibras de policaprolactona produzidas por electrospinning, de forma a que o fago ficasse com a sua cauda orientada para o exterior das nanofibras, mantendo a sua infectividade. Os resultados mais uma vez confirmaram que não só a presença de um antimicrobiano, mas também a forma como este está ligado, faz toda a diferença na estratégia de desenvolvimento de têxteis antimicrobianos.

Concluiu-se, portanto, que uma quantidade optimizada de “novos” compostos antimicrobianos alternativos a antibióticos e biocidas sintéticos, bem como uma orientação específica, traduz-se num melhor desempenho no contato e eliminação de bactérias, sendo crucial para o desenvolvimento de biomateriais para contacto com a pele e mucosas.

Palavras-chave

Têxteis bioativos, pensos hospitalares, L-Cisteína, Bacteriófagos, Péptidos antimicrobianos

Abstract

Nowadays, millions of people become infected with bacteria that cause hospital infections, which is a major cause of mortality in hospitals, killing 700,000 people per year in the world. It is even projected that the number of deaths in hospitals will grow to 10 million by 2050.

The use of antimicrobial textiles, especially in close contact with the patients and in the immediate and non-immediate surroundings, may significantly reduce the risk of infections. However, they should possess broad spectrum biocidal properties, be safe for use and highly effective against antibiotic resistant microorganisms, including those that are commonly involved in hospital-acquired infections.

Most nosocomial infections are primarily by opportunistic microorganisms, i. e., they rarely cause diseases in a healthy immune system, but seek to exploit any weaknesses in the body of immunocompromised patients, such as victims of burns, cancer patients or beddriden with open wounds, in order to cause infections. These strains have the ability to grow in any environment, present important virulence factors, and have resistance to a large variety of antibiotics.

Several antimicrobial agents have been tested in textiles. Quaternary ammonium compounds, silver, polyhexamethylene biguanides and triclosan have been used, with limited success. They have powerful bactericidal activity, however, the majority have a reduced spectrum of microbial inhibition and may cause skin irritation, citotoxicity, ecotoxicity and bacterial resistance. In addition, its incorporation in the textiles reduces their activity substantially and limits availability. Moreover, the biocide can gradually lose activity during the use and textile repeated laundering.

To overcome these disadvantages, natural compounds such as L-Cysteine (L-Cys), bacteriophages and antimicrobial peptides (AMPs), were tested in this work as antimicrobial agents for fibrous materials.

As such, in a first approach we carried out studies in order to confer antimicrobial properties on textile and polymeric surfaces in such a way that they could irreversibly attract, bind and eliminate microorganisms, paving the way to a dynamic protective barrier. For this purpose, the amino acid L-Cys and the AMPs Magainin I, LL-37, and Cys-LC-LL-37 were used in order to provide antimicrobial properties to cotton fibrous materials . L-Cys was selected due to its proven antimicrobial properties granted by its thiol group and also proved its capacity to ensure antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. Covalent and non-covalent immobilization strategies were tested on different fibrous materials and subjected to intensive washing cycles, such as cotton, silk, polycaprolactone, and polypropylene, in order to immobilize L-Cys in a durable manner. For a better understanding of the interactions material-L-Cys-bacteria, cotton textile substrates were chemically modified with N, N-carbonyldiimidazole (CDI) and subsequently functionalized with different concentrations of L-Cys. These studies revealed that there was a specific amount of CDI activator (4%) which would be ideal to more efficiently bind L-Cys (5%). These results revealed a higher antimicrobial efficiency, when compared to another study, in which the cotton substrate was non-covalently immobilized with Magainin I and LL-37. Cotton-L-Cys caused most death among bacteria, after washing cycles, due exclusively to its covalent bound that was able to immobilize L-Cys more permanently. In support of this hypothesis, a polymer difficult to modify - polypropylene - was grafted with L-Cys, which strengthened its nanostructure and endowed it with thiol groups that allowed to bind the peptide Cys-LC-LL-37 via disulfide bond (covalent). It was found that Cys-LC-LL-37 resisted to successive wash cycles, and the flexibility of this peptide was unique to the elimination of the microorganisms.

Subsequently, the knowledge acquired when using cotton and polypropylene were transferred to silk and polycaprolactone, in order to test the applicability of this developed concept to other fibrous structures potentially to be used as antimicrobial textiles. Different percentages of L-Cys were immobilized, by different chemical reactions, on samples of aforementioned polymers with biomedical potential, and X-ray spectroscopy (EDS), Fourier transform infrared spectroscopy (FT-IR), calorimetry (DSC), Ellman's reagent, and contact angle were used to chemically check L-Cys immobilization, as well as antimicrobial and cytotoxicity assays, so as to ensure that the applications would not be toxic to humans. Also, silk and polycaprolactone samples covalently bound by 1 and 5% L-Cys, respectively, eliminated very well the microorganisms. In addition, these samples retained L-Cys during several wash cycles.

At this stage, after the work developed and the knowledge acquired, enabled us to move into a new strategy of immobilization of bacteriophages in fibrous materials. The covalent coupling of the vB-Pae-Kakheti phage capsid to the surface of polycaprolactone nanofibers produced by electrospinning was performed, so that the phage had its tail facing outwards, maintaining its infectivity. The results again confirm that not only the presence of an antimicrobial, but also the way it is immobilized, makes all the difference in the development strategy of antimicrobial textiles.

It was concluded, therefore, that an optimized amount of "new" antimicrobial compounds alternative to antibiotics and synthetic biocides, as well as their specific orientation, consisted of a better performance upon contact and elimination of bacteria, being crucial for the development of biomaterials for contact with skin and mucosa.

Keywords

Bioactive textiles, wound dressing, L-Cysteine, Bacteriophages, Antimicrobial peptides

Thesis Overview

This thesis is structured in four main chapters.

In the first chapter, an introduction on antimicrobial compounds, from antibiotics to synthetic biocides and ending with the "new" antimicrobial agents is described. In the end, there is a brief contextualization on methods for modification of surfaces.

In the second chapter, the main purpose and specific goals of this thesis work are explained.

The third chapter presents the results and discussion stemming from this PhD work, in the form of international peer-reviewed scientific papers. This chapter is structured as follows:

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

PAPER II - Covalent Modification of Cellulosic-based Textiles: A New Strategy to Obtain Antimicrobial Properties

PAPER III - Antimicrobial and antioxidant surface modification toward a newsilk-fibroin (SF)-l-Cysteine material for skin disease management

PAPER IV – New garment proposal for prevention of spreading Gram-negative bacteria resistant to carbapenem antibiotic class under hospital settings

PAPER V – Amino Acid-Based Material for the Complementary Therapy of Decubitus Ulcers

PAPER VI - Immobilization of bacteriophage in wound-dressing nanostructure

PAPER VII – Electrospinning polypropylene with an amino acid as a strategy to bind the antimicrobial peptide Cys-LC-LL-37

In the fourth chapter there is a general discussion and conclusions of all developed scientific papers, perspectives and suggestions for future work.

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List of Scientific Publications

Peer-reviewed international scientific papers included in the thesis resulting from this Doctoral work

PAPER I – Comparison of the antibacterial activity of modified-cotton with Magainin I and LL-37 with Potential as Wound-Dressings.
Marta Pedrosa, Cláudia Mouro, Frederico Nogueira, Joana Vaz, Isabel Gouveia.
Journal of Applied Polymer Science. 2014, DOI: 10.1002/app.40997.(2014)

PAPER II - Covalent modification of cellulosic-based textiles: a new strategy to obtain antimicrobial properties.
Frederico Nogueira, Joana Vaz, Claudia Mouro, Erhan Piskin, Isabel C. Gouveia.
Biotechnology and Bioprocess Engineering. 2014, Volume 19, Issue 3, Pages 526-533, DOI 10.1007/s12257-013-0498-7

PAPER III - Antimicrobial and antioxidant surface modification toward a newsilk-fibroin (SF)-l-Cysteine material for skin disease management.
Frederico Nogueira, Luiza Granadeiro, Claudia Mouro, Isabel C. Gouveia.
Applied Surface Science. 2015, Volume 364, Pages 552-559, DOI 10.1016/j.apsusc.2015.12.174

PAPER IV - New garment proposal for prevention of spreading Gram-negative bacteria resistant to carbapenem antibiotic class under hospital settings.
Frederico Nogueira, Ana Gomes, Isabel C. Gouveia.
Journal of Industrial Textiles. 2018, Volume 47, Issue 6, Pages 1152–1165, DOI 10.1177/ 1528083716682919

PAPER V - Amino acid based material for the complementary therapy of Decubitus Ulcers.
Frederico Nogueira, Isabel C. Gouveia.
Journal of Microbiology and Biotechnology. 2017, Volume 27, Issue 4, Pages 747-758, DOI 10.4014/jmb.1609.09061

PAPER VI - Immobilization of bacteriophage in wound-dressing nanostructure.
Frederico Nogueira, Natia Karumidze; Ia Kusradze, Marina Goderdzishvili, Pilar Teixeira, Isabel C. Gouveia.
Nanomedicine: Nanotechnology, Biology, and Medicine. 2017, Volume 13, Issue 8, Pages 2475-2484, DOI 10.1016/j.nano.2017.08.008

PAPER VII - Electrospinning polypropylene with an amino acid as a strategy to bind the antimicrobial peptide Cys-LC-LL-37.
Frederico Nogueira, Pilar Teixeira, Isabel C. Gouveia.
Journal of Materials Science. 2018, Volume 53, Issue 6, Pages 4655-4664, DOI 10.1007/s10853-017-1841-8

List of Scientific Communications

Oral scientific communications resulting from this Doctoral work

New strategies for surface modification of Cotton and Silk textiles with antimicrobial properties
F. Nogueira, P. Teixeira, I. C. Gouveia
I Simpósio FibEnTech 2016 – Materiais Fibrosos e Tecnologias Ambientais, UBI, Portugal
https://www.ubi.pt/Ficheiros/Entidades/1020/Atas-I-SIMPÓSIO_FibEnTch-2016.pdf

New strategies for surface modification of Cotton and Silk textiles with antimicrobial properties
Frederico Nogueira, Erhan Piskin, Pilar Teixeira, Isabel Gouveia.
Journal of Biotechnology. 2014, Volume 185, Supplement , Pages S20, DOI 10.1016/j.jbiotec.2014.07.068

Covalent and non-covalent strategies for surface modification of different textile materials with antimicrobial properties
Frederico Nogueira, Marta Pedrosa, Pilar Teixeira, Erhan Piskin, Isabel Gouveia
International Conference on Antimicrobial Research, November 21-23, Lisbon, Portugal
http://repositorium.sdum.uminho.pt/bitstream/1822/25919/1/Pages%20from%20ICAR2012-5.pdf

A new medical textile- L-cysteine functionalized cotton, its mechanism of action and potential antimicrobial properties
Marta Pedrosa, Frederico Nogueira, Estela Caldeira, Filomena Silva, Ana Oliveira, Isabel Gouveia
International Conference on Antimicrobial Research, November 21-23, Lisbon, Portugal
http://www.formatex.org/icar2012/index.html

Biomaterials for the controlled delivery of bioactive agents in the chronic wounds regeneration
Frederico Nogueira, Marta Pedrosa, Estela Caldeira, Erhan Piskin, Isabel Gouveia
15th International Biotechnology Symposium and Exhibition, September 16-21, 2012, Daegu, Korea
http://www.ibs2012.org/

Chapter 1 - General Introduction

1. Antimicrobial textiles

Infections caused by bacteria have been a major cause of health disorders throughout human history. After the discovery of antibiotics arose a general belief that the problem of bacterial infections would be solved. However, pathogens have evolved sophisticated drug resistance mechanisms. The continuous use of antibiotics has resulted in multi-resistant bacterial strains worldwide having a higher incidence in hospitals where they coexist in an environment favorable to the development of various microorganisms (Mainous III & Pomeroy, 2001). As a result, antimicrobial resistance has emerged as one of the most serious health threats, which calls for concerted efforts to develop new antibacterial agents (Kazmierczak *et al.*, 2014). Consequently medical textiles have undergone a great development in recent times and have become an area of great importance and interest.

The spread of microorganisms, either through the environment or even through our body, contributes to contamination and proliferation in hospital textiles, being the main causes of hospital infections (Ristié *et al.*, 2011; Gouveia, 2010). Their elimination thus becomes essential (Ristié *et al.*, 2011; Gao & Cranston, 2008).

There are pathogenic and non-pathogenic microorganisms. Humans present naturally a wide spectrum of non-pathogenic microorganisms of great importance for health, which means that there is a fundamental interaction between human and microbiota (Gao & Cranston, 2008). However, there are also pathogenic microorganisms that are the main causes of diseases in humans. According to the level of virulence of these microorganisms and the human response to this imbalance, this can result in more or less serious diseases, and may bring some complications to the patient (Gouveia, 2010). Unfortunately, in immunocompromised patients, even non-pathogenic agents can cause serious complications, which is the main problem in hospital settings (Khan *et al.*, 2015).

Textile-based materials are found in different forms at the hospitals and are potential proliferators of microorganisms (Borkow & Gabbay, 2007). Depending on their applications, either at surgical, implant level, or at a more external level such as gowns, sheets, dressings, gauzes and others, textiles should have antimicrobial properties. The inhibition of microbial proliferation in hospital textiles is essential in order to reduce the risk of infections by non-pathogenic microorganisms. Antimicrobial textiles can significantly reduce the risk of infections, particularly when used in direct contact with or in the vicinity of the patient (Khan *et al.*, 2015).

Medical textiles are composed mainly by synthetic fibers, as they are resistant to most microorganisms. Among the most used synthetic fibers stays the polyester, which in its original state does not theoretically allow the development of bacteria due to its hydrophobicity (Purwar & Joshi, 2004). However, due to the porosity of the textile fabrics and the moisture between the fibers of the fabric results a humid environment conducive to the development of microorganisms (Gouveia, 2010).

The textile materials used at the medical level present specific needs according to their end use, such as biocompatibility, hydrophilicity, etc., which makes them suitable for use in medical applications. Furthermore, a new area has been developed in finishing of textiles, mainly against the growth and proliferation of microorganisms (Ristié *et al.*, 2011; Gouveia, 2010). The textile industry continually seeks new technologies to meet the demands of consumers by producing functional and intelligent textiles capable of recording changes in environmental conditions or functions of the body and respond to these changes. Likewise, consumers' attitude towards hygiene and active lifestyle has created a growing market for a wide range of biofunctional textiles with an antimicrobial activity (Borkow & Gabay, 2008; Singh *et al.*, 2005). Its application now ranges from underwear, sportswear, decoration, and even protective clothing in areas at high risk of infection by pathogens (hospitals, schools and hotels) (Singh *et al.*, 2005; Kramer *et al.*, 2006). As these textiles are capable of absorbing skin substances and releasing therapeutic components thereto, they have applications at the preventive level, such as surgical laboratory coatings or as (curative) wound dressings (Borkow & Gabay, 2008; Zilberman & Elsner, 2008). Thus, antimicrobial textiles are bent upon biomedical products (Kramer *et al.*, 2006; Zilberman & Elsner, 2008).

There are various ways of achieving antimicrobial properties in textile materials, such as the incorporation of antimicrobial agents directly upon the production of fibers, coating or adsorption of these agents on the textile fibers and immobilization of antimicrobials onto fibers by means of covalent or ionic bonds (Gao & Cranston, 2008).

Antimicrobial agents for textiles can be classified in three forms: antibiotics, compounds of synthetic origin and compounds of natural origin. Among the compounds of synthetic origin are metal salts and salts, quaternary ammonium compounds, silver compounds, PHMB (polyhexamethylene biguanide), triclosan, N-halamine, peroxyacid and regenerative dyes, however causing microbial resistance, non-resistant to laundry and cytotoxicity (Khan *et al.*, 2015; Borkow & Gabbay, 2007). Among the compounds of natural origin there are, for example, chitosan, natural dyes and natural plant extracts such as aloe vera. Yet, studies are still carried out on this type of compounds, such as eucalyptus oil (Khan *et al.*, 2015).

2. Antibiotics and bacterial resistance

There are different types of antibiotics with different functions and, in the past, its use increased the number of infectious diseases that could be prevented or cured. However, bacteria have an impressive ability to develop resistance to antibiotics.

Exposure to antibiotics, by inappropriate use, has promoted the development of severe antimicrobial resistances. With inappropriate use we understand unsafe prescription, antibiotics directed to fight viral infections, over-the-counter sale, lack of quality control in manufacturing, out of date, inadequate surveillance or defective susceptibility testing, animal feed and sprayed on plants to prevent infections and promote growth (Cantóna *et al.*, 2013).

For more than half a century, society relied entirely on antibiotics as if they were the only solution to eradicate microbial pathogenic infections. The misuses aforementioned have made antibiotics less effective, and researchers are desperately looking for new alternatives to antibiotics. In a frantic attempt to maintain the effectiveness of antibiotics, pharmaceutical industries have made multiple combinations of antibiotics, which work for a short period, quickly regaining microorganisms resistance to these combinations again. Interestingly, since the last 4 decades only 3 new classes of antibiotics have been invented, albeit only effective against Gram-positive (Coates *et al.*, 2011). The fact that there is no research works with textiles combined or immobilized with antibiotics only proves that antibiotics are not a huge wager.

In order to understand how antibiotics act, and how bacteria develop resistance, they are divided into 4 groups according to the site, in which they inhibit an essential function to the survival of the microorganism: (i) Inhibitors of cell wall synthesis (animal eukaryotes have no wall) (ii) Inhibitors of protein synthesis (Eukaryotic ribosomes are distinct from prokaryotes) (iii) Inhibitors of nucleic acid synthesis (iv) Anti-metabolites.

2.1. Inhibitors of cell wall synthesis

This is the most common mechanism of antibiotic activity. Most drugs that interfere with cell wall synthesis are classified as ß-lactams, because they have a common ß-lactam ring structure (penicillins, cephalosporins, carbapenems). This ß-lactam ring inhibits the function of the enzymes that make cell wall synthesis, so called penicilin-binding protein (PBP). These promote the construction of chains and the establishment of peptidic bridges – transpeptidase activity.

When in the presence of a ß-lactam, PBPs bind covalently to it, which causes an inhibition of the transpeptidase activity of PBPs. Subsequently, cell wall is degraded after the activation of a lysis system by autolysins, with consequent bacterial death. This is why ß-lactams are bactericidal.

Bacteria became resistant to these antibiotics by three general mechanisms. Firstly, bacteria could avoid interaction of the antibiotic with the target PBP by altering the porin-forming pores, changing the load and size of these channels and promoting exclusion of the antibiotic. Secondly, overproduction of PBP (rare event), acquisition of a new PBP (occurs in MRSA), modification of an existing PBP by recombination (resistance to penicillin in *S. pneumoniae*), or modification of existing PBP by point mutation (resistance to penicillin in *E. faecium*). Finally, some resistant bacteria could codify lactamases that caused the hydrolysis of the antibiotic. These lactamases may have specificity or may have a wide spectrum of action, for example the extended-spectrum B-lactamases ESBL (Wilke *et al.*, 2005).

ß-lactams are essentially active against Gram-positive bacteria. The action against Gram-negative bacteria is more critical because of the outer membrane barrier. Glycopeptides (eg. vancomycin), with a different mechanism of action, interact with the D-alanine-D-alanine end of peptidoglycan preventing cell wall synthesis, being only active against Gram-positive (Wilke *et al.*, 2005).

2.2. Inhibitors of protein synthesis

These drugs interfere with one of the bacterial ribosome subunits preventing protein synthesis. Aminoglycosides and tetracyclines interfere with the 30S subunit, whereas macrolides, oxazolidinones, lincosamides and streptogranins interfere with the 50S subunit.

Aminoglycosides are bactericidal and inhibit the formation of the initiation complex (fmet-tRNA). They bind irreversibly to 30S ribosomal proteins, with disruption of protein synthesis by premature release of the mRNA from the ribosome with production of aberrant proteins by erroneous reading of the mRNA. They have been uptaken to treat infections by many gram-negative bacilli (*Enterobacteriaceae*, *Pseudomonas*). However, resistance mechanisms such as mutation of the ribosome binding site, decreased antibiotic uptake, increased antibiotic outflow of the cell, and enzymatic modification of the antibiotic (the most common), have been observed. The enzymatic modification occurs through the action of several enzymes, namely phosphorylases, adenylases and acetylases.

Tetracyclines are broad-spectrum and bacteriostatic antibiotics. They inhibit protein synthesis by preventing the entry of tRNA into the ribosome (deform the 30S subunit). The mechanism of resistance involves the formation of efflux pumps, ribosome alteration and enzymatic modification of the antibiotic. Chloramphenicol is an antibiotic with a broad spectrum of action similar to tetracycline. It reversibly binds to the 50S ribosomal subunit blocking protein elongation. Its mechanism of resistance is observed in bacteria that produce a plasmid-encoded acetylase. This acetylase catalyzes the acetylation of the 3-hydroxy group of chloramphenicol, preventing binding of the antibiotic to the 50S subunit.

Macrolides, such as erythromycin, clarithromycin and azithromycin, once they inhibit protein synthesis are bacteriostatic antibiotics with a broad spectrum of action. They reversibly bind to the 23S rRNA of the 50S subunit, which blocks the protein elongation. There are two mechanisms of macrolide resistance acquisition: (a) expression of an efflux pump encoded by the mefA gene (macrolides efflux), which will characterize a resistance pattern demonstrated by the M phenotype; (b) modifying the target with methylation of the macrolide binding site to the 23S rRNA and stopping bacterial protein synthesis. In this process the erm (erythromycin resistance methylase) genes that encode methylases are involved, and methylation results in loss of binding of macrolides, lincosamides and streptogramin B (MLSB phenotype) (Tu *et al.*, 2005).

Clindamycin belongs to the family of lincosamides. It blocks the elongation of the peptidic chain by binding to the 50S subunit of the ribosome. It inhibits peptidyltransferase by interfering with the amino acid-acyl-tRNA complex. It is active against staphylococci and gram-negative anaerobes. The resistance reported arises by the methylation of the 23S rRNA, preventing the binding of antibiotic.

Aminoglycosides are bactericidal drugs primarily used in the treatment of Gram-negative infections, while macrolides and tetracyclines are bacteriostatic against Gram-negative, Gram-positive, Chlamydia and Mycoplasma. Oxazolidinones (linezolid), lincosamides (clindamycin) and streptogranins (quinupristin-dalfopristin) are used in Gram-positive infections resistant to other anti-bacterial agents (Wilson, 2014).

2.3. Inhibitors of nucleic acid synthesis

Quinolones, such as ciprofloxacin and levofloxacin, are synthetic chemotherapeutic agents that inhibit bacterial topoisomerase (gyrase). They are active against Gram-positive and negative and have the inconvenience of developing resistance rapidly. This enzyme is required for DNA replication, recombination and repair. In gram-negative the target is topoisomerase II and in gram-positive it is topoisomerase IV. As resistant bacteria undergo mutations in the chromosome in the structural genes of topoisomerase II and topoisomerase IV, there is a decrease in antibiotic entry by mutations in the permeability regulating genes, as well as overexpression of efflux pumps that actively eliminate the antibiotic.

Rifampicin inhibits DNA-dependent RNA polymerase and prevents the initiation of RNA synthesis, being bactericidal against Mycobacterium tuberculosis and Gram-positive bacilli. However resistances have already been reported with a genetic change in the ß subunit of bacterial RNA polymerase of *E. coli* and *Mycobacterium tuberculosis* (Goldstein, 2014).

Metronidazole is a cytotoxic compound that destroys bacterial DNA. It is active against anaerobic bacteria and various parasites, but with several mechanisms of resistance being increasingly reported, with mechanisms yet to elucidate (Dhand & Snydman, 2009).

2.4. Anti-metabolites

These drugs interfere with the synthesis of folic acid, an essential compound for certain microorganisms. Sulfonamides (sulfamethoxazole, sulfisoxasol) are analogues of paminobenzoic acid (PABA), an initial compound of bacterial metabolic pathway. These are less used today, once they cause high microbial resistance mediated by the horizontal transfer of genes of dihydropteroate synthase (Skold, 2000). Trimethoprim, on the other hand, inhibits the enzyme dihydrofolate reductase, which catalyzes a further step of the pathway. Both are effective against Gram-negative and Gram-positive bacteria. The combination trimethoprim-sulfamethoxazole (also called co-trimoxazole) is widely used and is also effective against the fungus *Pneumocystis jiroveci*. Resistance to co-trimoxazole in *S. pneumoniae* has though become a serious threat (Schmitz *et al.*, 2001).

3. Synthetic Biocides

3.1. N-halamines

N-Halamines are organic amine compounds, with a halogen bound to the nitrogen of the amine radical, which present great toxicity because all the halogens with the exception of the iodine are toxic (Sun & Sun, 2001). Although N-halamines can cause burns on the skin and in the airways, they have already been applied in cotton, keratinous fibers (Wool), and synthetic fibers (Nylon; Polyester). Its mechanism of immobilization was (i) grafting a halamine polymer precursor (acrylamide and methacrylamide) and applying them to the surface thereof; (ii) binding of halamine to cotton fibers via siloxane (covalent bonding). Their mechanism of bacterial killing is by the transfer of chlorine atoms from the N-Cl groups to the acceptor regions of the microorganisms. The N-Cl bonds are converted to N-H upon the process of killing microorganisms, in which enzymatic and metabolic processes become compromised, leading to inactivation of microorganisms. Inactive N-H bonds are recharged by bleach to restore their antimicrobial functions.

Although N-Halamines present a strong antibacterial activity and a regeneration capacity, it needs to be regenerated - it does not withstand washing cycles - which gives short-term antimicrobial capability. Furthermore, the high adsorption of chlorine on the surface of the material causes an unpleasant odor and change color of fabrics (Gao & Cranston, 2008).

3.2. Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are moderate to highly toxic, irreversibly damaging the nasal epithelium. The healing of wounds is also delayed. QACs were immobilized to polyester fibers containing carboxylic or sulfonate groups, and to fibers containing only carboxyls, such as wool, cotton and nylon 66. The immobilization mechanism is via the ionic interaction between the cationic ammonium and the anionic carboxylic groups of fibers (electrostatic bonding), or through silanization (covalent bonding). The mechanism of bacterial killing is by electrostatic interactions with the negative potential of bacterial membrane. Their hydrophobic chains are also capable of penetrating the membrane of microorganisms to disrupt their functions. The application of 5% QAC's in wool was reported to have their antimicrobial effect during 10 washes (Jennings *et al.*, 2015).

Although QACs are unlikely to gain antimicrobial resistance, they have little resistance to laundry or slightly better strength if it is covalently attached. Furthermore they deactivate in the presence of anionic detergents forming complexes, and large amount of softening agents are required, once the fabric becomes rough (Gao & Cranston, 2008).

3.3. Silver

Silver presents a low toxicity associated for humans and it is highly toxic to fish and accumulates in aquatic food chains. Even though there is no evidence for skin sensitization by bulk silver, a possible systemic absorption of silver particles due to a disrupt skin barrier has to be considered (Windler *et al.*, 2013). The mechanisms of silver immobilization in the fibers have been described as impregnation (embedding) or placed *in situ*. It has been immobilized in polyester, polyamide, wool, and regenerated cellulose. Its gradual release can lead to an extended period of biocidal activity. Silver eliminates microorganisms by binding to their membrane structures and eventually acts intracellularly, eliminating them. Its mechanisms of bacterial killing involve crossing the cell membrane through an ion exchange mechanism, linking to bacterial membrane proteins with thiols, causing their inactivation. Furthermore, silver can also link to DNA, because it has affinity for phosphorus, where it intercalates between the purine and thymine pairs, denaturing the DNA molecule. Antimicrobial textiles coated with silver show low propensity to develop resistance to bacteria. Further drawbacks are the cumulative costs of therapy that are limited to the initial costs of the silver coated textile, and it is not biodegradable (Gao & Cranston, 2008; Kramer *et al.*, 2006).

3.4. Poly hexamethylenebiguanide

Poly (hexamethylenebiguanide) (PHMB) has low toxicity associated, and has been used as a disinfectant in the food industry, as well as in pools. It has already been immobilized in cotton, polyester and polyamide fibers. PHMB can be adsorbed to cotton fibers through electrostatic attractions between its amines and carboxylic acid groups, which are formed after oxidizing the glucose rings of cellulose. PHMB is an active membrane agent that attacks bacteria that have negative membrane potential, compromising the integrity of the outer membrane and binding afterthen to phospholipids. PHMB presents disadvantages in terms of antimicrobial efficacy that is rapidly lost, there is a potential for bacterial resistance acquisition, and large amounts of PHMB are required in textiles to achieve adequate activity and durability (Gao & Cranston, 2008).

3.5. Triclosan

It has been shown that Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) weakened the muscle tissue cells of rats and fish when they were exposed to the same doses as humans are (Cherednichenko *et al.*, 2012). Moreover, relatively recent studies (Yueh *et al.*, 2014) indicate that Triclosan can induce liver cancer, because it stimulates the production of chloroform that is classified as a possibly carcinogenic agent (Yueh *et al.*, 2014). Other studies raise the possibility of causing hormonal changes in the thyroid, and influence the defenses of the skin (Stoker *et al.*, 2010). It has been used in tooth pastes, hand soaps, shower gel, and deodorants. Triclosan has already been combined with nylon, cotton, polyester, and also incorporated into yarns and fabrics. After 50 washing cycles, the antimicrobial activity decreased. To achieve a longer lasting finish, triclosan has been inserted into polymer films or microspheres. It is a broad-spectrum antimicrobial agent with a MIC (minimum inhibitory concentration) of 10 ppm against many common bacterial species. Unlike most other cationic biocides used in textiles, triclosan is not ionized in solution. Triclosan acts on the blockade of lipid biosynthesis, as well as on the interaction with the amino acid residues of the active sites of the membrane enzymes, causing bacterial lysis. It also inhibits the incorporation and metabolism of glucose by *S. mutans*, *S. sanguis* and *A. naeslundii*. Triclosan also affects the production of *Streptococcus sp.* and the activity of *Porphimonas gengivalis* proteases (Gao & Cranston, 2008; Kramer *et al.*, 2006).

4. "New" natural antimicrobial agents

Numerous researches have been carried out on the development of three groups of antibiotic-resistant antimicrobial agents of natural origin: bacteriophages (phages), bacterial cell wall hydrolases (BCWH), and antimicrobial peptides (AMPs). Phage-based therapies are highly efficient, specific and have a relatively low cost. However, caution should be exercised in the selection of phage candidates for therapeutic applications, such as the possibility of encoding unwanted toxins which, when integrated into the bacterial host genome, alter immunity and bacterial virulence. BCWHs are divided into three groups: lysozymes, autolysins and virolysins. Out of these, virolysins are the most promising candidates as they are highly specific and have the ability to rapidly cause lysis of antibiotic resistant bacteria. Finally, AMPs are a family of natural peptides produced by prokaryotic and eukaryotic or phage-encoded organisms. AMPs have a wide diversity in size, structure, mode of action and specificity and have high potential for clinical therapeutic applications (Dhand & Snydman, 2009).

4.1. Bacteriophages

Phages are non-living agents and intracellular parasites, so they have dependence on a living organism (bacteria) for its replication. Phages are filterable, incapable of producing energy or proteins independently of the host cell, and their genomes may be RNA or DNA but not both. As a consequence, they use the host cell to produce their components, such as mRNA, proteins and identical copies of their genome. They have to code certain proteins that may not be provided by the cell. Viral components have to self-assemble and phages must be infectious to persist in nature. Phages nucleocapsid can be icosahedric or filamentous.

4.1.1. Classification

The phage classification is performed by the ICTV (International Committee on Virus Taxonomy) and derives from the scheme proposed by Bradley in 1967 (Bradley, 1967). The classification depends mainly on the morphology and the type of nucleic acids that constitute them. Although attempts have been made to tailor the classification of phages to more recent methods based on the genome or proteome, morphological criteria prevail (Ackermann, 2001). The capsid is the structure that protects the genetic information and the tail has fibers or other structures that are involved in the recognition of binding of the Phage to the host bacterium.

In the phage genome there are genes encoding proteins responsible for DNA packaging and replication, transcription regulation, host lysis as well as structural proteins (Ackermann, 2001).

4.1.2. Phage-Bacteria Interaction

The contact of the phages with the host bacterium occurs via phage tails and tips that recognize specific receptors, such as carbohydrates, proteins, lipopolysaccharide molecules and flagella of bacteria (Welkos *et al.*, 1974). The most common bacterial receptors are lipopolysaccharide or surface proteins in the case of Gram-negative bacteria, while in Gram-positive, peptidoglycan elements, such as teichoic acids, lipoteichoic acids and associated proteins. After recognition (characterized by a reversible binding), the tail base is the correctly positioned, followed by irreversible attachment of another phage protein to a secondary bacterial receptor (Leiman, 2003). Most phages are highly specific, which limits their interaction with a reduced number of strains. There are, however, phages with broader action spectra that may encompass different strains of the same species or up to different species (multivalent phages) (Welkos *et al.*, 1974; Leiman, 2003).

4.1.3. Phage cycle

Phage life cycle can be lysogenic or lytic. Phages with lysogenic cycle integrate themselves into the chromosome or other replicative element of their host. This results in a lysogenic infection whereby phage genome is replicated synchronously with the host chromosome. During cell division the phage genome is passed from one generation to the next and under specific conditions, it spontaneously assembles and releases virions. On the other hand, phages with the lytic cycle go through 5 steps: (1) Attachment of the virion to a susceptible cell (2) Penetration (injection) of the virion or its nucleic acid into the cell (3) Synthesis of nucleic acid and proteins by virus-engineered cell metabolism early in infection. Later in infection, the structural proteins, which are subunits of the viral capsid, are synthesized (4) Assembly of capsomers (and membrane components in envelope viruses) and packaging of nucleic acid in the new virions (Hayes, 1968).

These steps in viral replication begin when the virions infect the cells. The timing of the replication cycle in bacteriophages varies between 20-60 min, depending on the phage and host. Because the virion release is a practically simultaneous process, the virions are said to undergo a one-step growth curve. Following the adsorption, the infectivity of the viral particles disappears, a phenomenon called eclipse. During this period, the nucleic acid of the virus separates from its coat of proteins (uncoating), and even if the cell blocks entry at this stage, the virion ceases to exist as an infectious entity. During the latent period, replication of nucleic acid and viral proteins occurs. The maturation begins as soon as the newly synthesized nucleic acid is packaged within protein coats (assembly). During the maturation stage, the titre of active virions within the cell increases dramatically. However, due to the fact that newly synthesized virions have not yet appeared outside the cell, the eclipse and maturation periods are also called the latent period. At the end of maturation, the release of the mature virions occurs, either as a result of cell lysis, budding, or excretion, depending on the virus. In average one phage infects one bacterium at growth stage. This bacterium will produce 50-200 new phages, which afterthen will infect adjacent bacteria (Carlton, 1999).

For the release of the bacteria, phages have an interesting mechanism known as protein clocks (Wang *et al.*, 2000). For the phages to be released from the host bacterium, they have to open a pore in the plasma membrane so as to allow the destruction of the peptidoglycan and thereby exit bacteria. For pore formation, the phages encode holins and to destroy the peptidoglycan encode virolysins. For the formation of the lesion it is necessary to form homo-oligodimers (minimum of 6 dimers) of effector of lysis S105. However, for every two S105 that are produced one S107 lysis inhibitor (ratio 2: 1) is also produced. All have affinity to combine with each other in the form of dimers. Thus, there is a slow accumulation of dimers in the cell membrane: active S105 / S105 and inactive S105 / S107. From the moment the first oligodimer is formed exclusively from S105, there is the formation of the first lesion, with consequent collapse of the membrane potential. At this time, the inhibitor S107 becomes the effector of lysis S105, and the heterodimers S105-S107 begin to participate in the formation of the HOLE, having a total intervention and there is a fast oligomerization, with finally a burst of membrane lesions and the collapse of the bacteria. The HOLE must be large enough to allow the passage of 70 kDa virolysins and is apparently non-specific because it allows the passage of heterologous virolysins.

Two apparently contradictory functions define the action of S107: It acts as an inhibitor while the membrane is polarized and actively contributes to the formation of the HOLE after membrane depolarization occurs. Concerning the advantages of the holin-inhibitor system, there is an accurate adjustment of the lysis moment (allows the accumulation of large numbers of phages); The lysis is fast and complete after the formation of the first HOLE (Grundling *et al.*, 2000).

4.1.4. Mechanisms of resistance

Some bacteria have developed systems to protect themselves against phages.

The first resistance system is at the adsorption level. To avoid phage adsorption, the bacteria can block the receptors on which the phage bind. *S. aureus* manages to mask its receptor of phage recognition by the production of Protein A, *E. coli* produces an outer membrane protein TraT that is able to mask or change the conformation of an OmpA porin, which is the receptor for many T-type phages. Another mechanism to prevent phage adsorption is the production of extracellular matrix, which ends up hiding the receptors of phage recognition. However, many phages have developed a system to recognize or even cleave these extracellular polymers. The last mechanism to avoid adsorption is the production of competitive inhibitors that will specifically bind to phage receptors and thus render them unavailable for phage recognition.

Another level of resistance to phage infection is the restriction-modification system of bacteria that can cleave the phage genome, rendering it unusable as soon as it enters the host.

Finally, there are abortive infection systems, which cause the infected cell to induce its own death upon phage replication, transcription or translation, for example the complex phage protein-DNA (Labrie *et al.*, 2010).

4.2. Antimicrobial Peptides

The innate immune response forms the first line of defense against various microorganisms. Thus, antimicrobial peptides are amongst the most important factors of this type of response (Téllez & Castaño, 2010).

AMPs are small molecules with extensive inhibitory activity against various microorganisms, such as gram-negative and gram-positive bacteria, yeasts, viruses and fungi (Izadpanah & Gallo, 2005; Smet & Contreras, 2005) differ in their molecular mass, composition of amino acid residues, charge, three-dimensional structure and biological characteristics (Brogden & Brogden, 2011). They are produced by different cell types, either in plants or animals, in which they express themselves in different ways depending on the organism and tissue in which they are present at the time of infection. It has a broad spectrum of action, including antifungal, antibacterial, antiviral, insecticide and anti-tumor activity and several mechanisms of action (Hancock & Diamond, 2000).

Antimicrobial peptides are classified into four groups according to their structure: (1) α-helix peptides; (2) β-sheet peptides; (3) peptides without defined, extended secondary structure and (4) β-hairpin peptides or loop peptides (Seo *et al.*, 2012).

The constituents of the major groups of peptides were classified mainly based on their biochemical characteristics. The most important resultant groups are the prokaryote derived AMPs, which are subdivided into cationic and anionic, eukaryotic, plant and animal species, either vertebrates or invertebrates (Marshall & Arenas, 2003).

In many cases, the purpose of these antimicrobial peptides is based on the defense and protection of various species against various invading pathogenic microorganisms.

4.2.1.Procaryotes and Eukaryotes

Among the prokaryotes, the antimicrobial peptides produced by gram-positive bacteria are the most relevant and are taxonomically classified as bacteriocins (Marshall & Arenas, 2003).

Prokaryotic AMPs, more precisely bacteriocins, were the first to be isolated and characterized. Although this type of peptides does not protect against infection, they have antibacterial properties as they contribute to the survival of individual bacterial cells by killing or inhibiting the growth of other bacteria, or even bacteria of the same species, that compete for nutrients in the same environment (Jenssen *et al.*, 2006).

The activity of the bacteriocins can be classified as narrow-spectrum, capable of eliminating the bacteria within the same species, or broad spectrum where they are able to target the bacteria of different species (Jenssen *et al.*, 2006). They form a group of several peptides with large variations in size, structure and mode of action.

Eukaryotic AMPs and bacteriocins, although constituting different groups of peptides, share some common characteristics, such as size (20-50a), and the fact that they contain cationic and hydrophobic peptides. These properties facilitate the initial interaction with the anionic cytoplasmic membrane (Hassan *et al.* 2012; Balciunas *et al.*, 2013). There are, however, key differences between them. Bacteriocins have a more powerful effect, since they require a lower concentration to act, in the order of nanomolar, whereas eukaryotic AMPs require a concentration in the order of micromolar to carry out their activity (Nissen-Meyer & Nes, 1997). Moreover, the spectrum of action of bacteriocins is narrow while eukaryotic AMPs have a broader spectrum and are less specific.

The eukaryotic AMPs role is defending and protecting the host from pathogenic microorganisms. On the other hand, bacteriocins do not have the role of protection, but rather guarantee the equilibrium and maintenance of microbial biodiversity (Maróti *et al.*, 2011).

Bacteriocins are considered as promising antimicrobial agents for various applications, such as food preservation and treatment of infections due to their high potency and specificity (Hassan et al., 2012).

Eukaryotic peptides can be divided into three major groups, eukaryotic AMPs from vertebrate animals, invertebrates and plants.

Antimicrobial peptides were initially isolated from invertebrate animals and subsequently further described in vertebrates (Li *et al.*, 2012). In vertebrates, they are found in phagocytic cells at sites that are daily in contact with pathogenic microorganisms, such as mucosal and skin surface, in secretions and body fluids such as saliva, sweat, urine and breast milk, as well as inside immune cells (Jenssen *et al.*, 2006). Cathelicidins form a vast and diverse group of vertebrate antimicrobial peptides. A second important group of these peptides are defensins which are subdivided into three groups based on the pairing of their cysteine residues with the disulfide bridges, are α-defensins, ß-denines and θ-defensins (Jenssen *et al.*, 2006).

Unlike vertebrates, invertebrates do not have an adaptive immune system, yet they have developed an innate immune system to protect against microbial infections (Li *et al.*, 2012). Insect AMPs are known to play an important role in humoral defense reactions. These peptides are synthesized in the fat body, which in mammals is equivalent to the liver, during the systemic response to the action of the pathogenic microorganisms, and are then secreted into the hemolymph (Li *et al.*, 2012; Bulet *et al.*, 1999). This type of AMP is found in hemolymph, in certain epithelial cells and in phagocytic cells (Jenssen *et al.*, 2006). These peptides are organized into five major groups, based on their antibacterial activity and their constitution by amino acids. They are, cecropins, defensins, proline-rich peptides and glycine-rich peptides and lysozyme (Li *et al.*, 2012; Hwang *et al.*, 2009).

Plants have developed several defense mechanisms causing a large production of antimicrobial peptides. These AMPs can be expressed in two ways, induced locally and systemically after the onset of pathogenic microorganisms, or constitutively occurring mainly in seeds, flowers and reproductive tissues particularly sensitive to infections (Maróti *et al.*, 2011). The two most relevant groups of plant-derived AMPs are defensins and thionines (Jenssen *et al.*, 2006).

4.2.2.Cationic Peptides

Cationic peptides represent the largest group of AMPs ever registered with their characterized antimicrobial activity (Gouveia, 2010; Marshall & Arenas, 2003).

These type of peptides have the ability to inhibit or even kill bacteria, such as gram-positive and gram-negative fungi, viruses and parasites. Cationic peptides are selective and thus normal host cells are relatively resistant to their action, they are generally positively charged, which allow them to interact with the cytoplasmic membrane whose charge is negative (Grubor *et al.*, 2006; Parisien *et al.*, 2008)

The major families of cationic peptides are defensins, catalytidines and histatins. The defensins are further divided into α-, ß-, and θ-defensins based on the position of their 6 cysteine residues on the disulfide bonds (Grubor *et al.*, 2006).

4.2.3. Anionic Peptides

Anionic peptides are a smaller group of molecules, compared to cationic peptides, which have been isolated primarily from mammals (Marshall & Arenas, 2003). They are small, hydrophilic and contain homopolymeric regions that are responsible for their negative charge (Gouveia, 2010; Grubor *et al.*, 2006).

Unlike cationic peptides, in order to present maximal bactericidal activity, anionic peptides need to utilize a cofactor that allows the peptide to overcome the negative charge on the surface of the microorganisms. The cofactor used is zinc (Gouveia, 2010; Grubor *et al.*, 2006).

These peptides are divided into various subfamilies such as: neuropeptide-derived molecules, aspartic acid-rich molecules, aromatic dipeptides and oxygen-binding proteins (Marshall & Arenas, 2003).

Like cationic peptides, the anionic ones have a broad spectrum of activity against gram-positive and gram-negative bacteria, yeasts, fungi and viruses (Gouveia, 2010).

4.2.4. Types of AMP Activity and Mechanisms of Action

AMPs exhibit an antimicrobial activity against several pathogenic microorganisms, such as bacteria, yeasts, viruses and fungi, presenting different mechanisms of action depending on the microorganism, such as antifungal activity, antiviral activity, and antibacterial activity (Smet & Contreras, 2005; Brogden & Brogden, 2011).

4.2.4.1. Antifungic Activity

The mode of action of antifungal peptides was initially described as being responsible for lysis or interference with fungal cell wall synthesis.

Most antifungal peptides, especially those that are isolated from plants, tend to be rich in polar and neutral amino acids (Jenssen *et al.*, 2006).

4.2.4.2.Antiviral Activity

The antiviral activity of AMPs is often related to the viral adsorption process and entry, or as a result of a direct effect on the viral envelope (Jenssen *et al.*, 2006). However, it is impossible to predict antiviral activity based mainly on secondary peptide structures (Jenssen *et al.*, 2006). Cationic AMPs demonstrate greater efficacy in inhibiting viral infections.

4.2.4.3.Antibacterial Activity

In general, antimicrobial peptides may inhibit some of important processes in the bacterial cell. AMPs range from membrane permeabilization to actions on various intracellular target molecules, including immunomodulatory activities.

The mechanisms by which the peptides permeabilize the bacterial membrane and the way they exert their antimicrobial activity are not yet fully defined, and so far several models have been proposed that describe the interactions between the peptides and the lipid bilayers. In general, there is an initial electrostatic attraction that is responsible for the association of the peptide with the bacterial membrane, then the AMPs undergo various changes in their conformation and expose their hydrophobic structure that will adapt to the specific conditions of the membrane, and form a pore/transient pore. Some of AMPs lead themselves into the bacteria where they contact with intracellular targets (Costa *et al.*, 2011).

4.2.5. Production of AMPs

AMPs can be obtained in three distinct ways: isolated directly from their natural sources, through bacteria, fungi and animals; chemical synthesis; and recombinant DNA expression (Hancock, 1997; Parachin *et al.*, 2012).

The isolation of AMPs from natural sources is not feasible economically due to high production costs (Hancock, 1997). This type of production has several drawbacks, not only due to the low concentrations of them in the organisms, but also due to its long isolation process that requires the use of numerous solvents causing a negative environmental impact. Thus, extraction directly from natural sources is not considered a good method to obtain high amounts of AMPs, although some peptides are obtained in this way, such as nisin and cyclotides (Hancock, 1997).

The chemical synthesis enables a natural, synthetic or semisynthetic production of AMPs. However, the costs of production are also high, especially for disulfide-bonded peptides (Hancock, 1997). In this type of production, peptides show complex post-translational modifications, such as brominations or glycosylations. Chemical synthesis is not considered a good method of large-scale production, being restricted to AMPs without post-translational modifications and low content of cysteine.

A major opportunity to produce AMPs in greater quantities has emerged through advances in recombinant DNA technology. This technology allows foreign genes to be cloned into specific vectors for expression in host cells, which may be eukaryotes or prokaryotes. This has been considered the most effective method due to its time-cost relationship. A recent study showed that the main hosts used for the production of AMPs are bacteria and yeast. *Escherichia coli, Bacillus subtilis, Propionibacterium freudenreichii* and yeasts *Saccharomyces cerevisiae, Pichia pastoris*.

Currently, plants have appeared as a promising host for the production of AMPs, since transgenic plants can be used directly for microbial control by expressing the peptide in the desired culture without the need of purification (Hancock, 1997).

4.2.6. Mechanisms of Resistance and Toxicity

Antimicrobial resistance is a natural biological phenomenon occurring in microorganisms, and it is associated with the inappropriate use of antibacterial agents (Parachin *et al.*, 2012).

The various types of microorganisms like fungi, bacteria and viruses have different types of resistance mechanisms. However, in general, the resistance to antimicrobial agents can be classified as intrinsic/natural, acquired and cross resistance (World Health Organization, 2012).

An AMP to reach its target site often needs to cross the outer layer and/or the membrane of the bacterium it will eliminate. The nature and composition of these layers varies with the type of microorganism and may work as a permeability barrier resulting in a reduced absorption of the AMP. Some enzymes may also cause degradation of the AMP. Intrinsic resistance is thus a chromosomally controlled property of bacterial cells that allows them to bypass the action of AMPs. Gram-negative bacteria tend to be more resistant than gram-positive bacteria (World Health Organization, 2012). The acquired resistance to AMPs may happen as a result of mutations, plasmids containing genetic information, and transposons (World Health Organization, 2012). When bacteria or other microorganisms develop resistance against a particular type of AMP, which often results in resistance to other AMPs with a similar chemical structure or mechanism of action, to which microorganisms may not have been exposed yet, is named cross resistance (McDonnell & Russell, 1999).

4.3. Bacterial cell wall hydrolases (BCWH)

BCWHs are enzymes that degrade peptidoglycan and cause bacteriolysis. These can be divided into three classes, according to their sources: Lysozymes, which belong to the components of innate immunity of eukaryotes and are broad spectrum; Autolysins, which are exogenous antimicrobial agents of bacteria with unkown spectrum; Virolysins that are phage-encoded lysins, coming from bacteriophages, and have narrow spectrum.

They are disadvantageous in that they have an absence of effect on most Gram negative bacteria as result of their outer membrane (Wyckoff *et al.*, 2012).

4.3.1. Lysozymes

They are found in tears, saliva, nasal secretions and sweat. For example, in the digestive tract the saliva that naturally contains lysozyme, acts as a barrier to agents that cause infection. Lysozyme is a glycosidase that hydrolyzes the β-(1, 4) bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), forming protoplasts (cells without cell wall). It has an effective defense mechanism against bacteria, especially for Gram-positive; Lysozymes are, on the other hand, non-functional for most Gram-negative owing to the protection offered by the outer membrane. Resistance mechanisms have already been reported in *Staphylococcus*, (i) with the O-acetylation of N-acetylmuramic acid of peptidoglycan, and (ii) attachment of other polymers (e.g. Polysaccharides) to its cell wall (Bera *et al.*, 2005). Furthermore, in Lactococcus lactis (iii) the D-aspartic acid incorporation was observed (Solopova *et al.*, 2006). Finally, (iv) the synthesis of lysozyme inhibitors to neutralize the enzymatic activity was also observed (Callewaert *et al.*, 2008).

4.3.2. Autolysins

Autolysins are less-studied, membrane-bound proteins. Each bacterial species contains one or more autolysins, which are involved in a variety of physiological cell functions, such as cell wall biosynthesis, cell separation, cell adhesion, and virulence. They break the β-(1, 4) bond between NAM and NAG and are able to provoke immunological responses against their corresponding bacteria (Smith *et al.*, 2000). Some strains of S. *aureus* have already developed resistance to the autolysin Lysostaphin by the modification of its bacterial wall, but, on the other side, this modification make bacteria less fit (Osipovitch *et al.*, 2015).

4.3.3. Virolysins

Virolysins arise as the most promising antibacterial among BCWH. They turn out to be the product of the isolation of dsDNA phage virolysins that act on peptidoglycan, which, for example, have affinity for choline that functions as a Gram-positive cell wall receptor. Since rapid bacterial lysis occurs, there is a very low probability of development of resistance by bacteria.

Unlike phage therapy, virolysins do not cause immunogenicity, toxicity or resistance issues. Phage therapy involves a process that takes approximately 30-minute – phage lytic life cycle - and it has 3 steps: infection; replication followed by assembly; and lysis of bacteria with release of new virions into the environment, which will subsequently infect new bacteria causing phage amplification. Virolysine therapy is, on the other hand, defined by 2 steps: purified virolysine binds to the target bacteria, and rapidly kills them by osmotic lysis. No resistance mechanisms have been reported for repeated exposure of bacteria to virolysins. Unfortunately, virolysins cannot bypass the outer membrane of Gram-negative bacteria, where phage therapy has advantage (Parisien *et al.*, 2008).

5. Comparison between Antimicrobial Compounds

Bacteria easily become resistant to antibiotics. Synthetic biocides show toxicity to the cells and to environment, and do not resist to laundry.

Bacteriophages are most highly specific towards both Gram-positive and Gram-negative bacteria, highly efficient, relatively cost-effective, and offer a century-old virus treatment known as Phage-therapy.

AMPs are broad-spectrum against bacteria and fungus, present low level of induced resistance, may cause toxicity at high doses in order to be efficient, and a major drawback is that they are costly to produce.

BCWHs have limitations towards Gram-negative bacteria, due to their outer membrane, and important Gram-positive pathogens like *S.aureus* are already resistant to lysozymes.

The use of phage therapy over antibiotic therapy has some advantages, namely that it is effective against multiresistant pathogenic bacteria, and the replacement of normal microbial flora does not occur because phages only kill its target bacteria. Phages can respond rapidly to the selected phage-resistant bacteria since mutation frequency of phage is significantly higher than that of bacteria. Furthermore, the cost of developing phage treatments is lower than that of new antibiotics, and side effects are very rare. However, there are some concerns regarding this type of treatment: rapid bacterial cell lysis can result in the release of large amounts of bacterial membrane endotoxins, some phages may encode toxins, the absence of pharmacokinetic data, neutralization of the phage by the host immune system may lead to the failure of therapy, and the conversion of lytic phage into lysogenic (prophage) leads to bacterial immunity attacks by the corresponding lytic phage and may also alter the virulence of the bacterium (Matsuzaki *et al.*, 2005).

6. Antimicrobial functionalization of fibrous materials

"Biomaterials science" is the physical and biological study of materials and their interaction with the biological environment. For a successful conception of devices one should control their interface with the biological environment. The surface of a material may be considered a type of planar defect, since the atoms on the surface are not connected at all sites to other atoms and there is extra energy associated with this region due to the valence band unfilled. This excess energy is called Surface Tension (γ). Once this step is thermodynamically unstable, there is a driving force to minimize surface tension by absorption of atoms or molecules. Surface modification of a biomaterial does not alter its mechanical properties: only a thin layer shall be modified (the outermost layer), be resistant to delamination, be simple and robust to allow easy commercialization. The interface between a material and biological medium is composed of ions, water, and proteins. It is to this coated surface that the system will react. Controlling protein adsorption to the surface of a material is the key aspect to ensure the success of the most biomaterials and medical devices (Ratner, 2004).

6.1. Factors affecting materials functionalization

Factors as wettability, charge, sterical repulsion, and roughness play an important role on materials functionalization strategies. Spreading or wetting a surface is related to its surface energy. Wettability is the total effect of attraction of droplet molecules to each other, and attraction or repulsion of droplet molecules to surface molecules. With this parameter, one can study adhesion, wettability, and surface energy. However, it has disadvantages when studying rough surfaces, surfaces that absorb the droplet, and heterogenous surfaces (Ratner, 2004). Surface charge can have the additional effect of attracting or repelling charged areas of proteins, namely allow the electrostatic crosslinking between compounds. Adding large and flexible hydrophilic polymer chains such as poly(ethyleneglycol) (PEG) to a surface will result in a decrease in protein adsorption, due to the large size of the carbon chain which functions as a broom preventing adhesion, and to its hydrophilicity which has the "non-fouling" effect of repelling protein adhesion. In addition, high degree of roughness may promote protein adsorption by physically "trapping" the proteins in the valleys on the surface.

6.2. Physicochemical modification

Materials can be surface-modified by 3 methods: physicochemical, biological, and mechanical.

The physicochemical possibilities of modification are depicted in figure 1.

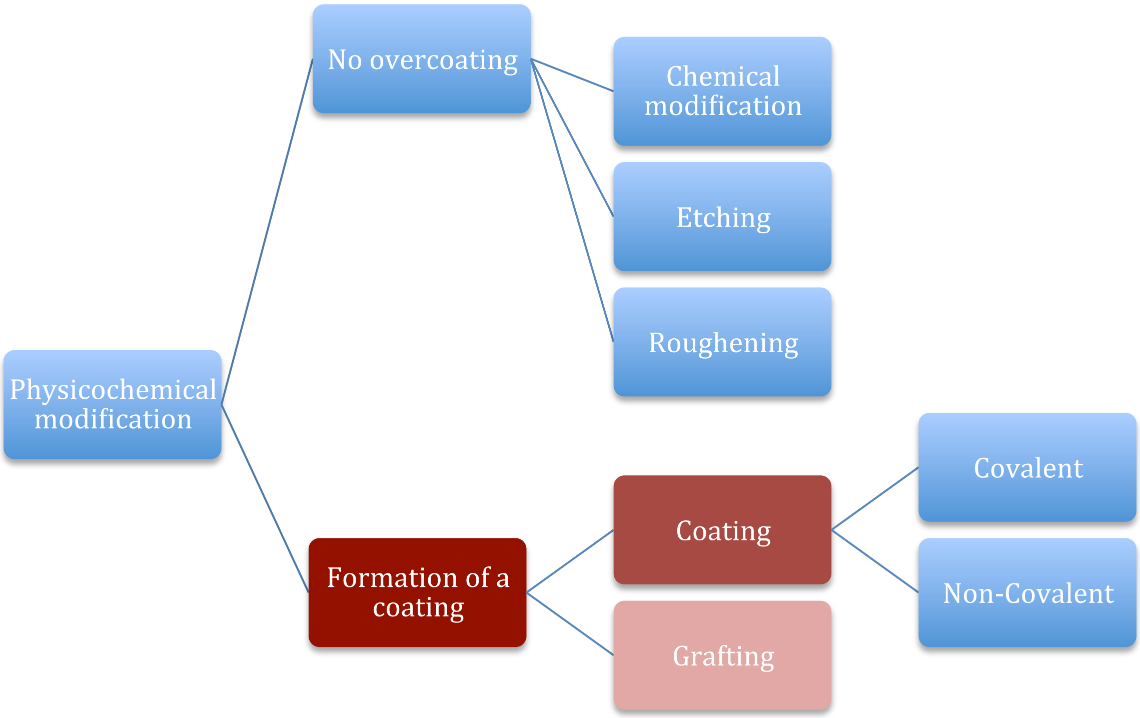

Figure 1. Representaion of different physicochemical modification strategies

In a covalent strategy specific chemical reactions are very specific and change only one functional group into another with high yield and few side reactions. On the other hand, non-specific chemical reactions leave a distribution of different functional groups at the surface, e.g. Plasma treatment. In covalent attachment it is required the presence of a reactive substrate surface with: OH, COOH, NH_2.

Biomolecules can be bound to the substrate either directly or via spacer arm. A spacer arm is an inert molecule that provides physical space between the molecule and the substrate, allowing rotational freedom to the immobilized biomolecule improving its bioactivity, and can be biodegradable when the local and slow release of the biomolecule is important.

Immobilized biomolecules must remain attached through covalent immobilization, maintain its biological activity and selectivity via spacer arms, present appropriate selectivity through non-fouling spacer arms - resist the adsorption of proteins and/or adhesion of cells due to its hydrophilicity - and non-fouling surfaces, and have an appropriate orientation with chemical biofunctionalization.

On the other hand, non-covalent strategies are performed due to electrostatic attraction between two compounds to be joined. In the Dipping technique the substrate is dipped in a solution containing the dissolved coating material (usually a polymer dissolved in an organic solvent). Layer-by-layer is a technique in which two different blocks are attracted to each other, typically by electrostatic interactions.

In the process of grafting, copolymers are blended, wherein block A is compatible with the interior of the polymer and block B has more affinity with surface. In this case, block A will serve as an anchor to hold the molecule inside, whereas surface characteristics will be ensured by block B. For example, if the medium where the material placed is aqueous, and an additive with hydrophilic group is added, it will migrate to the surface. If, on the other side, the material is in contact with air, the hydrophobic group will migrate to the surface.

Non-covalent attachment is performed when covalent bonds are unwanted or their chemistry is very complicated. In this case, adsorption of the biomolecules to the substrate is done via hydrophobic and electrostatic interactions, and subsequently crosslinking to improve the coating stability (Ratner, 2004).

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Chapter 2 – Aims of the Thesis

Aims of the thesis

The aims of this thesis lie on the use of selected “new” antimicrobial agents, namely L-Cysteine, antimicrobial peptides, and bacteriophages, immobilized through different covalent and non-covalent strategies on different textiles and polymers, in order to to provide novel, smoother, and increased surface-to-area fibrous and nanofibrous materials with antimicrobial properties, which can be used as part of chronic wound management and hospital garments.

The skin of patients with diabetes or immunodeficiency is particularly susceptible to infection by different microorganisms. The opportunistic pathogens are responsible for some lethal infections under hospital settings, being the cause of both acute and chronic skin infections and of the inability of chronic wounds to heal. In this way, our contribution may be considered an important part in wound/skin-infection management.

In this regard, new environmental friendly materials, safe, with strong antimicrobial properties and alternative to antibiotics are herein presented for potential use under hospital settings.

Chapter 3 – Scientific Papers

Paper I

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

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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

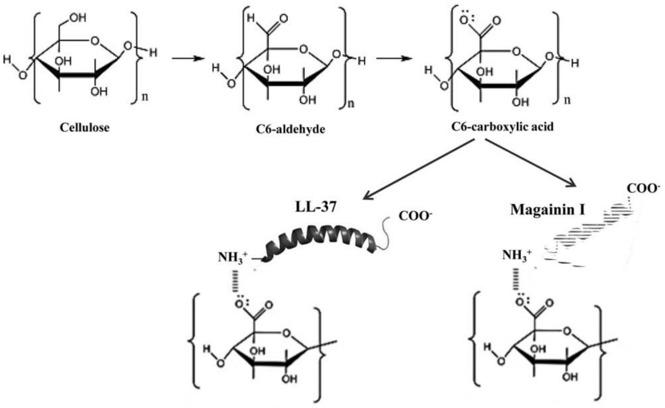

Figure 1. Schematic representation of the proposed mechanism of linkage of the antimicrobial peptides onto TEMPO activated cotton samples.

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 2×10^4 cells/well. Cells were used on the 20th passage.

Briefly, cells were seeded in 24-well plates (2×10^4 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 μL 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

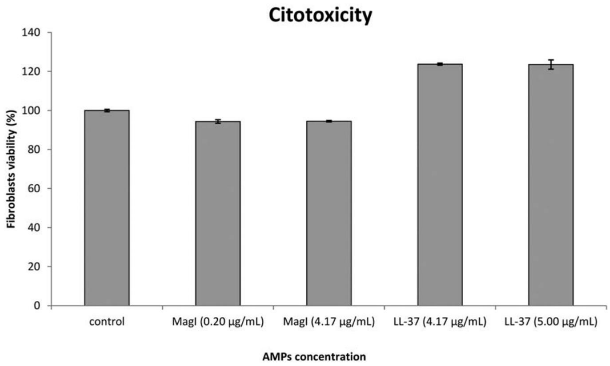

Figure 2. Fibroblasts viability percentage (mean ± SEM) when in contact with two concentrations of magainin I (MagI) and LL-37 and the untreated controls.

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 μg/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 μM (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 β(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/

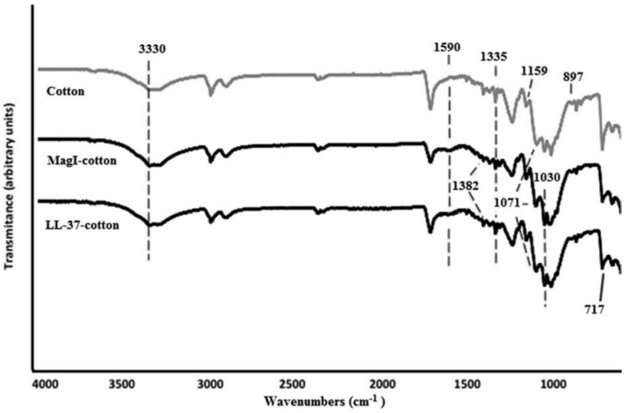

Figure 3. IR spectra of unmodified and modified cotton with LL-37 and magainin I (MagI).

mL), and presents higher number of amino acids in its structure (33), higher number of aromatic rings from its Phe aminoacids (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	1.55×10^3	0.00		3.30×10^7	1.41×10^6	
Magainin I	1.95×10^3	6.36×10^2	-	2.80×10^7	7.07×10^5	15
LL-37	2.23×10^3	1.10×10^3	-	3.63×10^6	3.54×10^4	89
S. aureus CFU/mL	Average of CFU/mL	Standard deviation	% of Bacterial reduction	Average of CFU/mL	Standard deviation	% of Bacterial reduction
Cotton	9.80×10^3	4.95×10^2		3.53×10^7	2.93×10^7	
Magainin I	8.23×10^3	2.47×10^2	16	1.49×10^7	1.20×10^6	58
LL-37	8.98×10^3	3.18×10^2	8.0	1.46×10^7	1.41×10^6	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.

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Paper II

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RESEARCH PAPER

Covalent Modification of Cellulosic-based Textiles: A New Strategy to Obtain Antimicrobial Properties

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Abstract In the past years the textile industry has witnessed new advances in the area of textile fiber chains engineering, which allow the modification of the structure of such chains so as to produce polymers responsive to changes in the environment, thus capable of attaching to cells and bioactive molecules. On the other hand, following our society's trend towards higher hygiene standards, the research and development of antimicrobial textiles has shown a remarkable increase. Applications of such textiles can nowadays be found in underwear, sportswear, home furnishing, protective clothing, wound-dressings and in microbial infection high risk settings, such as health care institutions. The present research aims at the development of a strong, durable and washable antimicrobial L-Cysteine (L-Cys)-functionalized cotton by means of a covalent mechanism. The covalent binding of L-Cys onto cellulosic fibers was assessed by Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy (SEM-EDS) and Fourier Transform Infrared Spectroscopy (FTIR) analysis. Antimicrobial assays showed that the functionalized cotton yielded strong microbial killing rates, exhibiting inhibition ratios of 89 and 83% against *K. pneumoniae* and *S. aureus*, respectively. These results demonstrate the effectiveness of the covalent modification of cotton fabrics with L-Cys adding antimicrobial properties to cotton fibers and thus open the door to a world of applications in the area of increased risk microbial infections.

Keywords: L-Cysteine, antimicrobial-textiles, *Staphylococcus aureus*, *Klebsiella pneumoniae*

1. Introduction

Throughout the past decades hospitals have been facing a major challenge concerning the growing multi-drug microbial resistance, especially in immunodepressed patients. The main cause of microbial resistance may lie in the abuse of antibiotics in the treatment of diseases, as well as in the raising of cattle and poultry for weight gaining purposes [1]. The development of antimicrobial textiles offers a promising solution in the prevention of infections in clinical settings since microbial shedding from our body contributes to microorganism spreading into a textile material, either directly in clothes or on surrounding textiles.

The use of some chemical antimicrobial agents in textiles has already been tested, as for example Quaternary Ammonium Compounds (QACs), Biguanidines, Silver, Triclosan, and N-Halamines. However, these have proved to be of limited clinical applicability. They exhibit some cytotoxicity causing some irritation of the skin, toxicity to the environment and, except for silver and N-Halamines, exhibit a reduced spectrum of microbial inhibition thus bringing about microbial resistance. Besides, with the exception of QACs, which establish very durable covalent bonds with textile fibers, they gradually lose their bioactivity with use and laundering [2,3].

Furthermore, the mechanisms already tested to covalently attach antimicrobial agents to cellulose-based textiles are

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few, and we quote here, amongst the most relevant: (i) the ionic binding of quaternary ammonium salts on cotton fabrics using a covalently bound adduct that increases anionic sites [4]; (ii) the covalent attachment to cotton of a chloromelamine derivative through nucleophilic substitution reactions [5]; (iii) the binding of a cyclodextrin derivative with cotton by a nucleophilic substitution reaction between the hydroxyl groups of the cellulose chain and the chlorotriazine ring [6]; (iv) the attachment of chitosan to cotton fabric *via* cross-linking agents [7]; and finally (v) trimethoprim and sulfamethoxazole antibiotics modified to act as reactive dyes and covalently bonded onto the cotton surface in order to provide it with antibacterial properties [8]. Actually, covalent bonding of a molecule on a low-reactive surface (as cotton) requires the prior activation thereof [9].

L-Cysteine (L-Cys), with a molecular weight of 121.16 g/mol, is a unique amino acid present in a number of food sources, which exhibits a sulphur group at the end of its variable side group. L-Cys exerts an antimicrobial effect on Gram-positive and Gram-negative bacteria, by means of an antimicrobial mechanism which relies on metabolic disturbance and some degree of membrane depolarization [10], what makes it an appealing alternative to antibiotics [11]. On account of the high reactivity of its –SH group, which causes a sharp decrease in enzymatic activity and bacterial metabolism, L-Cys targets the bacterial membrane, making it hardly possible for microorganisms to develop resistance, as this would require a change of their membrane composition and/or organization, which would be highly demanding and energetically unrewarding.

In addition, because of their similarity with macromolecules which the biological environment is prepared to recognize and deal with metabolically, natural polymers show few adverse reactions, excellent humidity control, biocompatibility and low-allergic responses. Cotton is mainly composed of cellulose, which is a natural biodegradable biopolymer, a β-1,4-linked glucan, with –OH at positions 2, 3, and 6. Furthermore, it exhibits important hydrophilic characteristics, such as high moisture and fluid retention properties [12], low impurity content, antistatic behavior and good mechanical properties [13], wherefore cotton textiles are widely adopted in hospitals and other health-care institutions. However, since cotton fibers do not have an antimicrobial effect by themselves, the development of new strategies and processes to provide cotton materials with lasting and effective antimicrobial properties is of the utmost importance [2,3,12].

Hence, this work aims at the development of a process which allows the bonding of L-Cys on cotton fibers through the use of N,N-carbonyldiimidazole (CDI) at different concentrations, a surface-activator of hydroxyl groups. In addition, the antimicrobial properties of such covalently functionalized textiles are analyzed. Because of its covalent bond to a cellulose-based substrate, L-Cys was expected to act as a by-contact antimicrobial, thus preventing it's leaching from the substrates to the environment, and consequently minimizing the development of resistance by microorganisms and enhancing its resistance to laundering.

2. Materials and Methods

2.1. Materials

A cotton surface activator solution was prepared by dissolving N,N-carbonyldiimidazole (CDI) (Sigma) in Anhydrous Tetrahydrofuran (THF) (Sigma) at an absolute absence of water in a glove box chamber with nitrogen replacing atmosphere. Samples were then functionalized in a L-Cysteine (L-Cys) (Sigma-Aldrich) solution (ddH$_2$O) at pH 9.5. *S. aureus* (ATCC 6538) and *K. pneumoniae* (ATCC 4352) strains were grown on Nutrient Agar (NA) and Nutrient Broth (NB) (Panreac).

2.2. Methods

2.2.1. Determination of minimal inhibitory concentration (MIC) of L-Cys

MIC determination was performed in accordance with the macrodilution method. In this procedure we followed the Standard M07-A6 applied to *S. aureus* (ATCC 6538) and *K. pneumoniae* (ATCC 4352), the recommended strains for antimicrobial assays on textile fibers, as described in Standard JIS L 1902:2002. In brief, the minimal inhibitory concentration was determined by serial dilutions of 1:2 in Mueller-Hinton Broth (MHB) (Sigma-Aldrich) with concentrations of L-Cys ranging from 9.7×10^{-2} to 2.3×10^{-5} g/mL. Bacterial inoculums were prepared from fresh overnight liquid cultures and its turbidity adjusted to 0.5 McFarland (approximately $1 \sim 2 \times 10^8$ CFU/mL) with sterilized water, and then diluted to give a final concentration of 1×10^5 CFU/mL. 1 mL of inoculum was added to each tube containing 1 mL of L-Cys. After 24 h incubation at 37ºC antibacterial activity was assessed.

2.2.2. Cotton preparation

Cotton was first washed in a 1 g/L solution of ECE Reference Detergent for 30 min at 40ºC, rinsed, and dried afterwards at 30ºC until used, in order to remove impurities and soils.

2.2.3. Functionalization

In order to perform the surface activation of cellulose (as shown in Fig. 1), prior to L-Cys covalent binding, surface activator solutions were prepared using 0, 4, 40, and 60%

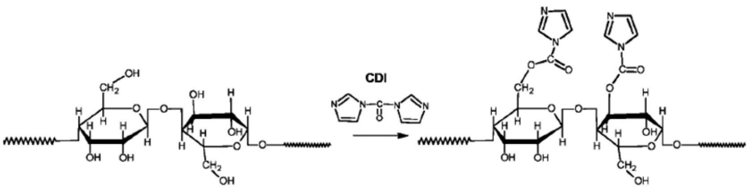

Fig. 1. CDI-mechanism for the activation of cellulose.

(w/V) N,N-carbonyldiimidazole (CDI) (Sigma) concentrations, by dissolving the latter in Anhydrous Tetrahydrofuran (THF) (Sigma) at an absolute absence of water in a glove box chamber with nitrogen replacing atmosphere. The cotton samples were immersed in this solution for 2 h at 15 rpm, and then rinsed in THF. The obtained samples were then functionalized by means of a 24 h long exhaustion process at 40ºC, 15 rpm, in a Mathis-BFA12 finishing machine (adapted from [20]). Concentrations of 5 and 7.5% over the weight of the fiber (owf) L-Cys were used. Finally, samples were rinsed three times with ddH_2O and dried at 30ºC. Afterwards, the samples were washed in a 1 g/L solution of AATCC 1993 Standard Reference Detergent WOB, in 5 washing cycles performed at 40ºC during 60 min - a method adapted from the international standard EN ISO 105-C06:2010 - and then dried at 37ºC for approximately 4 h. This soaping procedure was performed on the samples treated with L-Cys, prior to their subsequent investigation and assessment of antibacterial activity, in order to make proof of the resistance to washing of the L-Cys bond to the cotton surface. All samples were produced in duplicate.

2.2.4. Cotton characterization

Cotton samples were characterized using different surface analyzing techniques.

2.2.4.1. Scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS)

The surface morphology of the samples was analyzed with a HITACHI S 2700 scanning electron microscope. Images of the details of the cotton fibers were formed through secondary electrons. The high voltage used was 20 KV. An EDS attachment was used to ascertain the sulphur presence on the L-Cys-cotton fibers.

2.2.4.2. Fourier transform infrared spectroscopy (FT-IR)

The chemical composition of cotton and L-Cys-cotton was analyzed by means of FT-IR. Measurements were done with a Thermo-Nicolet is 10 FTIR spectrophotometer. Each sample was scanned 64 times, with a spatial frequency resolution of 4 cm^{-1}.

2.2.5. Antimicrobial activity

The antimicrobial behavior of cotton samples successfully functionalized with L-Cys (samples pre-activated at 4, 40, and 60% CDI) was evaluated against two bacterial strains: Gram-negative *K. pneumoniae* (ATCC 4352) and Gram-positive *S. aureus* (ATCC 6538). The selection of these strains as well as the entire procedure were carried out according with the Japanese Industrial Standard JIS L 1902:2002, as described by ourselves in a previous work [11]. Briefly, after preparation of the inoculum and bacterial cultures, both functionalized samples and controls were tested. Antimicrobial activity was evaluated by two different methods: (i) a qualitative method performed in duplicate, in which samples were placed on petri dishes seeded with the respective bacteria embedded in broth and incubated at 37ºC for 24 ~ 48 h; (ii) a quantitative method performed in triplicate, to determine the percentage of bacterial growth inhibition rates of the antimicrobial textiles (% of Inhibition) at 0 and 24 h:

$$\%Inhibition = \frac{C-A}{C} \times 100$$

where C represents the average value of Colony Forming Units (CFU-µg/mL) of non-modified textile, and A represents the average value of CFU of functionalized textile [14].

The qualitative assays were performed only on the samples exhibiting the best functionalization conditions, in order to evidence the formation of an halo around the test-specimen resulting from the covalent binding of L-Cys onto cellulose. The qualitative Test Agar diffusion, was used to test textile samples of a Pattern-determined size in direct contact with a bacterial suspension ($1 \times 10^6 \sim 1 \times 10^7$ CFU/mL) blended with Nutrient Agar (Cultimed) in Petri dishes. These samples were placed in the center and pressed down with the aid of sterile tweezers to ensure the intimate contact of the whole surface with the medium. Finally, the plates were incubated at 37ºC for 18 ~ 24 h. On account of the non-leaching properties of the L-Cys-cotton bond, there was no formation of an halo usually used to describe the antimicrobial effect of the samples, which could however be evaluated by analyzing the (absence of) bacterial growth in the area in contact with the sample.

2.2.6. Statistical analysis

SPSS Statistics 21.0 software was used to perform the statistical analysis of the data using One-Way ANOVA, with LSD as Post-Hoc tests for establishing multiple comparisons between samples in this study. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Minimal inhibitory concentration (MIC) of L-Cysteine (L-Cys)

MIC values of 48 and 6 mg/mL were obtained for *K. pneumoniae and S. aureus,* respectively.

3.2. Energy dispersive X-ray spectroscopy (EDS)

The L-Cys presence on cotton samples varies with the surface activation and functionalization conditions and can be indirectly assessed through elemental analysis of the sulphur amount on each sample. Cotton, a cellulosic fiber, does not contain sulphur in its native structure. Thereby, the amount of surface sulphur correlates with the effectiveness of the functionalization process with L-Cys (Table 1).

Table 1 shows the activation at 4% (w/V) to be more prone to retain higher amounts of L-Cys, namely 0.32% wt.(percentage by weight) for 5% L-Cys owf and 0.44% wt. for 7.5% L-Cys owf. As can be concluded from the aforementioned Table 1, the highest retention of sulphur on cotton surface after five washing cycles, for concentrations of functionalization of both 5 and 7.5% owf of L-Cys, was obtained with 4% (w/V) of CDI activator. In the absence of surface pre-activation (0% (w/V) CDI) only small percentages of sulphur, 0.21% wt. for 5% L-Cys owf and 0.23% wt. for 7.5% L-Cys owf, were detected, which suggests the existence of physical or electrostatic bonds of L-Cys onto the cotton surface, as described previously [10].

3.3. Fourier transform infrared spectroscopy (FT-IR)

The infrared spectra of unmodified cotton is shown in Fig. 2. 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

Table 1. Energy dispersive X-ray spectroscopy.

Cotton functionalized with 5% L-Cysteine		*Cotton functionalized with 7.5% L-Cysteine*	
Condition	*S (Elements [C(atom)])*	*Condition*	*S (Elements [C(atom)])*
Blank	0.00	*Blank*	0.00
0% CDI	0.21	*0% CDI*	0.23
4% CDI	0.32	*4% CDI*	0.44
40% CDI	0.12	*40% CDI*	0.30
60% CDI	0.17	*60% CDI*	0.10

Functionalization assays with L-Cysteine concentrations of 5 and 7.5% owf, using different concentrations of surface-activator (CDI), 0, 4, 40, and 60% (w/V) and the blank (non-activated sample).

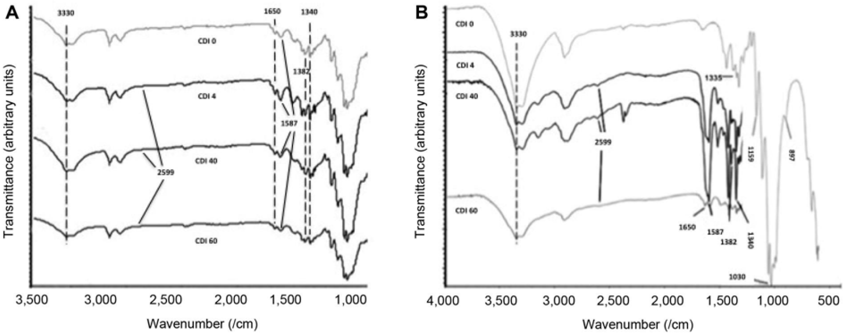

Fig. 2. IR spectra of unmodified and modified cotton with different concentration of surface-activator at (A) 5 and (B) 7.5% owf L-Cys functionalization.

deformation vibrations. The peaks related to the β(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} [15].

Structural changes on cotton after functionalization were also assessed by FT-IR. At L-Cys concentrations of 5 and 7.5% owf, Figs. 2A and 2B, the presence of L-Cys was confirmed by the observed peaks of the amides, namely at 1650 cm^{-1} due to the amide I band C=O stretching, at 1587 cm^{-1} due to N-H bending of primary amines, at 1340 cm^{-1} due to the C-N stretching vibrations of the amide III, as well as a peak at 1382 cm^{-1} associated with –CH_3 symmetrical angular deformation. Particularly worthy of note is the peak at 2599 cm^{-1} associated with the the S-H stretching of –CH_2SH functional groups [15]. At 5% owf, Fig. 2A, IR spectrum suggest an increased L-Cys immobilization at surface-activator concentration of 4% (w/V) of CDI and a reduced immobilization at 40 and 60% (w/V) of CDI. Furthermore, at 7.5% owf, Fig. 2B, IR spectra also suggest an increased L-Cys immobilization at surface-activator concentrations of 4 and 40% (w/V) of CDI, and a reduced immobilization at 60% (w/V) of CDI. These trends can be seen from the increased peaks associated with C=O stretching, N-H bending of primary amines, vibration of amide III, and –SH stretching. At 0% (w/V) of activator there were no peaks suggesting any covalent bond formation.

3.4. Antimicrobial assays

3.4.1. Quantitative method (suspension)

Cotton activated surfaces showed good positive microbial inhibition results, as shown in Fig. 3. It can be observed that the lowest concentration of activator tested (4% CDI) was enough to promote the greatest antimicrobial inhibition in both bacterial strains after 24 h – ~ 88% against *K. pneumoniae* and ~ 83% against *S. aureus*. Furthermore, as the activator concentration was raised, the percentage of inhibition decreased. As an example, for *K. pneumoniae* (A) it was observed an inhibition of 89.39% for an activation with 4% (w/V) and functionalization with 5% owf L-Cys (p < 0.05). However, when the activator concentration was raised to 40% (w/V), growth inhibition dropped to 63.47% (p > 0.05).

Results also showed that L-Cys exhibit good inhibition properties at its lowest concentration (5% owf). For example, in case of *S. aureus* strain (B) samples activated with 4% (w/V) CDI and functionalized with 5 and 7.5% owf L-Cys showed a significant CFU reduction with 5% owf L-Cys concentration (p < 0.05), whereas with 7.5% owf L-Cys concentration, the reduction was not significant (p > 0.05). Generally, microbial inhibition rates showed to be always higher after bacterial adsorption onto L-Cys-cotton samples activated with 4% (w/V) of CDI.

3.4.2. Qualitative method (halo)

Complementarily, qualitative tests were performed concerning the antimicrobial activity of cotton activated with 4% CDI (w/V) and functionalized with L-Cys at 5 and 7.5% owf, in order to obtain a visual perception of microbial inhibition. This activator percentage was chosen based on the previously obtained results which showed 4% (w/V) CDI to be the most efficient activator concentration to cross-link L-Cys with the fiber and exhibited the best antimicrobial inhibition results (quantitative method) for both concentrations of functionalization.

Actually, halo assays results (Agar diffusion tests) (Figs. 4 and 5) obtained during testing against *K. pneumoniae* and *S. aureus*, confirmed that there was an antimicrobial activity of L-Cys-functionalized cotton. The antimicrobial effect can be visualized in the inhibition zone of microbial growth underneath the cotton sample. This kind of inhibition pattern normally occurs when the antimicrobial agent doesn't have the capacity to migrate. It follows that, the

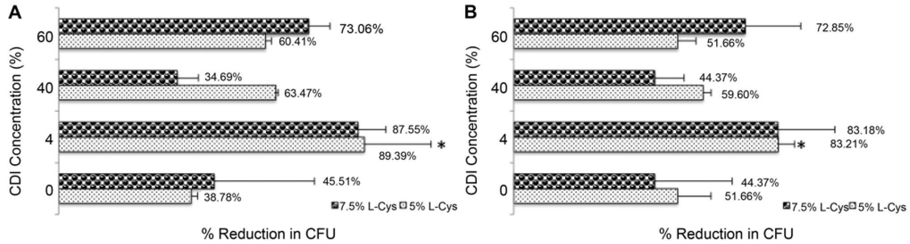

Fig. 3. Percentage of microbial inhibition against *K. pneumoniae* at 24 h (A) and *S. aureus* at the same time point (B) – all of these values were obtained by comparison with results of non-functionalized cotton. *Statistically significant different from 0% CDI 5% L-Cys (p < 0.05, LSD test).

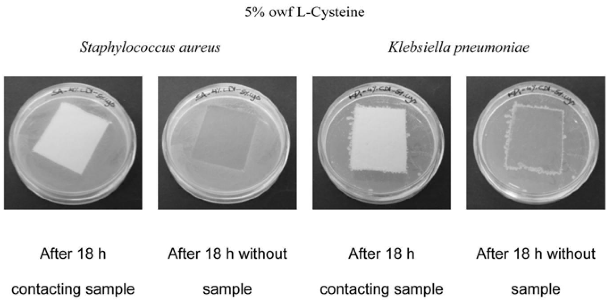

Fig. 4. Halo Assay: inhibitory effect of samples functionalized with 5% owf L-Cysteine and activated with 4% (w/V) CDI, against *S. aureus* and *K. pneumoniae*, after 18 h of incubation.

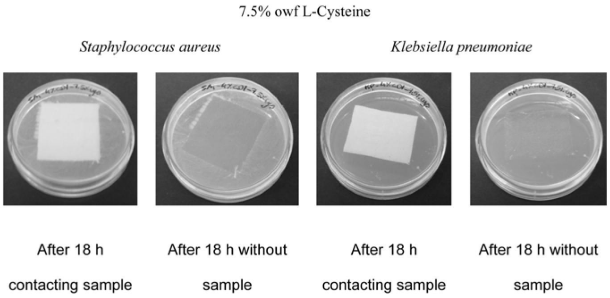

Fig. 5. Halo Assay: inhibitory effect of samples functionalized with 7.5% owf L-Cysteine and activated with 4% (w/V) CDI, against *S. aureus* and *K. pneumoniae*, after 18 h of incubation.

inhibition zone is restricted to the antimicrobial textile and the area that is in intimate contact with it [11].

4. Discussion

A previous study carried out by Morris *et al*. [16] postulated the antimicrobial effect of thiol compounds through their reaction with sulphydril groups located at the membrane of Gram-positive and Gram-negative bacteria [16]. Gouveia *et al.* [11] established that besides arginine, amino acids containing cysteine residues with free sulphydril groups (-SH) seem to have an important role in antibacterial activity [11]. Finally, L-Cysteine (L-Cys) was chosen for its low cost, its antimicrobial activity, its non-cytotoxicity [10] and insomuch as it seems to be very attractive to use on cotton surface modification [10,11].

For fiber modification purposes, our strategy focused on the primary amine of L-Cys linkage to cellulose, which is not important for its antimicrobial activity in this work, once the antimicrobial effect relies on the free –SH groups of L-Cys covalently bonded onto cotton. Actually, the cotton modification strategy (activation and functionalization) promoted the covalent bonding between the primary amine of L-Cys and free hydroxil groups (-OH) of cellulose by means of zero-length covalent bonding chemistry [17]. CDI converted free hydroxil groups of cellulose into the intermediate imidazole-carbamate groups, which ultimately reacted with N-nucleophile of the free amines of L-Cys [17].

MIC values of 48 mg/mL for *K. pneumoniae*, and 6 mg/mL for *S. aureus* obtained with L-Cys are significantly higher than those obtained with other compounds, such as chitosan (0.05 mg/mL in the case of *S. aureus* and *K. pneumoniae*) and triclosan (6 mg/mL in the case of *S. aureus*) [18,19]. However, the mechanism of L-Cys bonding onto the cotton fibers with their free sulphydril groups pointing outward proved to require much lower concentrations of L-Cys to kill bacteria (5% over the weight of the fiber (owf) in a ratio solution of 1/50 g of fiber per 50 mL; thus, 1 mg/mL), when comparing with L-Cys in an aqueous solution. L-Cys molecules dissolved in aqueous solutions can create undesirable S-S bonds, thus losing L-Cys reactive groups. Hence, the chosen bonding strategy proved to be more efficient, thereby requiring less concentrate L-Cys solutions to grant cotton fibers the desired antibacterial activity.

Cotton fibers with different concentrations of CDI (0, 4, 40, and 60 mg/mL) and with 5 and 7.5% owf L-Cys were tested. Energy Dispersive X-ray Spectroscopy (EDS) results showed that the best L-Cys adsorption was achieved with the activator at 4% (w/V) CDI, at either 5 or 7.5% owf functionalization. In addition, EDS at 7.5% exhibited a slightly higher adsorption of L-Cys. FT-IR spectra confirmed that L-Cys bonded well onto the cotton fibers, especially at CDI 4% (w/V) at functionalization of 5% owf, as well as at 4 and 40% (w/V) of CDI at L-Cys functionalization of 7.5% owf. Furthermore, at the other CDI concentration activated surfaces, smaller sulphur peaks were still detected. Hence, this chemical mechanism proved to be suitable to introduce the desired modifications in cellulose. In accordance with the chemical surface data, the highest killing ratio of bacteria in contact with samples after 24 h incubation occurred with the lowest concentration of activator tested, 4% (w/V) CDI (for 5 and 7.5% owf L-Cys), enough to promote the best antimicrobial inhibition rates against both bacterial strains. The relatively high antimicrobial inhibition trend of the highest concentration of surface activator used at 60% (w/V) CDI (for 7.5% owf L-Cys) might be due to the formation of non-specific physical adsorption reactions aside from the covalent bond obtained. Martins *et al.* [20] also stated that when fewer concentrations of pKL (selective to heparin) were bonded onto self-assembled monolayers (SAMs) (using the lowest activator concentration) heparin binding was more effective. Contrarily, with higher amounts of pKL on the surface (due to more activator usage), heparin binding was no more selective due to steric hindrance of packed chains or high degrees of activator that could bind several lysines of pKL [20]. In conclusion, both concentrations of L-Cys showed to inhibit these two reference strains at all antimicrobial assays, although 5% owf L-Cys proved to be the most effective amount of L-Cys used, together with an activation of surface of 4% (w/V) CDI.

5. Conclusion

This study showed that cotton fibers covalently functionalized with L-Cysteine using a zero-length crosslinking chemistry were obtained, as demonstrated by Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy (SEM-EDS) and Fourier Transform Infrared Spectroscopy (FT-IR), after five washing cycles according with the international standard EN ISO 105-C06:2010.

Functionalized cotton presented high microbial killing rates, exhibiting inhibition ratios in the order of magnitude of 89 and 83% against the tested strains *K. pneumoniae* and *S. aureus*, respectively.

Thereby we report hereby a new process to obtain cotton textile material with strong antimicrobial properties with great potential to be used in several applications, especially in a health care context.

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APPENDIX

Table A.1. Full EDX

	Cotton – 5% L-Cys		
	Elements [C(atom)]		
	O	**S**	C
BL_5%	44.31	**0**	55.54
0% CDI	44.85	**0.15**	54.94
4% CDI	49.23	**0.32**	50.45
40% CDI	45.43	**0.12**	54.44
60% CDI	42.34	**0.17**	57.49

	Cotton – 7.5% L-Cys		
	Elements [C(atom)]		
	O	**S**	C
BL	46.27	**0**	53.52
0% CDI	48.63	**0.22**	51.14
4% CDI	49.70	**0.44**	49.87
40% CDI	45.75	**0.30**	53.94
60% CDI	37.64	**0.10**	62.26

Table A.2. Suspension Tables

Klebsiella pneumoniae – 24 h		
Sample	Average CFU/mL	% Inhibition
Cotton-control	2.45×10^8	---------------------------------------
5% L-Cysteine		
0% CDI	1.50×10^8	38.78%
4% CDI	2.60×10^7	89.39%
40% CDI	8.95×10^7	63.47%
60% CDI	9.70×10^7	60.41%
7.5% L-Cysteine		
4% CDI	3.05×10^7	87.55%
40% CDI	3.20×10^8	69.38%
60% CDI	6.60×10^7	73.06%

Staphylococcus aureus – 24 h		
Sample	Average CFU/mL	% Inhibition
Cotton	7.55×10^8	---------------------------------------
5% L-Cysteine		
4% CDI	1.27×10^8	83.21%
40% CDI	3.05×10^8	59.60%
60% CDI	3.65×10^8	51.66%
7.5% L-Cysteine		
4% CDI	1.27×10^8	83.18%
40% CDI	4.20×10^8	44.37%
60% CDI	2.05×10^8	72.85%

	Average CFU/mL_24 h
S. aureus	2.11E+08
K. pneumoniae	6.48E+07

Paper III

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Antimicrobial and antioxidant surface modification toward a new silk-fibroin (SF)-L-Cysteine material for skin disease management

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ABSTRACT

A novel dressing material – silk fibroin fabric (SF)-L-Cysteine (L-Cys) – is here developed to be used as standard treatment for atopic dermatitis (AD), which combines comfort, thermic, and tensile strength properties of silk materials with antioxidant and antimicrobial effects of L-Cys. A careful understanding about the linking strategies is needed in order not to compromise the bioavailability of L-Cys and deplenish its bioactivity. Durability was also addressed through washing cycles and compared with hospital requirements, according to international Standard EN ISO 105-C06:2010. The present research also analyze the interactions between *Staphylococcus aureus* and SF-L-Cys under simulating conditions of AD and demonstrated the effectiveness of a double covalent grafting, with the importance of SF tyrosine (Tyr) covalent linkage with L-Cys (SF-g-L-Cys/Tyr-g-L-Cys) even after several washing cycles, twenty five, whereas for a disposable application a single covalent mechanism of grafting L-Cys proved to be sufficient (SF-g-L-Cys). Results showed effective antimicrobial activities exhibiting higher inhibition ratios of 98.65% for SF-g-L-Cys after 5 washing cycles, whereas 97.55% for SF-g-L-Cys/Tyr-g-L-Cys after 25 washing cycles, both at pH 9.5 grafting strategy. Furthermore, it is also reported a non-protumoral effect of L-Cys. A new advance is herein achieved at the world of medical antimicrobial textiles tailored to address wound moisture environment and exudate self-cleaning, which may open novel applications as complementary therapy for AD disease.

1. Introduction

Silk, produced by silkworm *Bombyx morii*, consists of fibroin and sericin. Fibroin, which is the structural protein of silk fibers, comes out from the silkworm gland in the form of filaments enclosed in a water-soluble sericin gluelike coating [1]. Silk fibroin (SF) is composed of heavy (350 kDa) and light chain (25 kDa) polypeptides linked by a disulfide bond [2,3]. Its structure is mainly composed by the aminoacids glycine (46%), alanine (29%), and serine (12%), which are aminoacids having small side chains. Also, SF presents tyrosine (4.8%), which has an aromatic side chain [4]. They form flexible and stretching resistant secondary structure beta-sheets, which render silk its characteristic mechanical strength [5–7]. Silk is non-toxic, non-immunogenic, biocompatible, and hydrophilic. Although it is stated that the itching of AD is exacerbated by spiky fibers of wool and by the wild texture of cotton [8,9], silk, on the other hand, with its extremely smooth and its comfortable properties reduces these effects and therefore has been increasingly researched for applications related to atopic dermatitis (AD). Furthermore, Sugihara et al. [10] postulated that wounds treated with a silk dressing healed 7 days faster, by promoting the enhanced synthesis of collagen and epithelialization [10].

AD, also known as eczema, is a chronic and recurrent inflammatory pruritic skin disorder which causes lichenification, papules, and excoriations. AD has been increasing over the past decades, reaching prevalence of about 20% in children and 3% in adults worldwide [11]. A combination of genetic, environmental, and immunological factors may be the main causes for the development of AD, whose consequences can be psychologically traumatic, leading to emotional stress and decreasing substantially the quality of life. AD is characterized by a combination of T-helper type (Th) 2 immune activation, skin infection/colonization by *Staphylococcus aureus*, and skin barrier dysfunction. AD prevalence rise may be explained by today's lifestyle and nutrition [11]. The cleaner environment in upbringing as well as antibiotics abusing during early childhood has induced more often Th 2 immune responses,

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which are related to allergic responses, instead of Th 1 immune responses, which respond to infections [12]. Likewise, immune system responds to superantigens of cell walls of *S. aureus* inducing IgE allergic response [13]. After an AD trauma, an uncontrolled rising in body temperature and dehydration can lead to heart failure and death [14]. AD is a 2-step pathogenesis. First an allergic antigen-antibody (IgE) reaction takes place, and then a microbial infection takes over and maintains or exacerbates the disease [15]. AD patients often have more *S. aureus* ratio on the skin, diseased and normal, when comparing to non-diseased individuals. Furthermore, wounded skin can be colonized up to 1000× more *S. aureus* when compared to normal skin [16].

S. aureus is surrounded by a capsule that inhibits phagocytosis by polimorphonuclear leukocytes, and a slime layer composed of monosaccharides, peptides, and proteins, which allow its attachment to different tissue receptors and other external devices, such as catheters, suture and prosthesis. Also, *S. aureus* produces a variety of toxins that work as virulent factors [17]. Clinical diseases are triggered by *S. aureus* producing toxins or direct invasion and destruction of tissues. Notwithstanding that clinical manifestations of disease rely almost exclusively on toxin activity, in minor situations it can also be due to bacterial proliferation that forms abscess and tissue destruction. It also presents resistance against the currently used antimicrobials [18].

In the present work we have chosen L-Cysteine (L-Cys), which is a α-amino acid with a thiol group at the end of its variable side group, which is nucleophilic and has a wide spectrum of bioactivity [19], relying its activity on metabolic disturbance and membrane depolarization [20]. It targets the bacterial membrane and causes a great decrease in enzymatic activity and bacterial metabolism. Owing to its ability to form conjugates with free radicals or trace elements [21], L-Cys may also render our final application antioxidant properties.

This research work consisted on the development of two processes that allowed the covalent grafting of L-Cys onto SF: (i) functionalization of SF carboxylates (degraded amide groups) and cross-linking with the amine compounds of L-Cys; (ii) functionalization of SF Tyr with L-Cys, with the formation of Schiff base intermediates that were chemically stabilized by reductive amination.

This allowed to draw important conclusions regarding the best way to pursuit for a disposable application, as well as when designed for a multi-patient with laundry-resistant capabilities (after 25 washing cycles).

2. Materials and methods

2.1. Materials

Silk was obtained from the cocoons of larvae of the silkworm *B. morii* (Brazil). Silk was knitted at a rectilinear needle loom with 7 Picks per inch, with a Jersey knit structure and a linear density of 21.68 number metric (Nm). Its sericin was removed with dichloromethane in a soxhlet apparatus. Silk fibroin (SF) was then functionalized in a L-Cysteine (L-Cys) (Sigma–Aldrich) solution (ddH$_2$O) at pH's 5.5 and 9.5. Covalent bonds at tyrosine (Tyr) were performed with NaCNBH$_3$ dissolved in NaOH:HCl buffers (Panreac). *S. aureus* (ATCC 6538) strain was grown on Nutrient Agar (NA) and Nutrient Broth (NB) (Panreac).

2.2. Methods

2.2.1. Minimal inhibitory concentration (MIC) evaluation

Minimal inhibitory concentration (MIC) determination was performed with the microdilution method in accordance with M07-A6 guidelines applied to *S. aureus* (ATCC 6538) – Standard JIS L 1902:2002. Briefly, L-Cys was serial diluted in Mueller-Hinton Broth (MHB) (Sigma–Aldrich) to yield concentrations from 0.3 to 20 mg/mL. Overnight liquid *S. aureus* cultures were turbidity-adjusted to 0.5 McFarland (1×10^7 CFU/mL for *S. aureus*) with sterile H$_2$O, from which 500 μL were blended with 4500 μL MHB, forming the bacterial work suspension. 50 μL of work suspensions – L-Cys dilutions and stock solution – were added into 96-multi well plate. Samples were incubated for 24 h at 37 °C. Deposited microorganisms (dot-shaped) in the bottom of each well were examined. The last well in the dilution series that showed deposit (microbial killing) corresponded to MIC of the antimicrobial agent. Triplicates were used.

2.2.2. Antioxidant activity: determination of DPPH (2,2-diphenyl-1-picrylydrazyl) radical scavenging activity

The antioxidant activity of L-Cys was determined by the method of radical scavenging activity using DPPH$^{\circ}$. DPPH$^{\circ}$ is stable nitrogen radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH$^{\circ}$ bears a deep purple color, but over the course of the reaction becomes colorless, when the odd electron of its nitrogen atom is reduced by receiving a hydrogen atom from the antioxidant testing compound L-Cys. The DPPH$^{\circ}$ assay was adapted according to the procedure described by [22]. Briefly, the stock solution was prepared by dissolving 4 mg DPPH with 100 mL methanol. The percentage of remaining DPPH$^{\circ}$ for each concentration of L-Cys was determined using the equation of the obtained calibration curve and absorbance values measured at 517 nm, at 30 min time point:

$$\%\mathrm{DPPH}^{\circ}{}_{\mathrm{REM}} = [\mathrm{DPPH}^{\circ}]_{\mathrm{T}}/[\mathrm{DPPH}^{\circ}]_{\mathrm{T=0}} \times 100\% \qquad (1)$$

Efficient concentration (EC$_{50}$ μg/mL) was defined as substrate concentration to produce 50% reduction of the DPPH$^{\circ}$.

2.2.3. Tumoral activity assay

The cytotoxicity of L-Cys was performed, adapting the procedures from Standard E DIN EN ISO 10993-5. It was selected the concentration of L-Cys chosen for this work, 1%, and its half 0.5%, on breast cancer cell line (NCI-PBCF-HTB22 (ATCC® HTB-22$^{\mathrm{TM}}$)). Incubation was done at 37 °C for 48 h under a humidified atmosphere containing 5% CO$_2$. MTT viability assay was performed. Solvent, positive and negative controls were included.

2.2.4. Silk substrate preparation

2.2.4.1. Silk material. 20 g of SF knitted fabric was boiled for 4 h in dichloromethane in a soxhlet apparatus to extract residual lipids, and then rinsed thoroughly with distilled water. It was then washed according to BS 4923: 1991 – methods for individual domestic washing and drying for use in textile testing, in a 4 g/L solution of ECE Reference Detergent (the formulation of the detergent is given in BS 1006:CO6 (ISO 105:CO6)) for 60 min at 40 °C, rinsed, and dried afterwards at 30 °C until used, in order to remove impurities and soils.

2.2.4.2. Grafting/functionalization processes. SF knitted fabric samples were functionalized by means of an exhaustion process at 90 °C, 15 rpm, in a Mathis-BFA12 finishing machine. Single covalent strategy (SF-g-L-Cys): samples were immersed in a 1% over the weight of the fiber (owf) L-Cys solution (ddH$_2$O) adjusted to pH's 5.5 and 9.5, for 3 h. Fig. 1 A. Double covalent strategy (SF-g-L-Cys/Tyr-g-L-Cys): samples were immersed in a 1% over the weight of the fiber (owf) L-Cys solution (ddH$_2$O) adjusted to pH's 5.5 and 9.5, for 3 h, and were added 0.3 (w/V) NaCNBH$_3$ solution from the beginning of second hour. Samples were rinsed three times with ddH$_2$O and dried at 30 °C (Fig. 1B).

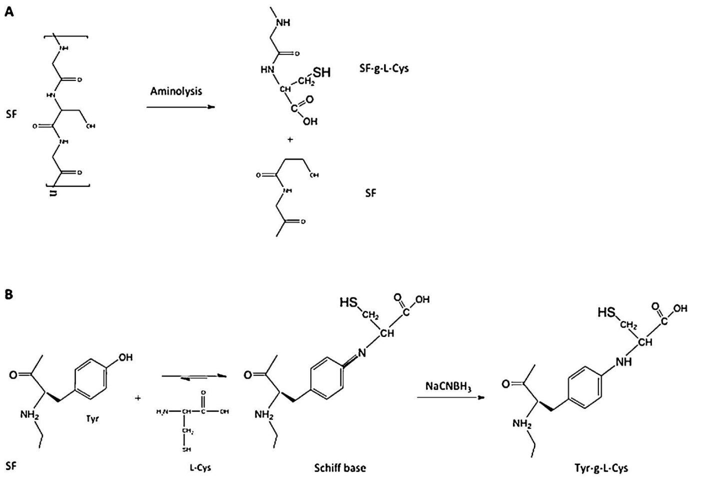

Fig. 1. Strategies for the activation of silk fibroin. (A) SF-g-L-Cys; (B) SF-g-L-Cys/Tyr-g-L-Cys.

Firstly, SF knitted fabric samples were functionalized on its carboxylates (degraded amide groups) and were cross-linked with the amine compounds present in L-Cys solution (SF-g-L-Cys). Secondly, SF knitted fabric samples were functionalized on its Tyr ketone groups, with the formation of Schiff base intermediates that were chemically stabilized by reductive amination, forming ultimately a covalent bond (SF-g-L-Cys/Tyr-g-L-Cys).

2.2.4.3. Washing cycles assay – resistance to laundry. Samples were (i) washed in a 4 g/L solution of AATCC 1993 Standard Reference Detergent WOB for 5 washing cycles performed at 40 °C during 30 min each, and (ii) washed in a 4 g/L solution of AATCC 1993 Standard Reference Detergent WOB for 25 washing cycles performed at 40 °C during 30 min each. These methods were adapted from the international standard EN ISO 105-C06:2010. Washing cycles were done prior to all studies in order to assess the resistance to washing of L-Cys bonds on SF surface.

2.2.5. Grafted-silk characterization

SF samples were characterized using different surface analyzing techniques against appropriate controls.

2.2.5.1. Contact angle. Contact angles were measured using an OCAH-200 set-up by Dataphysics equipped with a high-speed video system with CCD video camera with a resolution of 752 × 484.

2.2.5.2. Fourier transform infrared spectroscopy (FT-IR). The chemical composition of SF, SF-g-L-Cys, and SF-g-L-Cys/Tyr-g-L-Cys was analyzed by means of FT-IR. After three rinses (no washing cycles), 5 washing cycles, and 25 washing cycles FT-IR spectra were analyzed. 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}.

2.2.5.3. Exhaustion rates: degree of functionalization. In order to assess the efficiency of the functionalization, i.e. the quantity of L-Cys that the substrate SF was able to crosslink, exhaustion rate of functionalization assays were performed for each condition. Ellman's reagent, (5,5′-dithio-bis-(2-nitrobenzoic acid/DTNB) (Sigma) produces a yellow color when bonded to free thiol groups in solution. After reacting with free thiol groups (of L-Cys) there is formation of a dissulphydric compound and a colored species – 2-nitro-5-thiobenzoic acid (TNB). This colorimetric assay was performed by measuring and comparing the functionalization solution before and after functionalization, after doing a calibration with known concentrations of L-Cys. For each sample 250 μL was added to a test tube containing 2.5 mL of sodium phosphate (pH 8) and 50 μL of Ellman's reagent. Test tubes were vortexed and were incubated for 15 minutes before their absorbance being read at 412 nm.

2.2.5.4. Tensile strength assays. In order to evaluate the degradation caused on SF by the functionalization processes, several quality control assays were performed. Resistance to rupture assays were done in all samples, with and without L-Cys, to subsequent comparison. Assays were performed on a Dynamometer, according to Standard EN ISO 2062, at 20 ± 2 °C, under 60% relative humidity. Samples were strip-cut 5 cm × 50 cm, also string cut, and placed between dynamometer tweezers. A defined pre-tension was set in the beginning, and the test ended up with samples rupture.

2.2.6. Antimicrobial activity assessment

The antimicrobial behavior of SF samples functionalized with L-Cys (-g-L-Cys and -g-L-Cys/Tyr-g-L-Cys) was tested according to Japanese Industrial Standard JIS L 1902:2002, specific for textile samples. Gram-positive *S. aureus* (ATCC 6538) strain was chosen once it plays an important role in atopic dermatitis (AD). Briefly, inoculum of *S. aureus* was prepared with a final concentration of 1–5 × 10^6, bacteria/mL. 200 μL of *S. aureus* were inoculated on 0.4 g square samples. Samples were incubated for 24 h. Antimicrobial activity was assessed by a quantitative method to determine the percentage of bacterial growth inhibition (% of Inhibition) at 24 hours:

$$\%\text{Inhibition} = \frac{C - A}{C} \times 100$$

where *C* represents the average value of Colony Forming Units (CFU-μg/mL) of non-modified SF, and A represents the average value of CFU of grafted SF.

Table 1
Concentration of L-Cys versus % of remaining [DPPH].

Concentration µg/mL	% of remaining [DPPH]
0	100
0.05	8.459185758
0.04	47.30720708
0.03	48.37492768
0.02	76.5334419
0.1	4.770788268
EC50	0.029957282

2.2.7. Scanning electron microscopy (SEM)

Adsorbed bacteria were reticulated with 1.5% glutaraldehyde overnight at 4 °C. Samples were then dehydrated with an increasing ethanol/water gradient (50–99%, v/v), and subjected to critical point drying (K850, EMITECH). After being sputtered with a thin layer of gold, samples were examined by SEM (Hitachi S2700).

Images were formed through secondary electrons using a high voltage of 20 kV. Five fields per sample were obtained using a magnification of 20 000 and 75 000×.

2.2.8. Transmission electron microscopy (TEM)

The morphology of adhered *S. aureus* on SF knitted fabric functionalized with L-Cys (-g-L-Cys and -g-L-Cys/Tyr-g-L-Cys) was addressed by TEM. Bacteria were stained with 2% uranyl acetate for 5 min, and mounted on a mesh with thin bar copper grids covered with formvar. Each sample was examined at 30 000× magnification using HITACHI HT7700 TEM at an accelerating voltage of 80 kV.

2.2.9. Statistical analysis

SPSS Statistics 21.0 software was used to perform the statistical analysis of the data using One-Way ANOVA, with Tukey as Post Hoc tests for establishing multiple comparisons between samples in this study. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Minimal inhibitory concentration (MIC) of L-Cys

Minimal inhibitory concentration (MIC) of L-Cysteine (L-Cys) against *S. aureus* was found to be 5 mg/mL.

3.2. Antioxidant activity

3.2.1. Determination of DPPH (2,2-diphenyl-1-picrylydrazyl) radical scavenging activity

The reduction capability of the DPPH$^{\circ}$ radical was determined by the decrease on its absorbance at 517 nm, induced by Cysteine. Table 1 shows that as the concentration of Cysteine increased, the percentage of remaining DPPH$^{\circ}$ diminished.

The percentage of remaining DPPH$^{\circ}$ was proportional to the antioxidant concentration of L-Cys. The concentration that caused a decrease in the initial DPPH$^{\circ}$ concentration by 50% (EC50) was found to be 0.029957 µg/mL, which implies that L-Cysteine have antioxidant activity.

3.3. Tumoral activity assay

Soluble L-Cys did not show any effect on breast cancer cell line MCF-7. Values were not significantly different from control, with 4% and 9% of growth above at doses of 1 and 0.5 mg/mL L-Cys, respectively (Fig. 2). Only an alteration above 30% in comparison with controls is considered pro-tumorigenic [23].

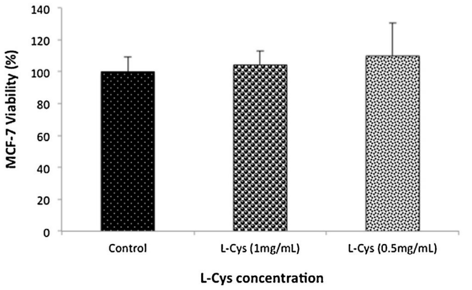

Fig. 2. MCF-7 breast cancer cell line viability percentage when in contact with two concentrations of L-Cys and the untreated controls.

3.4. Silk characterization

3.4.1. Contact angle

The wettability of silk fibroin (SF) was assessed by the measurement of the contact angle between the surface and water using the sessile drop technique. Water contact angles measured at 10 s showed a hydrophilic SF surface; 21.1 ± 1.5°. Therefore grafted SF samples proved to be able to absorb exudates and provide wound moisture, which render them usable as healing wound dressings against AD.

3.4.2. Fourier transform infrared spectroscopy (FT-IR)

The infrared spectra of unmodified SF is shown in Fig. 3a. Peaks at 3300 cm^{-1} and 3100 cm^{-1} are associated with amides A and B, respectively. At 1650 cm^{-1} vibrations are due to C=O stretching vibrations of amide I, and the peak at 1520 cm^{-1} is related to N—H in plane bending together with the out-of-phase C—N stretching of amide II.

SF covalent structural modification after 25 washing cycles was also assessed by FT-IR. IR spectra confirmed L-Cys bonded on SF surface, as shown by the increase of peaks associated with L-Cys immobilization on a surface, around 3300, 3100 and 1650 cm^{-1}, as shown in Fig. 3B. Amide I is related to vibrations of the peptide bonds. However, the peak at 1520 cm^{-1} related to amide II diminishes, which tells the information of SF protein secondary structure modified/unfolding based on the extent of L-Cys cross-linking to SF. Furthermore 3300 cm^{-1} is also associated with OH stretching related to free —OH in SF aromatic tyrosine [24]. When covalent bond formed at Tyr residue the vibration assigned to —OH decreased. This might be the reason why the sample SF-g-L-Cys/Tyr-g-L-Cys at pH 9.5 90 °C (spectrum e) showed significant decrease of the absorption band at 3300 cm^{-1}.

3.4.3. Efficiency of functionalization/grafting

The exhaustion efficiency of L-Cys on SF fabric was assessed after 3 h of functionalization at 90 °C. All samples gave rise to figures of over 80% exhaustion rate, once L-Cys readily adsorbed to SF chemistry. SF-g-L-Cys/Tyr-g-L-Cys double covalent bonding showed 80.35% (pH 5.5) and 80.71% (pH 9.5), whereas SF-g-L-Cys single covalent bonding rates were 87.09% (pH 5.5) and 85.94% (pH 9.5).

3.4.4. Tensile strength assays

Tensile tests were done to evaluate the effect of single covalent and double covalent treatments on the mechanical properties of samples, and are shown in Table 2.

A worthy maximum stress and elongation were observed for SF thread and strip at different conditions of functionalization; Results

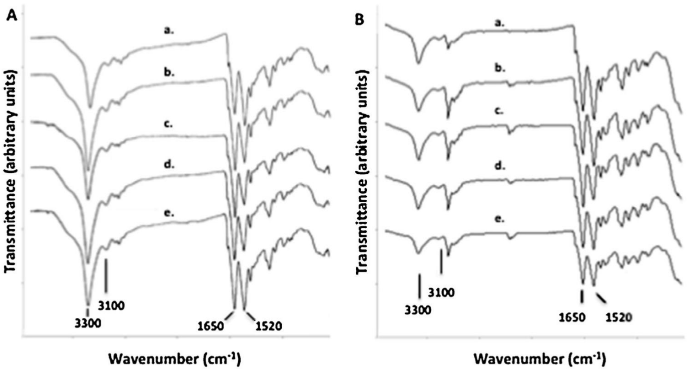

Fig. 3. IR spectra of unmodified (a) and modified silk fibroin with different parameters of surface-activation after (A) 0 washing-cycles and (B) 25 washing-cycles, with 1% owf L-Cys functionalization. (b) SF-g-L-Cys at pH 5.5 90 °C; (c) SF-g-L-Cys at pH 9.5 90 °C; (d) SF-g-L-Cys/Tyr-g-L-Cys at pH 5.5 90 °C; (e) SF-g-L-Cys/Tyr-g-L-Cys at pH 9.5 90 °C.

Table 2
Mechanical characterization. Resistance to rupture assays in thread and strip.

	Material	Stress at break (Newton)	Elongation (%)
Thread	SF	31.08 ± 4.06	15.37 ± 4.15
	pH 5.5 SF-g-L-Cys	28.59 ± 4.28	13.69 ± 3.45
	pH 5.5 SF-g-L-Cys/Tyr-g-L-Cys	27.10 ± 5.83	13.99 ± 4.16
	pH 9.5 SF-g-L-Cys	33.69 ± 0.36	18.14 ± 0.02
	pH 9.5 SF-g-L-Cys/Tyr-g-L-Cys	31.15 ± 12.7	16.49 ± 6.72
Strip	SF	620.7 ± 54.9	57.49 ± 4.75
	pH 5.5 SF-g-L-Cys	640.6 ± 55.2	59.15 ± 4.47
	pH 5.5 SF-g-L-Cys/Tyr-g-L-Cys	486.6 ± 80.5	55.25 ± 4.37
	pH 9.5 SF-g-L-Cys	566.0 ± 46.3	58.02 ± 8.39
	pH 9.5 SF-g-L-Cys/Tyr-g-L-Cys	709.3 ± 52.2	64.13 ± 6.24

were not significantly different from controls, which proves the covalent modifications set up in this work did not compromise SF.

3.5. *Antimicrobial assays*

3.5.1. *Quantitative method (suspension)*

SF activated surfaces showed good microbial inhibition results, as shown in Fig. 4. Following three rinses after functionalization, no significant differences ($p < 0.05$) between covalent/double covalent treatments could be seen regarding microbial killing, except that all of them were highly microbicidal. After 24 hours of incubation, results of microbial reduction were close to 100%.

After 5 washing cycles, SF-g-L-Cys treatment at pH 9.5 showed to be significantly ($p < 0.05$) more microbicidal for *S. aureus* – 98.65% – against 89.33% of SF-g-L-Cys pH 5.5. Also, SF-g-L-Cys/Tyr-g-L-Cys modifications were 77.38% for functionalization at pH 9.5, and 78.40% for pH 5.5.

On the other hand, after 25 washing cycles SF-g-L-Cys/Tyr-g-L-Cys double covalent modification proved to be worthwhile, once it showed such a great amount of microbial reduction, which proved to form bonds/structural conformations strong enough to resist to several washings. *S. aureus* when adsorbed to SF-g-L-Cys/Tyr-g-L-Cys, modified under pH 9.5 with 1% owf L-Cys, showed a significant CFU reduction of 97.55% ($p < 0.05$), whereas when adsorbed to SF-g-L-Cys bacterial reduction was not so prominent, with results averaging 67.39%.

3.6. *Scanning electron microscopy (SEM)*

For any condition after 25 washing cycles, there was a decrease in the number of *S. aureus* adsorbed. However, for SF-g-L-Cys/Tyr-g-L-Cys functionalized at pH 9.5, an outstanding killing effect showed almost no bacteria adsorbed to fabric surface (Fig. 5).

3.7. *Transmission electron microscopy (TEM)*

Following 25 washing cycles, Fig. 6 shows that there was a fierce degradation of *S. aureus* membranes, when adhered to SF-g-L-Cys/Tyr-g-L-Cys, especially at pH 9.5 (Fig. 6e). Considering the effect of SF-g-L-Cys/Tyr-g-L-Cys modified at pH 5.5 (Fig. 6c), it showed adhered bacteria with their membranes slightly harmed, when comparing to controls.

4. Discussion

This work compared single covalent against double covalent immobilization of a bioactive agent L-Cysteine (L-Cys) on silk fibroin (SF) knitted fabric to originate an effective antimicrobial wound dressing (bioactive material) against atopic dermatitis (AD) disease. Furthermore, end applications of laundry resistance versus disposibility, are herein discussed.

L-Cys amino acid, which has a wide spectrum of microbial activity and well-known safety [10–12], was for the first time functionalized at its lowest concentration – 1% over the weight of the fabric (wof). In previous works the minimum amount used with efficiency of microbial killing was 5% owf, in combination with either wool [23], or cotton [10,12]. This L-Cys fall is understandable, in that recently new approaches relying on removal of lipids from the surface of fibers have exposed their surface with a variety of reactive moieties. In this sense, covalent treatments can be directed straightforward to these reactive sites, rather than physically-directed, which offers higher durability and resistance to laundry [25].

For L-Cys immobilization onto SF fabric two processes were considered in order to potentiate its activity. The first process relied on modification of 87% of SF structure, namely the covalent bonding on glycine, alanine, and serine amino acids. L-Cys was blended with SF at both low and high pH's. The strong acid HCl plus heat (90 °C), as well as strong base NaOH plus heat (90 °C), made SF amides hydrolize to carboxylic acids through reverse of Fischer

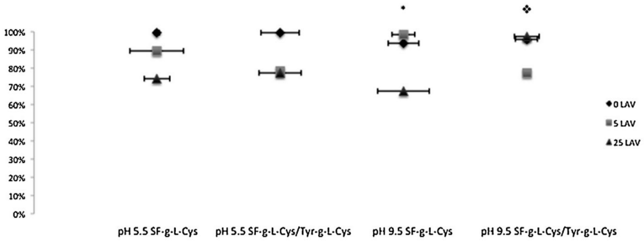

Fig. 4. Percentage of microbial inhibition against *S. aureus* at 24 h – all of these values were obtained by comparison with results of non-functionalized silk fibroin. * statistically significant for 5 washing cycles. ❖ statistically significant for 25 washing cycles ($p < 0.05$).

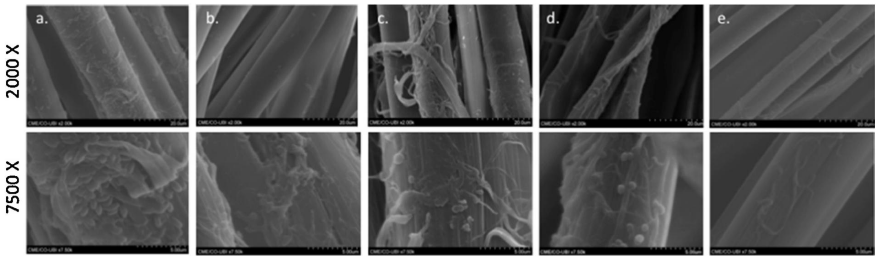

Fig. 5. SEM images of adsorbed *S. aureus* on silk fibroin surface at different conditions. (a) Control; (b) SF-g-L-Cys at pH 5.5 90 °C; (c) SF-g-L-Cys/Tyr-g-L-Cys at pH 5.5 90 °C; (d) SF-g-L-Cys at pH 9.5 90 °C; (e) SF-g-L-Cys/Tyr-g-L-Cys at pH 9.5 90 °C.

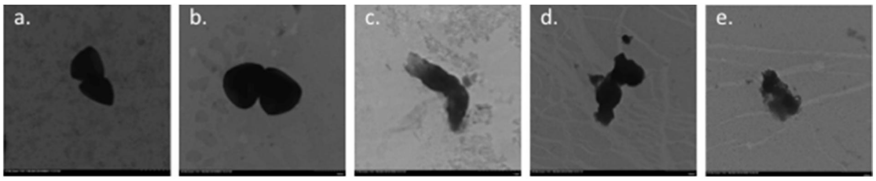

Fig. 6. TEM images of detached *S. aureus* on silk fibroin surface at different conditions. Magnification of 10 000×. (a) Control; (b) SF-g-L-Cys at pH 5.5 90 °C; (c) SF-g-L-Cys/Tyr-g-L-Cys at pH 5.5 90 °C; (d) SF-g-L-Cys at pH 9.5 90 °C; (e) SF-g-L-Cys/Tyr-g-L-Cys at pH 9.5 90 °C.

esterification and "saponification", respectively. Also, the presence of further carboxylates in SF is also described in literature [26]. Amino groups of L-Cys were then covalently linked by reacting with these carboxylic acids by aminolysis (SF-g-L-Cys). The second process relied on covalent modification of further 4.8% of SF structural tyrosine aromatic side chain. Aldehydes of tyrosine side chains were blended with the amines of L-Cys, forming unstable Schiff base intermediates (or imines). These intermediates were chemically stabilized by reductive amination with $NaCNBH_3$ [27], forming ultimately a covalent bond. In the first process, Schiff base intermediates also formed at these sites but reverted, once they were not stabilized by reduction. Furthermore, in this process of grafting L-Cys on Tyr, important issues as Tyr localization, and its influence on backbone conformation and packing were regarded [4]. L-Cys carries a thiolate group in its molecular structure which kills microorganisms through disulfide bridge establishment with some bacterial wall structural components [28] or even intracellular components if internalized.

The ability of L-Cys to inhibit the growth of microorganisms in solution was given by Minimal Inhibitory Concentration (MIC) studies of soluble L-Cys against *S. aureus* which was 5 mg/mL. This value is high when compared with chitosan polymer (0.05 mg/mL) and, on the other side, lower than triclosan (6 mg/mL) [29,30]. When we compare these former MIC results with regard of settling the same molecule (L-Cys) on a surface, data demonstrated immobilization advantageous with the requirement of lower amount of L-Cys, namely 1% owf (1% over the weight of the fiber (owf) in a ratio of 1 g fiber/50 mL solution; thus, 0.2 mg/mL). L-Cys molecules in solution bind and gather each other through S-S bonds, losing their main reactive thiolates. On the other side, immobilizing L-Cys onto a surface prevented its agglomeration and allowed the use of lesser quantity to coat the surface, in order to grant SF fibers antibacterial activity.

It has already been demonstrated that topical application of antioxidants decreased wound healing time [31]. The total antioxidant capacity of L-Cys was assessed by using spectrophotometric methods with DPPH° (2,2-diphenyl-1-picrylydrazyl) [22]. The L-Cys concentration that caused a decrease in the initial DPPH° by 50% (EC_{50}) was found to be 0,029957 μg/mL. These results showed that L-Cys has antioxidant activity. Sánchez-Moreno [32] showed EC50 values of a few other antioxidants, as gallic acid (26 μg/mL), tannic (59 μg/mL), caffeic acid (69 μg/mL), ascorbic acid (76 μg/mL), queratin (84 μg/mL), BHA (93 μg/mL) and rutin (102 μg/mL). These values show that albeit L-Cys is antioxidant, its antioxidant power is low but can be increased by rising the L-Cys concentration, if necessary (customized).

The concentration of L-Cys that was used for SF functionalization did not interfere with tumoral cells growth rate, which proves the safety of using this application in neoplastic patients. Furthermore, L-Cys has already been studied for its cytotoxicity against fibroblasts, proving to be safe, hence not cytotoxic [20].

SF hydrophilic surface, with a contact angle of 21.1 ± 1.5°, besides absorbing AD wound exudate and providing wound hydration which opens the way to important factors for proper healing, showed to attract *S. aureus* present in an AD disease environment, allowing *S. aureus* to contact and be eliminated by the grafted L-Cys. J. Ji and W. Zhang stated that a hydrophilic surface attracted *S. aureus* [33] whereas H. Yang and Y. Deng indicated that a hydrophobic surface reduced its adhesion [34].

The formation of silk structure is driven by Tyr residues, which have a strong effect not only locally but also long-range intermolecular interactions [4]. Upon its double functionalization with L-Cys, SF structure might have adopted such an altered folding due to covalent bonding at Tyr residue. This might be the reason why the sample SF-g-L-Cys/Tyr-g-L-Cys functionalized at pH 9.5 90 °C showed significant decrease of the FT-IR absorption band at 3300 cm^{-1}. Nevertheless, it is possible to conclude that the diffusing capacity of L-Cys in the fiber was high, >80%, which means L-Cys has the ability to bind covalently to the fiber surface easily and strongly. Also, high exhaustion rates meant a low quantity of L-Cys required to achieve an effective antimicrobial activity.

Accordingly, FT-IR results confirmed that L-Cys was well immobilized to SF fibers at both pH's of functionalization. Furthermore, as washing cycles were performed, effects at 5 and 25 washing cycles showed SF-g-L-Cys/Tyr-g-L-Cys grafting to be multiple washing resistant along with SF-g-L-Cys grafting at pH 9.5. In accordance with the chemical surface analyses of SF fiber surfaces, microbiologic data after 24 h of incubation, showed for 5 washing cycles the highest killing of bacteria (98.65%) happened at pH 9.5 through L-Cys grafted on a single covalent way (SF-g-L-Cys) ($p < 0.05$). This high microbicidal reduction might be due to the structural/conformational accessibility of L-Cys on SF when comparing to SF-g-L-Cys/Tyr-g-L-Cys. Furthermore, after 25 washing cycles the optimal bacterial killing effect (97.55%) happened at pH 9.5 through double covalent grafting SF-g-L-Cys/Tyr-g-L-Cys ($p < 0.05$). This high microbicidal reduction might be related to the exposing of erstwhile structural folding-protected L-Cys due to multiple washing cycles. A qualitative analysis was also performed through Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) by characterizing *S. aureus* according to its shape when adsorbed on SF surface after it had been washed 25 times (25 washing cycles). Data demonstrated that few bacteria adhered to the background fibers and the ones adhered were in a bad shape/morphology at the condition of SF-g-L-Cys/Tyr-g-L-Cys functionalized at pH 9.5. As expected, acidic conditions of functionalization yielded poorer grafting of L-Cys on SF fibers. On the other hand, according to Hornsey and Pepper [35] the higher pH condition of functionalization led to higher effectiveness of grafting [35] once protonated amines of L-Cys were more attracted to the SF negative carboxylate groups when under the alkaline pH 9.5. Also, SF high exhaustion of L-Cys is due to its structural reactivity, which translated in higher retention after several washing cycles, leading to increased antimicrobial properties of SF fibers, when compared to cotton [20]. Mechanical properties of SF showed high, and were not significantly altered with any treatments in this study, which means SF structure never got compromised by used functionalization processes.

5. Conclusions

This research work describes both single covalent and double covalent grafting methods for the functionalization of silk fibroin (SF) with antibacterial and antioxidant agent L-Cysteine (L-Cys). The developed SF-L-Cys materials have the potential to inhibit medically relevant *S. aureus* bacterial species and as such to be used to control atopic dermatitis since no toxicity was found. Moreover, the durability of the fabric was significantly improved when the active antibacterial agent L-Cys was double covalently attached to SF tyrosine. It was demonstrated the effectiveness of a double covalent grafting, with the importance of SF tyrosine (Tyr) covalent linking with L-Cys (SF-g-L-Cys/Tyr-g-L-Cys) for several washing cycles, whereas for a disposable application a single covalent mechanism of grafting L-Cys proved to be sufficient (SF-g-L-Cys).

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Paper IV

Article

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New garment proposal for prevention of spreading Gram-negative bacteria resistant to carbapenem antibiotic class under hospital settings

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Abstract

Sensitive skin diseases, including atopic dermatitis, skin inflammation and bedsores, leave patients vulnerable under hospital setting. It is important for the development of a hospital gown with "soft hand" properties and at the same time as a protector against nosocomial infections. *Klebsiella pneumoniae* has developed resistance to antibiotics in the carbapenem antibiotic class, known as carbapenem-resistant *K. pneumoniae* (CRKP). CRKP is resistant to nearly all antibiotics and can kill up to 50% of infected patients.

This work consisted in the development of a washable recycled silk fibroin-based gown covalently linked with an amino acid L-Cysteine(L-Cys), focused on prevention of *K. pneumoniae* establishment, proliferation and spreading to community, for use under hospital settings. With the growing problem of resistance to antibiotics and few new therapies on the horizon, gowns adsorbed with L-Cys show to function as a barrier to the establishment and proliferation of microorganisms, providing user protection from infectious disease. This gown was knitted at a rectilinear needle loom with a Jersey knit

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structure. Then it was cross-linked with L-Cys, subjected to laundry, and subsequently characterized by energy-dispersive X-ray spectroscopy, Fourier transform infrared spectroscopy, contact angle, free energy of adhesion, scanning electron microscopy and transmission electron microscopy. Results presented a bactericidal effect against *K. pneumoniae* of 94.92% after three rinses and 88.88% after five washing cycles, with the few adhered bacteria with an altered and compromised morphology.

Keywords

L-Cysteine, antimicrobial-gown, nosocomial infections, aminolysis, *Klebsiella pneumoniae*

Introduction

The microbiota comprises all microorganisms usually found in healthy individuals. These microorganisms live on skin and mucosae of all persons after birth and are renewed until their death. Indigenous microbiota benefits host through early stimulation of immune system, prevention of colonization of pathogenic microorganisms, and synthesis of essential compounds (e.g. Vitamin K). On the other hand, various studies suggested that the development of potential pathogenic microorganisms upon alteration of local conditions, antimicrobial therapeutics, and immunosuppresion may happen [1]. Healthcare-associated infections, such as pneumonia, bloodstream infections, wound or surgical site infections, and meningitis can provide conditions for *Klebsiella pneumoniae* establishment and infection development [2]. *Klebsiella pneumoniae* that belongs to Enterobacteriaceae family makes up comensal flora and causes opportunistic infections. It is a Gram-negative rod, non-motile, and it is characterized by a prominent polysaccharide capsule, which is responsible for its mucoid appearance and virulence [3]. Aside from colonization of gastro-intestinal tract, it colonizes skin and nasopharynx. One-third of individuals carry *K. pneumoniae* in stool; however, this number increases to 90–100% in hospitalized individuals and/or the ones taking antibiotics on a regular basis, as well as children [4]. These complications are likely to occur in immunocompromised patients under hospital settings, as elderly, malnourished, bedridden or paralyzed, and individuals who have a diminished pulmonary function [4]. The misuse of antibiotics has increased antibiotic resistance. Jim O'Neill stated that if nothing was done, 10 million annual deaths by infections caused by resistant bacteria would happen in the world, with an associated cost of 90 billion euros by 2050. One of contributing factors to emergence of resistance includes its widespread use in animals [5]. Hence, *K. pneumoniae* presents a risk of conversion to carbapenem-resistant *K. pneumoniae* (CRKP), especially in hospital settings. CRKP has become resistant to essentially all antibiotics, and can kill up to 50% of infected patients [6] Moreover, when it becomes established, it proliferates and can spread rapidly throughout the community, through its fimbriae and polyssacharide capsule to adhere and cause virulence [7]. These events add urgency to develop new strategies at the preventive level rather than at the curative level with antibiotics.

Gowns are a type of underwear worn by inpatients and should be changed once a day. The microbial contamination and probability of infection with CRKP increase as the day goes on, which implies microbial movement or transfer from gown to rashes or blisters developed in skin-sensitive patients. A gown can absorb an amount of sweat and microorganisms from human body and environmental contaminants in hospital settings, which is the ideal growth environment for microbial growth and *K. pneumoniae* transformation to CRKP.

Silk fibroin (SF) is a non-toxic hydrophilic natural textile, tissue compatible, with high tensile strength, being a product already approved under Food and Drug Administration (FDA) for clinical applications. In order to develop a silk recycled waste-based gown, four dimensions were considered: (i) material, where consumers have preferences for innovative raw materials; (ii) ecological, stimulating the rapprochement between cultures and nature as well as the criterious usage of resources; (iii) economic, with the use of recycled silk waste of cocoons as a low-cost process; (iv) antimicrobial, preventing *K. pneumoniae* establishment and colonization [8,9]. As antibiotics are ineffective and even counterproductive in fighting drug-resistant *K. pneumoniae*, L-Cys amino acid proves promising, since no microbial resistance has been reported.

In this research, L-Cysteine (L-Cys) was used to covalently modify silk fibroin surface through aminolysis. L-Cys is a common α-amino acid coming from a variety of food sources, and its thiol group at the end of variable side group is nucleophilic and provides a wide spectrum of bioactivity [10]. L-Cys activity is based on metabolic disturbance and membrane depolarization [11]. L-Cys targets the bacterial membrane, decreases its enzymatic activity, and compromises bacterial metabolism.

The present research reports on the study of recycled SF gown that embodies antimicrobial properties. More specifically, its covalent linking with L-Cys shows laundry-resistance and prevents the establishment, spreading, and convertion of *K. pneumoniae* to CRKP under hospital settings.

Experimental

Materials

Bacteria cultivation: Klebsiella penumoniae (ATCC 4352) strain was cultivated on Nutrient Agar (NA) and was then grown on Nutrient Broth (NB) (Panreac).

Silk fibroin (SF) samples preparation: SF recycled waste was obtained from silkworm Bombyx morii (Brazil), Mn 30000, knitted at a rectilinear needle loom with seven needles per inch, with a Jersey knit structure and a linear density of 21.68 number metric (Nm). Its sericin was removed using a soxhlet apparatus with dichloromethane, as a standard procedure in our lab (Figure 1).

Covalent modification on SF samples surface was performed via aminolysis with 1% (w/V) L-Cysteine (L-Cys) (Sigma-Aldrich), for 3 h, under pH 9.5, at 90°C (Figure 2).

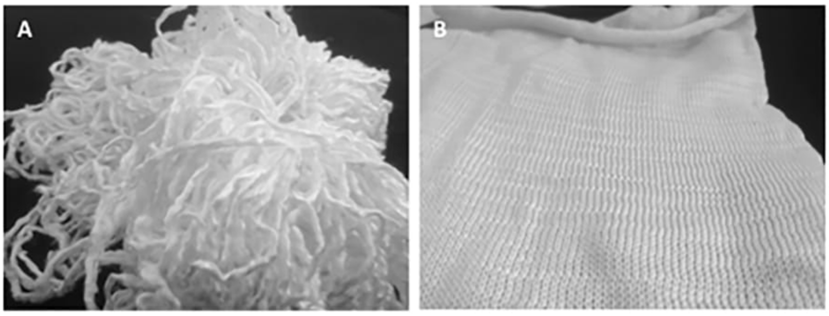

Figure 1. Silk recycled waste products (a) as raw materials for the development of Silk knitted structure (b).

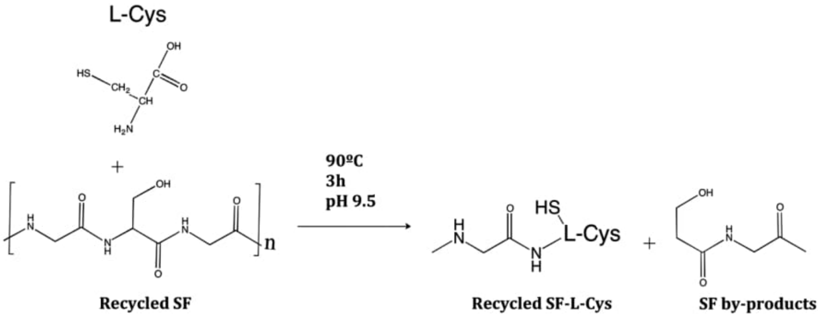

Figure 2. Schematic representation of the mechanism of covalent bonding of L-Cys on recycled SF (SF-L-Cys). Chemical structures were designed in CS ChemDraw Ultra.

Five washing cycles were done according to standard EN ISO 105-C06:2010, in a 4 g/L solution of AATCC 1993 Standard Reference Detergent WOB for 30 min each, at 40°C, rinsed, and dried afterwards at 30°C until used.

Methods

Minimal inhibitory concentration: The susceptibility of *K. pneumoniae* to L-Cys was performed with the microdilution method following M07-A6 guidelines applied to *K. pneumoniae* (ATCC 4352) – Standard JIS L 1902:2002. Briefly, a serial dilution of L-Cys was carried out in Mueller-Hinton Broth (MHB) (Sigma-Aldrich) to yield concentrations from 0.3 mg/mL to 20 mg/mL. Overnight liquid *K. pneumoniae* culture was adjusted to 0.5 McFarland (1×10^8 CFU/mL for *K. pneumoniae*) with sterile H_2O, from which 500 µL was added to 4500 µL MHB – bacterial work suspension. 50 µL of work suspension and L-Cys dilutions were added to wells in a 96-multi-well plate. Samples were incubated for 24 h at 37°C. A smear of

Table 1. Energy-dispersive X-ray spectroscopy.

Condition	C (Elements [C(atom)])	N (Elements [C(atom)])	O (Elements [C(atom)])	S (Elements [C(atom)])
A	46.48	21.69	31.83	0.00
B	47.33	21.31	31.29	0.07
C	47.89	21.12	30.92	0.07

Note: Control SF (A); SF-L-Cys after 0 washing cycles (B); SF-L-Cys after five washing cycles (C).

microorganisms in the bottom of wells demonstrate microbial killing and constitute the minimal inhibitory concentration (MIC). Triplicates were used.

Energy-dispersive X-ray spectroscopy: The sulphur of adsorbed L-Cys was quantified using an energy-dispersive X-ray spectroscopy (EDS) attachment of a HITACHI S 2700 scanning electron microscope, with a high voltage of 20 kV (Table 1).

Fourier Transform Infrared Spectroscopy: The chemical composition of native SF and SF functionalized with 1% L-Cys after five washing cycles was analyzed by Fourier transform infrared spectroscopy (FTIR) (Thermo-Nicolet is10). Each sample was scanned 64 times, with a spatial frequency resolution of 4 cm^{-1}.

Contact angle: Contact angles were measured with an OCAH-200 set-up device (Dataphysics) outfitted with a high-speed video system with CCD video camera with a resolution of 752 × 484.

Free energy of adhesion: The surface tension of SF samples was determined (equation (1)) by the sessile drop contact angle method at RT, for three different types of components: the apolar diiodomethane and both polar water and ethylene glycol, whose surface tension components have already been determined.

$$(1+\cos\theta)\gamma_{\mathrm{SF}}^{\mathrm{TOT}} = 2\left(\sqrt{\gamma_{\mathrm{S}}^{\mathrm{LW}}\gamma_{\mathrm{SF}}^{\mathrm{LW}}} + \sqrt{\gamma_{\mathrm{S}}^{+}\gamma_{\mathrm{SF}}^{-}} + \sqrt{\gamma_{\mathrm{S}}^{-}\gamma_{\mathrm{SF}}^{+}}\right) \tag{1}$$

where θ is the contact angle, and $\gamma^{\mathrm{TOT}} = \gamma^{\mathrm{LW}} + \gamma^{\mathrm{AB}}$. The parameter γ^{LW} corresponds to the Lifshitz-van der Waals component of the surface free energy, and γ^{+} and γ^{-} the electron acceptor and electron donor parameters, respectively, of the Lewis acid–base component γ^{AB}, being $\gamma^{\mathrm{AB}} = 2\sqrt{\gamma^{+}\gamma^{-}}$ [12].

The interaction between *K.* pneumoniae and SF was predicted in terms of total interaction energy, and it is represented in the equation 2 by KP and SF, respectively.

$$\Delta G_{\mathrm{Adhesion}}^{\mathrm{TOT}} = \left(\sqrt{\gamma_{\mathrm{KP}}^{\mathrm{LW}}} - \sqrt{\gamma_{\mathrm{SF}}^{\mathrm{LW}}}\right)^{2} - \left(\sqrt{\gamma_{\mathrm{KP}}^{\mathrm{LW}}} - \sqrt{\gamma_{\mathrm{W}}^{\mathrm{LW}}}\right)^{2}$$

$$
\begin{aligned}
&-\left(\surd\gamma_{\mathrm{SF}}^{\mathrm{LW}}-\surd\gamma_{\mathrm{W}}^{\mathrm{LW}}\right)^{\mathrm{KP}}+2\Big[\surd\gamma_{\mathrm{W}}^{+}\left(\surd\gamma_{\mathrm{KP}}^{-}+\surd\gamma_{\mathrm{SF}}^{-}-\surd\gamma_{\mathrm{W}}^{-}\right)\\
&+\surd\gamma_{\mathrm{W}}^{-}\left(\surd\gamma_{\mathrm{KP}}^{+}+\surd\gamma_{\mathrm{SF}}^{+}-\surd\gamma_{\mathrm{W}}^{+}\right)-\surd\gamma_{\mathrm{KP}}^{-}\gamma_{\mathrm{SF}}^{+}-\surd\gamma_{\mathrm{KP}}^{+}\gamma_{\mathrm{SF}}^{-}\Big]
\end{aligned}
\tag{2}
$$

If $\Delta G_{\mathrm{Adhesion}}{}^{\mathrm{TOT}} < 0$, *K. pneumoniae* adhesion was expected favorable. On the contrary, adhesion would be unfavorable if $\Delta G_{\mathrm{Adhesion}}{}^{\mathrm{TOT}} > 0$ [12].

Antimicrobial activity: The antimicrobial properties of SF samples modified with L-Cys were tested according to JIS L 1902 Standard test method for assessing antimicrobial activity of incorporated antimicrobial agent(s) in textile materials. The Gram-negative strain *K. pneumoniae* (ATCC 4352) was chosen because its resistance to recommended antibiotic treatments has been raised over time, most recently becoming resistant to carbapenem antibiotic class, known as carbapenem-resistant *K. pneumoniae* (CRKP).

Briefly, a *K. pneumoniae* suspension of $1–5 \times 10^6$ bacteria/mL was inoculated into SF samples, and added with NaCl plus surfactant. After 0 and 24 h of incubation, samples were vortexed for 20 s to release any adsorbed *K. pneumoniae*. The antimicrobial activity was calculated by a quantitative method in order to determine the percentage of bacterial reduced growth rate (% of reduction) at 0 and 24 h

$$\mathrm{Reduction} = (\mathrm{C} - \mathrm{A})/\mathrm{C} \times 100 \tag{3}$$

where C represents the average number of Colony Forming Units (CFU/mL) of non-modified SF samples, and A represents the average number of CFU of modified SF.

In order to elucidate whether SF-L-Cys presents a bacteriostatic or bactericidal effect, equations (4) and (5) were resolved [13]

$$\text{Bacteriostatic activity} = \mathrm{Mb} - \mathrm{Mc} \tag{4}$$

$$\text{Bactericidal activity} = \mathrm{Ma} - \mathrm{Mc} \tag{5}$$

where Ma = log10 of the average of three replicas at T0h controls, Mb = log10 of the average of three replicas at T24h controls, and Mc = log10 of the average of three replicas at T24h SF-L-Cys.

Scanning electron microscopy: Adsorbed *K. pneumoniae* were reticulated overnight with 1.5% glutaraldehyde under 4°C. Samples were then serial dehydrated with an increasing ethanol–water gradient (50–99% v/v) for 10 min each, and subjected to critical point drying (K850, EMITECH). Then, they were sputtered with a thin layer of gold and examined by scanning electron microscopy (SEM) (Hitachi S2700).

The magnifications used were 2000 × and 7500 × with accelerating voltage of 20 kV.

Transmission electron microscopy: The morphology of adsorbed *K. pneumoniae* was observed by transmission electron microscopy (TEM). They were stained with 2% uranyl acetate for 5 min, and mounted on a mesh with thin bar copper grids covered with formvar. Each sample was examined at a magnification of 30,000 × in a HITACHI HT7700 TEM with accelerating voltage of 80 KV.

Statistical analysis: SPSS Statistics software, version 21.0, was used for statistical analysis with comparisons between results (*t*-test). $p < 0.05$ was considered significant.

Results and discussion

The reduced functional capacity of immunocompromised patients and complex crosstalk between Enterobacteria make the phenomenon of multidrug resistance even worse [14,15] which can open the door to establish infection and bacterial proliferation resulting in patients fighting a losing battle.

This research paper describes the development and testing of a silk-based gown made from cocoon of silkworm *Bombyx morii* recycled materials, by focusing on prevention of disease, with the compound (L-Cys) that does not trigger microbial resistance, rather than treatment with antibiotics, against an *in vitro* infection caused by *K. pneumoniae*.

Bacteria cultivation

In order to perform the simulation of adhesion assays (1×10^8 *K. pneumoniae*/mL), *K. pneumoniae* was grown in its optimal condition with vigorous shaking until reaching exponential growth. During this stage, the bacterial metabolic machinery is entirely adapted to the medium (Nutrient Broth), in which each bacterial cell divides into two cells.

Susceptibility testing

MIC results of L-Cys against *K. pneumoniae* were 10 mg/mL. These results suggested that L-Cys in aqueous solutions would require 10 mg/mL to kill *K. penumoniae*.

Silk fibroin (SF) samples preparation

Antimicrobial textiles show that besides protecting user, they can also prevent the formation of malodors released from bacterial growth. The developed silk fibroin (SF) gown was covalently modified by aminolysis, with the amino acid L-Cys. SF structural amides were hydrolized to carboxylic acid under alkaline environment (pH 9.5) and heat (90°C) through "saponification", in which the amino groups of L-Cys were then cross-linked. Amino acid L-Cys, with antimicrobial properties, was chemically bound onto textile fibers, which improved the orientation of the bound molecule (SF-NH_2-L-Cys-SH), decreased the quantity required (1% owf (over the weight of the fabric, in a m/V solution of 1 g fiber/50 mL; hence, 0.2 mg/mL)), and improved laundry resistance, and its mechanism of action only interfered with microorganisms when they contacted each other [16].

Energy-Dispersive X-ray spectroscopy

L-Cys covalent reaction onto SF fibers can be indirectly detected by the presence of sulphur, which did not decrease after five washing cycles. Furthermore, SF does not have sulphur in its native chemistry. After functionalization and three rinses, the amount of L-Cys was 0.07 wt.%. In addition, after five washing cycles, the amount of L-Cys was maintained at 0.07 wt.%. Controls confirmed no presence of sulphur (Table 1). EDS showed that L-Cys was grafted to SF fibers, as well as its maintenance after five washing cycles, which demonstrated the effective mechanism of functionalization.

Fourier transform infrared spectroscopy

The infrared spectra of unmodified SF is shown in Figure 3(a). Peaks at 3300 cm^{-1} and 3100 cm^{-1} are associated with amides B and A. At 1650 cm^{-1} vibrations are due to C = O stretching vibrations of amide I, and the peak at 1520 cm^{-1} is related to N–H in-plane bending together with the out-of-phase C–N stretching of amide II.

SF covalent structural modification before and after five washing cycles was also assessed by FTIR. IR spectra confirmed L-Cys bonded on SF surface, as shown by

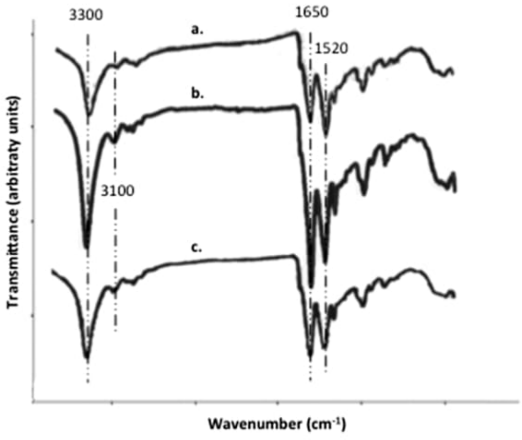

Figure 3. FTIR. Control SF (a). SF-L-Cys after 0 washing cycles (b). SF-L-Cys after five washing cycles (c).

the increase of peaks associated with L-Cys immobilization on a surface, around 3300, 3100 and 1650 cm^{-1}, as shown in Figure 3(b) and (c). Amide I is related to vibrations of the peptide bonds. However, the peak at 1520 cm^{-1} that is related to amide II diminishes, which shows that SF protein secondary structure was modified/unfolded based on the extent of L-Cys cross-linking to SF. Bound L-Cys on SF before (Figure 3(b)) five washing cycles was higher than after laundry (Figure 3(c)) because the peak at 1650 cm^{-1}, due to vibrations of bound L-Cys, was higher than the peak at 1520 cm^{-1}, which is related to the modification of SF [17].

EDS and Fourier transform infrared spectroscopy (FTIR) both showed that L-Cys was successfully grafted to SF fibers. Results also demonstrated the maintenance of cross-linked L-Cys after five washing cycles, which confirms the efficient mechanism of functionalization showed by EDS.

Contact angle

The wettability of SF (silk fibroin) and SF covalently modified samples (SF-L-Cys) at pH 9.5 was measured by Contact Angle Measuring System between their surface and water drop by the sessile drop method, at 10 s. The native/control SF fiber surface showed to be at the interface hydrophobicity–hydrophylicity, $111.83^\circ \pm 21.10^\circ$. On the other hand, after five washing cycles, SF-L-Cys became hydrophilic, $71.30^\circ \pm 4.41^\circ$.

This gown showed the capacity to transport moisture away from the skin, which improves the comfort in patients with sensitive skin [18].

Free energy of adhesion

A thermodynamic methodology of the free energy [12] was used to predict the *K. pneumoniae* binding potential to gown, according to van Oss [19,20]. SF altered from the hydrophobic–hydrophilic interface to hydrophilic character after its covalent treatment. Results showed that these modifications changed SF-L-Cys into a more favorable garment to bind to *K. pneumoniae*, therefore a method of killing bacteria. When *K. pneumoniae* was exposed to SF-L-Cys ($\Delta G_{\text{Adhesion}}{}^{\text{TOT}} = -7.37$) and SF-L-Cys after five washing cycles ($\Delta G_{\text{Adhesion}}{}^{\text{TOT}} = -3.96$), the $\Delta G_{\text{Adhesion}}{}^{\text{TOT}}$ decreased when compared to control SF ($\Delta G_{\text{Adhesion}}{}^{\text{TOT}} = 8.91$), meaning that the adhesion trend of SF-L-Cys to *K. pneumoniae* increased with the introduced modification. SF with an isoelectric point (pI) of 4.2 is negatively charged at neutral physiologic pH's [21]. SF fibers took for granted their affinity with water molecules in an environment which exposes the –OH and –SH groups to environment. This is important to enhance hydrophilic properties of gown and its self-cleaning. The negatively charged membrane potential of *K. pneumoniae* makes it repelled by native SF. In spite of the ubiquity of microorganisms, the ubiquity of microorganisms, even on hydrophobic surfaces, a gown whose fibers demonstrate an ability to quickly bind and kill microorganisms would seriously increase their odds of success as adjuvant in the prevention of establishment of *K. pneumoniae*.

Antimicrobial assays - quantitative method (JIS L 1902 2002)

Antimicrobial assays were conducted under recommended conditions for evaluating antimicrobial finishes on textiles [13]. The SF-L-Cys gown showed significant reduction of microorganisms when compared to controls ($p < 0.05$), as shown in Figure 4. This image illustrates how *K. pneumoniae* load diminished 94.92% after three rinses ($p = 0.0001$) and 88.88% after five washing cycles, none of the previous two significantly different from each other ($p = 0.2705$). Although L-Cys was functionalized using a very low quantity of 1% owf (0.2 mg/mL), most bacteria were eliminated, even after five washing cycles. Furthermore, bacteriostatic and bactericidal activity values were calculated, which showed to be −0.855 and 0.34, respectively. According to JIS L 1902 [13], bacteriostatic and bactericidal activity values for *K. pneumoniae* (ATCC4352) cannot be inferior to 2.0 and 0 [13], respectively. Likewise, our results support the theory that SF-L-Cys has a bactericidal activity. Furthermore, *K. pneumoniae* was not able to develop resistance to L-Cys, which has the capacity to disrupt enzymatic activity as well as bacterial metabolism [11]. This microbicidity of SF-L-Cys may cause bacterial lysis, after its hydrolytic action against peptidoglycan. L-Cys potentiated its –SH exposition against the external environment (bacteria) when cross-linked by their amines to SF fibers' surface. Otherwise, L-Cys in solution would require 10 mg/mL to kill *K. penumoniae*, as reported by MIC results. In accordance with latter results, qualitative experiments – SEM and TEM – showed low bacteria adhesion and their morphology compromised, when comparing to controls.

Scanning electron microscopy

Fiber topography and adhered *K. pneumoniae* were observed for controls and SF-L-Cys after five washing cycles. As illustrated in Figure 5, and contrasting to

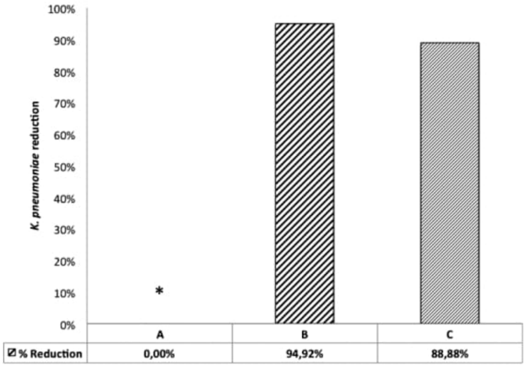

Figure 4. Percentage of *K. pneumoniae* reduction at 24 h ($n = 6$) – Control SF (a), SF-L-Cys after 0 washing cycles (b), SF-L-Cys after five washing cycles (c). *Statistically significant ($p < 0.05$).

controls, SF-L-Cys fiber surface became homogeneous and clean, as a consequence of a fall in the number of adhered *K. pneumoniae* after 24 h of incubation. Also, the presence of cross-linked SF fibers with L-Cys may prevent microbial contamination of the gown.

Transmission electron microscopy

The morphology of *K. pneumoniae* was also observed after five washing cycles under TEM. Results showed membrane-compromised *K. pneumoniae* when adsorbed to SF-L-Cys (Figure 6).

People experiencing sensitive skin, including atopic dermatitis, skin inflammation, bedsores, and related diseases, can benefit from the ‘‘softer hand’’ properties unique to this silk-based gown. Furthermore, it addresses fluid retention and self-cleaning due to L-Cys properties (hydrophilicity) [22], as well as bactericidal activity against *K. pneumoniae* after five laundering [23–25].

Hence, this hospital gown was constructed so that the comfort of sensitive skin sufferers and healing of the patients’ skin were enhanced beyond the requirements that hospitals meet. Furthermore, it may help prevent rashes and other skin irritations, due to a unique combination of silk and L-Cys properties.

According to published work, fabrics treated with antimicrobial compounds, namely anthranic acid, copper, silver, triclosan, polyhexamethylen biguanid (PHMB), quaternary ammonium compounds, lavender oil and most nanoparticles, have demonstrated leaching over laundering and/or cytotoxicity for skin [26–30].

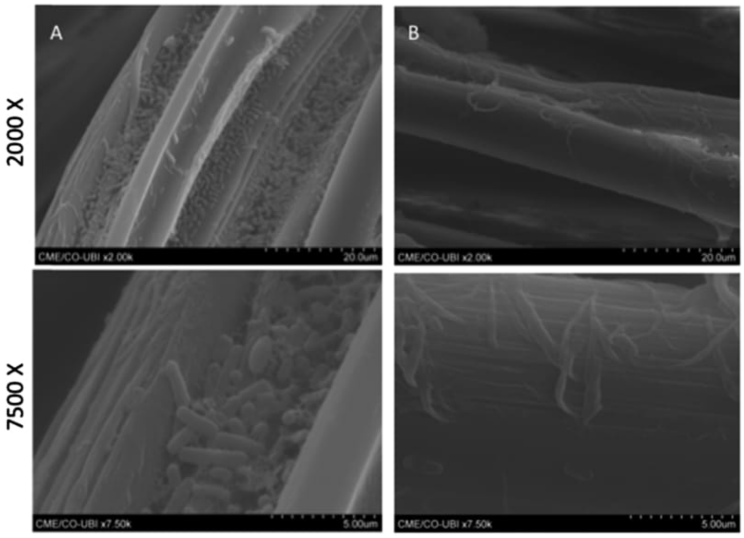

Figure 5. SEM images of control SF (a) and SF-L-Cys after five washing cycles (b).

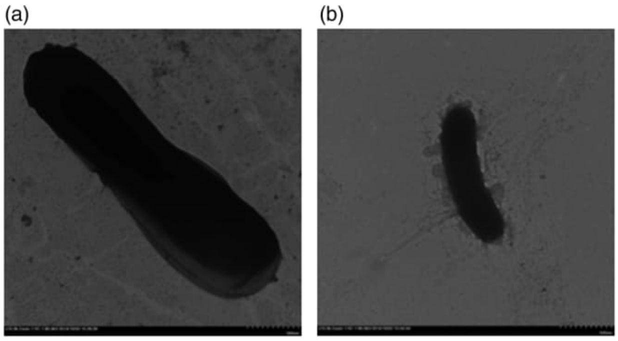

Figure 6. TEM images of *K. pneumoniae* that was in contact with control SF (a) and SF-L-Cys after five washing cycles (b).

Conclusions

Hospital gowns are a type of underwear worn by inpatients which should be changed once a day. The microbial contamination and probability of infection of patients affected by the problem of sensitive skin, with carbapenem-resistant *K. pneumoniae* (CRKP), increase as the day goes on. CRKP has become resistant to essentially all antibiotics, and can kill up to 50% of infected patients. Moreover, when it becomes established, it proliferates and can spread rapidly throughout the community, through its fimbriae and polyssacharide capsule to adhere and cause virulence. These events add urgency to develop new strategies to prevent antibiotic resistance from spreading.

This research paper describes the development and testing of a silk-based gown made from cocoon of silkworm *Bombyx morii* recycled materials, by focusing on prevention of disease, with the compound (L-Cys) that does not trigger microbial resistance, rather than treatment with antibiotics, against an *in vitro* infection caused by *K. pneumoniae*. There was a reduction of 94.92% after three rinses and 88.88% after five washing cycles, concomitantly with an altered morphology of adherent bacteria.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Paper V

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Amino Acid-Based Material for the Complementary Therapy of Decubitus Ulcers

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Chronic wounds, pressure sores, lesions, and infections of microbial origin in bedridden, paralyzed, or malnutrition patients remain the object of study of many researchers. A variety of factors behind the development of these disorders are related to the patient's immune system, making it unable to respond effectively to the treatment of the wound. These factors can be properly controlled, giving particular importance to the ethiology and stage of the wound, as well as the time periods corresponding to the replacement of the dressings. The present research reports a novel foam/soft material, L-Cys-g-PCL, with an application for decubitus/pressure ulcers, especially for wounds with a difficult healing process due to infections and constant oxidation of the soft tissues. During this work, the interactions between *S. aureus* and L-Cys-g-PCL foam were studied under conditions that simulate decubitus ulcers; namely, pH and exudate. The effects of duration of grafting (1 or 8 h) and pH (7.0 and 8.9) on wettability, surface energy, swelling, and porosity were also evaluated. Results showed an effective microbicidal activity exhibiting an inhibition ratio of 99.73% against *S. aureus*. This new L-Cys-g-PCL soft material showed saftey to contact skin, ability to be shaped to fill in sunken holes (craters) – pressure ulcers stage III – and to act as a smart material responsive to pH, which can be tailored to develop better swelling properties at alkaline pH where exudates are normally higher, so as to address exudate self-cleaning and prevention of desiccation.

Keywords: L-Cysteine, antimicrobial agents, chronic wounds, aminolysis, *Staphylococcus aureus*

Introduction

Decubitus ulcers (DUs), also called pressure ulcers or bedsores, are vascular disorders that are the result of prolonged pressure, friction, or shear over skin, especially at bony prominences [1, 2]. DUs are more likely in the elderly, ill-fed, or individuals who stay immobile for a long while, which include the bedridden and paralyzed [2, 3]. Frequent repositioning to scatter the weight on the surface of the skin and the use of special beds and cushions that reduce pressure through redistribution of load over a larger area of the equipment are essential parameters to prevent and control DUs [1]. Its prevalence has been increasing over the past decade, affecting about 7.7 million people worldwide [4]. Smoking, cold weather, hypertension, diabetes, hypercholesterolemia, and ischemic heart disease are contributors to the spread of pressure ulcers, with clinical long-term consequences, such as wounds that cannot heal within 3–4 months [5] – chronic wounds. Furthermore, patients can develop ischemia in the areas of inefficient blood circulation, a condition in which blood products, such as circulating cells, nutrients, and oxygen are delivered to the tissue below a fair amount. Hypoxia is, in this way, a direct consequence of ischemia [6]. The healing tissue demands no homeostatic but higher energy, and hence, a more oxygenated microenvironment, to promote adequate regenerative complexes that work for tissue repair [7]. Oxygen has a major role in the synthesis of collagen, induction of blood-vessel growth, regeneration of the epithelium, and fighting off infections. Although acute and mild hypoxia are described as angiogenic in a tumor context [8], extreme chronic wound hypoxia is not compatible with tissue repair [7]. Furthermore, tissues in a near-anoxic state can progress towards necrosis, which may spread to surrounding tissue, leaving the body highly vulnerable to microbial infections [9]. After a trauma, immune system triggers vasodilation and consequently vessel permeability with wound exudate as a final outcome. This fluid presents cells, key factors for healing, and a great ability to acquire microorganisms [10]. In chronic wounds, persistent inflammation and microbial infections can become established. Chronic exudates have a different composition and appearance than acute ones, and have deleterious effects on growth factors and peri-wound skin [11]. In chronic inflammation, the expression of mediators of inflammation is out of control; for instance, heparin-binding protein, with enhanced vascular permeability and a consequent abnormal production of exudate [12, 13]. Bacteria that colonize chronic wounds, most of them normal skin microbiota, also contribute to histamine release [14]. Moreover, resistant bacteria can also become established and have enough time to proliferate [15], which may kill the patient and spread to the community.

The success of treatment of a chronic wound involves elimination of bacterial colonization, proper oxic conditions provided by wound-dressing structure, temperature above 33ºC, growth factors, wound hydration (optimum moisture balance), exudate absorption (hydrophilicity), necrosis tissue avoidance, and prevention of maceration of surrounding tissue [16−18].

DUs are a huge reservoir of *S. aureus*, which can also be present in human skin without causing any infection. It is enclosed in a capsule that prevents engulfment by PMNs, along with a slime layer made up of biopolymers that can develop attachments to various human receptors as well as external biomedical devices. Additionally, 11 capsular serotypes are identified. *S. aureus* produces a range of toxins that operate as virulence factors: 5 cytolytic that detroy the cellular membrane, 2 exfoliative, 20 enterotoxins, and 1 TSST [19]. *S. aureus* causes clinical disease when it releases different toxins, carries out colonization, and brings about the necrosis of tissues. Although manifestation of the disease relies largely on the action of toxins, in a few situations the cause lies in the *S. aureus* growth that makes over abscess and tissue necrosis. Likewise, it provides resistance to the commonly used (and essentially every) antibiotics [20], a serious cause for concern in DUs [21]. The good progress in antimicrobial wound dressings provides a reliable solution to the adjuvant antimicrobial therapy for chronic disease.

The polyester polycaprolactone (PCL) is a non-toxic hydrophobic synthetic tissue-compatible polymer, being one of the five degradable polymers that the Food and Drug Administration officially approved to meet the needs of healthcare [22]. Pressure ulcers stage III are very difficult to treat and may require years to heal [1]. The polyester PCL has two highly characteristic properties especially suitable for pressure ulcers stage III: (i) its slow the biodegradability rate, which renders PCL an attractive drug delivery device for more than a year (2–3 years) [23]; (ii) and its low tensile strength, yet very high elongation at breakage turn PCL fascinatingly elastic. These are the desirable characteristic features for the regeneration of skin and vascular tissues [24]. On the other hand, other materials, such as hydrogels, bring lower stability: they require environmental control of humidity, nutrients, and microorganisms, which seem unrealistic to control under in vivo conditions; they are susceptible to enzymatic and microbial degradation, have low mechanical resistance, and last no more than a few weeks [22]. As with all biodegradable polymers, PCL is more or less degraded according to its own physicochemical composition; namely, molecular weight, degree of crystallinity, and geometry (form, dimension, porosity/void fraction, and ratio of surface to volume) [25].

This work describes the innovative use of L-Cysteine (L-Cys) that is part of diverse food sources. This α-amino acid is thiol-ended, which encompasses a wide range of antimicrobial activity [26], acting as a disrupter of metabolism and the membrane [27]. L-Cys aims particularly at the microbial membrane and compromises its enzymatic and the metabolic machinery. Its capability for conjugating with reactive chemical species and trace elements [28] adds the ability to counteract the damaging effects of oxidation in human tissues.

The present work takes account of the development of a process that granted the PCL foam with the covalent linkage of L-Cys (L-Cys-g-PCL), which also converted it into a hydrophilic material for exudate absorption purposes. Herein, we present new findings claiming the development of a new foam-shaped wound dressing with an amino acid for DUs. The development of biomaterials that provide comfort, antioxidant, antimicrobial, and non-cytotoxic properties, has become a priority of research.

Materials and Methods

Reagents

Polycaprolactone and 1,4-dioxane as solvent (>99%) were from Sigma (USA).

Staphylococcus aureus, strain ATCC 6538, was cultivated in solid nutrient agar and liquid nutrient broth (Panreac, Spain), with vigorously stirring.

Foam Preparation

A solution of PCL, M_n 45,000, 3% (w/v) in dioxane solvent, was prepared in a fume hood, stirring 2 h until complete dissolution. The solution was then placed in a freezer at −80ºC overnight, and subsequently freeze-dried for 48 h. Foam cross-sections were made and stored in a desiccator until use.

Foam samples were covalently functionalized via aminolysis with amine compounds present in 5% (w/v) L-Cysteine (L-Cys) solution (Sigma-Aldrich, Germany), for 1 and 8 h, under pH 6.5 at 40ºC.

Methods

The foam specimen was evaluated by surface/core analyzing procedures and further investigated for antimicrobial activity and swelling profiles to estimate its behavior and potential as a wound dressing for DUs.

Contact Angle

OCAH-200 (Dataphysics, USA) was used to record contact angles, using a high-speed video hardware with a resolving power of 752 × 484.

Free Energy of Adhesion

The surface tension of samples was ascertained using the sessile drop method at room temperature, using one non-polar component (diiodomethane) and two polar solvents (water and ethylene glycol), whichever surface tension components are known:

$$(1+\cos\theta)\, \gamma 1^{TOT} = 2\left(\sqrt{\gamma s^{LW}}\, \gamma 1^{LW} + \sqrt{\gamma s^{+}}\, \gamma 1^{-} + \sqrt{\gamma s^{-}}\, \gamma 1^{+}\right) \tag{1}$$

The symbol θ means the contact angle; also $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$. The parameter γ^{LW} corresponds to the Lifshitz-van der Waals element of the surface free energy; γ^{+} is the electron acceptor, and γ^{-} is the electron donor of the Lewis acid-base γ^{AB}, knowing that $\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}}$ [29].

The total interaction energy $\Delta G_{Adhesion}^{TOT}$ was predicted from the interactions between *S. aureus*[2] and foam[1]:

$$\Delta G_{Adhesion}^{TOT} = \left(\sqrt{\gamma_2^{LW}} - \sqrt{\gamma_1^{LW}}\right)^2 - \left(\sqrt{\gamma_2^{LW}} - \sqrt{\gamma_W^{LW}}\right)^2 - \left(\sqrt{\gamma_1^{LW}} - \sqrt{\gamma_W^{LW}}\right)^2 + 2\left[\sqrt{\gamma_W^{+}}\left(\sqrt{\gamma_2^{-}} + \sqrt{\gamma_1^{-}} - \sqrt{\gamma_W^{-}}\right) + \sqrt{\gamma_W^{-}}\left(\sqrt{\gamma_2^{+}} + \sqrt{\gamma_1^{+}} - \sqrt{\gamma_W^{+}}\right) - \sqrt{\gamma_2^{-}\gamma_1^{+}} - \sqrt{\gamma_2^{+}\gamma_1^{-}}\right] \tag{2}$$

If $\Delta G_{Adhesion}^{TOT} < 0$, the expected adhesion of *S. aureus* is considered favorable. Conversely, the expected adhesion is not considered favorable if $\Delta G_{Adhesion}^{TOT} > 0$ [29].

Energy Dispersive X-ray Spectroscopy (EDS)

An EDS interconnected to a scanning electron microscope (HITACHI S 2700, Japan) was used to quantify the sulfur presence on the samples.

Ellman's Assay (Datacolor)

Ellman's reagent, (5,5′-dithio-*bis*-(2-nitrobenzoic acid) (Sigma, USA) turns yellow immediately upon conjugating with free thiol groups, where it forms a dissulfidric compound called 2-nitro-5-thiobenzoic acid. This test was conducted to measure thiolates of L-Cys-modified PCL samples, allowing their quantification through determined *K/S* values. Briefly, samples were immersed in a bath ratio of 1/60 of Ellman's reagent at 40ºC for 45 min, under 15 rpm stirring, using a Mathis-BFA12. Color measurement was performed using the spectrophotometer Spectraflash SF300 (Datacolor, Switzerland). Reflectance (*R*) was measured at 412 nm wavelength of the visible light zone and it was intimately linked to concentration with the Kubelka-Munck formula:

$$K/S = (1\text{-}R)\hat{}2/2R \tag{3}$$

R represents reflectance, *K* constitutes the absorption coefficient, and *S* stands for the diffusion coeficient, because

$$K/S = \alpha\, C$$

where *C* is the dye concentration in the fiber and α a constant.

Structural Analysis

The chemical contents of PCL foam, as well as foams grafted with L-Cys for 1 and 8 h, were determined with a Thermo-Nicolet is10 Fourier transform infrared spectrophotometer (FT-IR). The polarized spectra were scanned 64 times, with a spatial frequency resolving power of 4 cm^{-1}.

X-Ray Diffraction (XRD) Analysis

The crystal structure of the samples was assessed by XRD, after being crushed to powders. Scans were performed between 2θ values of 5º and 90º.

Differential Scanning Calorimetry

The crystallinity of the 1 h L-Cys-g-PCL was further investigated with differential scanning calorimetry (DSC) (DSC 204 Phoenix (Netzsch, Germany)) with samples completely filled in small aluminum containers. Nonisothermal scans were carried out from 30ºC to 300ºC at a heating rate of 2ºC per minute, with a nitrogen-replacing atmosphere. The melting point (T_m) and enthalpy of fusion (ΔH_m) were obtained.

Swelling Ratio

Pre-weighted foam samples (*W*1) were immersed in a solution ratio of 0.1 g sample weight: 50 ml of ddH$_2$O adjusted to pH 7 and 8.9, under 25ºC. At 0, 2, and 24 h, samples were taken from the solution, wiped, and weighed (*W*2). The amount of swelling was determined according to Eq. (4).

$$\text{Swelling ratio (\%)} = (W2 - W1) \times 100\ /\ W1 \tag{4}$$

Porosity and Density

The density was determined based on Archimedean Principle, by weighing the samples at the room environment and immersed in a liquid (ddH_2O) at pH 7 and 8.9. Providing that the principle of density determination is $\rho = m(g)/V$ (cm^3), the sample density is

$$\rho_2 = (A/(B-A)) \times \rho_1 \quad (5)$$

where A represents the weight of the sample in the room environment, B stands for the weight of the submerged sample, and ρ_1 is the density of the test liquid (water) at the temperature of 37ºC. The density measurements provided information about the porosity, where

$$\% \text{ porosity} = [1 - (\text{foam density}/\text{bulk density})] \times 100 \quad (6)$$

The bulk density of PCL is 1.15 g/cm^3 (Sigma Aldrich).

Anti-*S. aureus* Activity

An assay was carried out to determine the bacteria viability reduction with the application of the L-Cys-functionalized foam samples, according to *E 2180-01 Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) in Polymeric or Hydrophobic Materials*. The gram-positive bacterial strain *Staphylococcus aureus* (ATCC 6538) was chosen as it is a common pathogen present in nosocomial infections, especially in bedsore ulcers. Briefly, inoculum of *S. aureus* culture was prepared with NaCl and agar-agar in order to form a agar slurry with 1−5 × 10^6 bacteria/ml, which was poured (1 ml) onto 3 × 3 cm square samples, with subsequent incubation for 24 h. The antimicrobial efficiency was quantitatively evaluated, where the percentage of bacterial reduction (%R) was calculated at 0 and 24 h using:

$$\%\text{Reduction} = \frac{C-A}{C} \times 100 \quad (7)$$

where C is the CFU (colony forming units) on the control sample and A is the CFU on the functionalized foam (L-Cys-g-PCL).

In order to ascertain whether the samples have a bacteriostatic or bactericidal effect, the following equations were used [30]:

$$\text{Bacteriostatic activity} = M_b - M_c \quad (8)$$

$$\text{Bactericidal activity} = M_a - M_c \quad (9)$$

where M_a is $\log_{10}$ of the average of three replicas of T_{0h} control samples, M_b corresponds to $\log_{10}$ of the average of three replicas at T_{24h} control samples, and M_c relates to $\log_{10}$ of the average of three replicas at T_{24h} treated samples.

Scanning Electron Microscopy (SEM)

A fixation solution of 1.5% gluteraldehyde was prepared and added to the adsorbed bacteria on samples, which were then incubated overnight at 4ºC. Afterwards, the samples were dehydrated at percentage values starting from 50% until 99%, with ethanol plus water, and then dried (K850 Emitech, UK). After covering the samples with a thin coating of gold, they were examined under a scanning electron microscope (Hitachi S2700, Japan) at a high voltage of 20 kV.

The purpose of SEM analysis was to quantify and compare *S. aureus* attachment after 24 h, with an initial innoculum of 10^7 *S. aureus*/ml, as well as the size of the pores. Pore sizes were measured using the equation: $d = l \times h$ [31], where l means the average maximum pore length and h is the average minimum pore length.

Cytotoxicity Assay

The testing of cytotoxicity of L-Cys-g-PCL on mouse fibroblast cell line BALB/c 3T3 was done based on an adaptation of E DIN EN ISO 10993-5. Two different treatments were performed: (i) sterilized L-Cys-g-PCL discs (0.56 cm^2) were poured floating at the center of each well; and (ii) perspiration extracts of 4.4%, 6.6%, 9.9%, 14.8%, 22.2%, and 33.3% were added. The samples were incubated for 24 h and at 37ºC, under 5% CO_2. The MTS test of cell viability was done, including solvent and negative controls.

Statistical Analysis

The significant values ($p < 0.05$) were calculated according to the multiple comparisons between results, with one-way ANOVA and post-hoc Tukey tests (SPSS 21.0).

Results

Contact Angle

The wettability of PCL foam, together with PCL grafted foams at 1 and 8 h (L-Cys-g-PCL), was calculated according to the angle between the samples surface and the water drop. These angles were recorded at 10 sec, because they proved stability and allowed us to observe a native hydrophobic PCL foam surface; 109.9° ± 5.0°. Interestingly, L-Cys-g-PCL for 1 and 8 h were found to be significantly different from the latter, turning to hydrophilic surfaces of 86.5° ± 10.0° and 58.1° ± 16.1°, respectively.

Free Energy of Adhesion

The interaction potential for bringing *S. aureus* closer to the samples was determined by a thermodynamic approach of the free energy [29]. It was determined that the ability of the *S. aureus* strain studied was unfavorable to bind to the control, which consisted of PCL ($\Delta G_{\text{Adhesion}}^{\text{TOT}} = 7.17$). When the same bacterium was exposed to 1 h L-Cys-g-PCL ($\Delta G_{\text{Adhesion}}^{\text{TOT}} = 19.14$) and 8 h L-Cys-g-PCL ($\Delta G_{\text{Adhesion}}^{\text{TOT}} = 19.99$), the $\Delta G_{\text{Adhesion}}^{\text{TOT}}$ increased, meaning that the adhesion capacity of *S. aureus* to grafted PCL decreased with the introduced modifications.

Energy Dispersive X-Ray Spectroscopy

The amount of sulfur groups present in the grafted samples was calculated with EDS analysis, which is used as

Table 1. Color strength (*K/S*) correlated to functionalization by L-Cys of controls, 1 h L-Cys-g-PCL, and 8 h L-Cys-g-PCL.

Foam samples	*K/S* (400 nm)
Blank	0.03 ± 0.02
1 h L-Cys-g-PCL	0.26 ± 0.09
8 h L-Cys-g-PCL	0,16 ± 0.06

an analytical technique for elemental analysis and chemical characterization. The graft presence varied with the modification time. The absence of sulfur in the PCL native structure helped us to determine indirectly the success of the grafting. The 1 h of grafting was bound to bind an increased load of L-Cys (*i.e.*, 1.64% wt. (percentage by weight)). On the other hand, 8 h of grafting retained only 0.32% wt. In the absence of grafting (controls), no sulfur traces were detected.

Colorimetric Assay (DataColor)

Additionally, to confirm the correlation of sulfur content to thiolates, colorimetric assay with Ellman's reagent was performed. Results of the color strength based on the amount of functionalized L-Cys are presented in Table 1.

Structural Analysis (FT-IR)

The native PCL spectrum is presented in Fig. 1a. This shows the expected characteristic peaks C-H, associated with CH_2 stretching vibrations at 2,943 cm^{-1} and 2,865 cm^{-1}, and with carbonyl adsorption at 1,721 cm^{-1} [32].

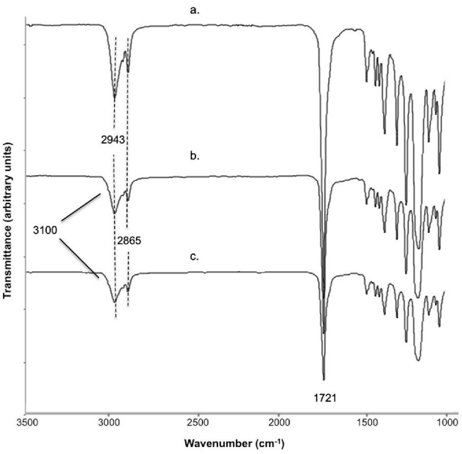

Fig. 1. IR spectra of unmodified polycaprolactone (PCL) foam (a), 1 h L-Cys-g-PCL (b), and 8 h L-Cys-g-PCL (c) both with 5% (w/V) L-Cys.

Structural differences accomplished on PCL after its covalent modification, at two times of grafting (1 and 8 h), were also estimated. Infrared spectra confirmed the PCL chain grafted with L-Cys, marked by the rise of the peak owing to its hydroxyl functional group, around 3,100 cm^{-1}, as shown in Fig. 1b and 1c.

X-Ray Diffraction Analysis

A careful look at the XRD spectra allowed us to see the phase composition and crystal structure of the PCL foam control (Fig. 2a), 1 h L-Cys-g-PCL (Fig. 2b), and 8 h L-Cys-g-PCL (Fig. 2c). The PCL foam and grafted ones showed their main peaks typical of an orthorhombic crystalline polyethylene-like unit cell at 21.5º and 23.5º, corresponding to (110) and (200) reflections, respectively [33]. Although the peak relative intensity values for 2θ of 21.5º and 23.5º were similar for the control (a) and 8 h L-Cys-g-PCL (c), the intense diffraction peak at 21.5º for 1 h L-Cys-g-PCL (b) appeared with no shift, albeit with a higher intensity than those of the latter, indicating the same crystal form with a high-ordered structure. This evidence could suggest a rather more crystalline structure for 1 h L-Cys-g-PCL, when comparing with the control and 8 h L-Cys-g-PCL.

Differential Scanning Calorimetry

Based on XRD evidence, the effect of 1 h L-Cys-g-PCL and its thermal properties were investigated. Melting temperatures (T_m) for the control, 1 h L-Cys-g-PCL, and L-Cys were obtained from the heating scan, and were 63ºC, 62ºC, and 182ºC, respectively (Fig. 3). These values suggest that the covalent introduction of 5% (w/v) L-Cys graft affected the PCL crystallinity degree. Covalent bonds do not break on melting or dissolving. Moreover, molecules are held together by weak intermolecular forces in covalent solids. When a solid melts, the forces that are overcome are the weaker ones, keeping the covalent bonding intact. This may explain why 1 h L-Cys-g-PCL decreased its melting point. Furthermore, its decreased enthalpy of fusion (0.58 mW/mg) might be due to first the latter reason, and second the aminolysis reaction, where the PCL chain is split in two parts. The enthalpy of fusion for the control was 0.67 mW/mg.

Swelling Ratio

The swelling ratio (%) of the studied samples was determined in pH 7 (Fig. 4A) and pH 8.9 (Fig. 4B), at a ratio

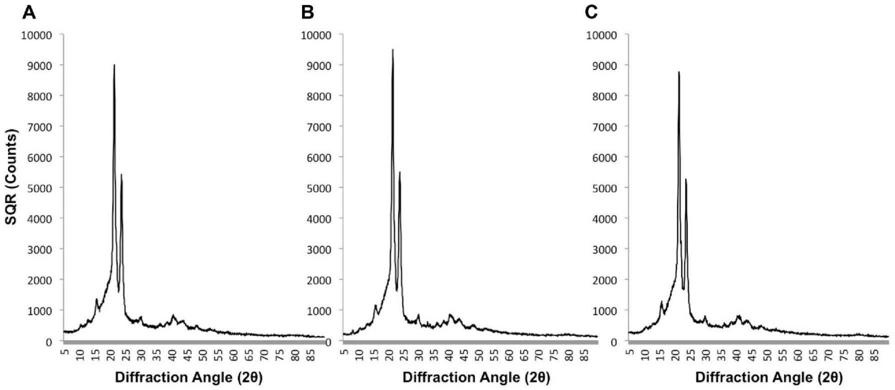

Fig. 2. XRD spectra of sample discs for unmodified polycaprolactone (PCL) (**A**), 1 h L-Cys-g-PCL (**B**), and 8 h L-Cys-g-PCL (**C**), both with 5% (w/v) L-Cys.

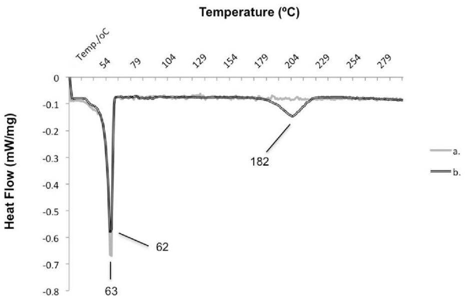

Fig. 3. Differential scanning calorimetry spectra of control polycaprolactone (PCL) foam (a) and the selected 1 h L-Cys-g-PCL (b).

solution of 0.1 g/50 ml, 37ºC, at 0, 2, and 24 h. Calculations were according to Eq. (4). All three samples, PCL foam (Fig. 4a), 1 h L-Cys-g-PCL (Fig. 4b), and 8 h L-Cys-g-PCL (Fig. 4c), showed time dependence of water absorbency. Furthermore, the absorbency was always the highest for 1 h L-Cys-g-PCL (Fig. 4b), and the lowest for PCL foam (Fig. 4a), except at pH 8.9 where 8 h L-Cys-g-PCL (Fig. 4c) after 24 h of incubation in solution was even lower. At pH 8.9, the absorbency was incredibly high for 1 h L-Cys-g-PCL (Fig. 4b), reaching swelling ratios of 382% after 2 h of incubation in solution, and 431% after 24 h of incubation in solution.

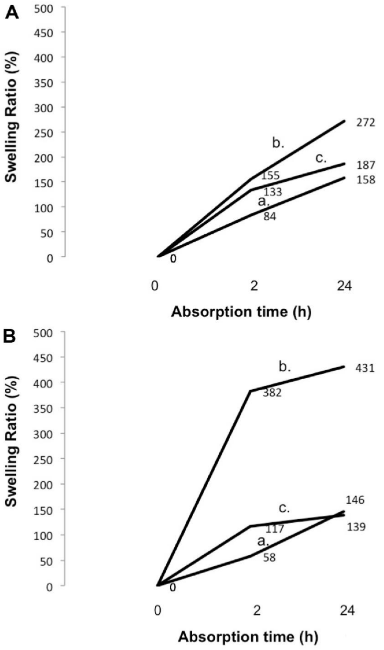

Fig. 4. Swelling (%) of samples of polycaprolactone (PCL) (a), 1 h L-Cys-g-PCL (b), and 8 h L-Cys-g-PCL (c), before and after incubation in buffers at pH 7 (**A**) and 8.9 (**B**).

Table 2. Densities and porosities of samples after 24 h of incubation under pH 7 and 8.9.

Foam samples (24 h)	Density (g/cm^3)		Porosity (%)	
	pH 7	pH 8.9	pH 7	pH 8.9
Blank	0.63	0.68	44.87	40.51
1 h L-Cys-g-PCL	0.37	0.23	68.01	79.82
8 h L-Cys-g-PCL	0.54	0.72	53.40	37.30

Porosity and Density

The foams were produced with a porous structure. Their density and porosity were assessed after 24 h, and are shown in Table 2. For both pH's, the porosity was always the highest for 1 h L-Cys-g-PCL, and the lowest for control PCL foam, except at pH 8.9 where 8 h L-Cys-g-PCL was inferior. At pH 8.9, porosity was very high for 1 h L-Cys-g-PCL, averaging 79.82%. This might be explained by the ionized L-Cys –OH and –SH groups at pH 8.9, which repel each other.

Anti-*S. aureus* Assays – Quantitative Method (Suspension)

This assay evaluated the *S. aureus* viability reduction when in contact with the L-Cys grafted PCL. The results are displayed in Fig. 5. It is possible to observe that after modification, both materials were antimicrobial, either at 0 h (Fig. 5A) or at 24 h (Fig. 5B). On immediate contact with grafted foams (0 h), 1 h L-Cys-g-PCL (b) showed 97.93% of significant bacterial reduction, against 77.86% of 8 h L-Cys-g-PCL (c). For 24 h the same pattern was observed: 1 h L-Cys-g-PCL (b) was significantly higher (99.67%) than the other (c) of 84.40%.

For 1 h L-Cys-g-PCL, bacteriostatic and bactericidal activity values were calculated, and were 2.70 and 1.38, respectively. On the other hand, for 8 h L-Cys-g-PCL, the bacteriostatic and bactericidal activity values were -0.57 and 0.75, respectively. According to JIS L 1902 [30] bacteriostatic and bactericidal activity values for *S. aureus* (ATCC6538P) should be close to 2.3 and 1.5, respectively. The 1 h L-Cys-g-PCL proved to have bactericidal effect, since its bactericidal value was close to the value of 1.38.

Scanning Electron Microscopy

The topography of the cross-sectioned lyophilized foams was analyzed by SEM. Their pore sizes were then measured on SEM images, whose average was obtained using the equation $d = \sqrt{(l \times h)}$ [31], where l means the maximum pore length and h is the minimum pore length. As illustrated in Fig. 6, PCL foams showed a homogeneous, highly interconnected porous structure. Furthermore, the low TIPS temperature led to low-sized pores and fibers with decreased thickness (thin). The average pore size was 54.65 ± 10.99 μm.

There was a significant decrease in the number of adherent *S. aureus* for both conditions of modified samples, after 24 h. For 1 h L-Cys-g-PCL, almost no bacteria was observed. Regarding 8 h L-Cys-g-PCL surfaces, they demonstrated, on the other hand, just a small decrease in the number of adherent bacteria when comparing with controls (Fig. 6).

Cytotoxicity Assay

The 1 h L-Cys-g-PCL was considered a safe antimicrobial

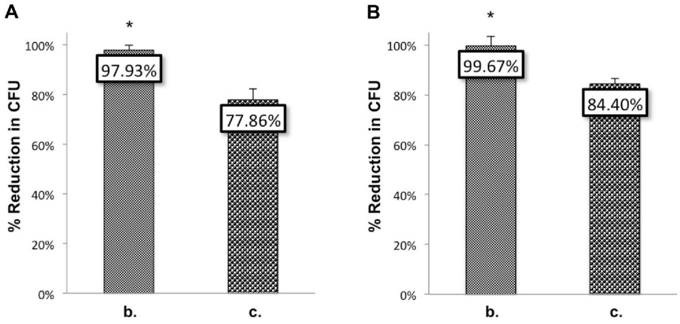

Fig. 5. Percentage of microbial inhibition against *S. aureus* at 0 h (**A**) and 24 h (**B**).

All of these values were obtained by comparison with results of native polycaprolactone (PCL). Samples tested were 1 h L-Cys-g-PCL (b) and 8 h L-Cys-g-PCL (c). * Statistically significant ($p < 0.05$).

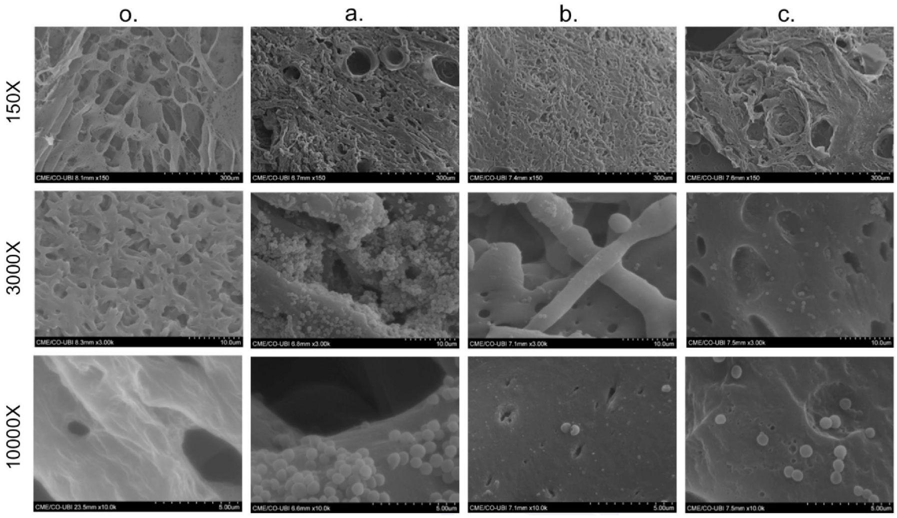

Fig. 6. SEM images of dried polycaprolactone (PCL) foam without bacteria (o).
Adsorbed *S. aureus* on control PCL (a), 1 h L-Cys-g-PCL (b), and 8 h L-Cys-g-PCL (c) after 24 h.

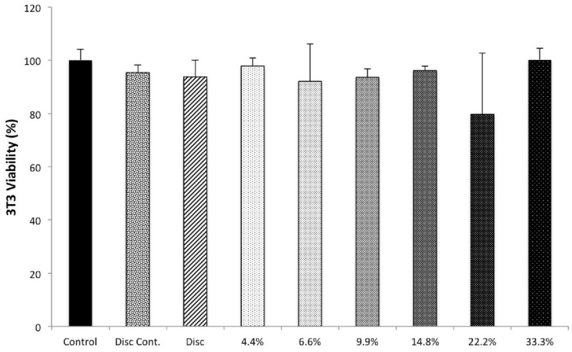

Fig. 7. Mouse fibroblast cell line BALB/c 3T3 viability percentage when in contact with L-Cys-g-PCL disc functionalized for 1 h and the perspiration extract of the same condition in different concentrations.

agent on mouse fibroblast cell line BALB/c 3T3 (Fig. 7). Results did not differ beyond 30% from controls, as shown in Fig. 7. Only a growth inhibition of more than 30% in comparison with the solvent control is assessed as a clear cell-toxic effect. On the other hand, a growth proliferation of more than 30% would be considered pro-tumorigenic (E DIN EN ISO 10993-5). In accordance with this, it can be concluded that no cytotoxic substances were released from these samples, avoiding the risk of skin irritation.

Discussion

This work aims to develop pH-responsive L-Cysteine grafted polyester PCL-based foams for DU treatment. The novelties of this work arise with the preparation of L-Cys-PCL conjugates with high antibacterial activity and the ability to be shaped to fill in sunken holes (craters) – pressure ulcers stage III – acting as a smart material responsive to pH, tailored to develop better swelling properties at alkaline pH where exudates are normally higher, so as to address exudate self-cleaning and prevention of desiccation. The generated soft materials efficiently inhibited *S. aureus* bacterial growth by the effect of the thiol groups grafted on the polyester.

In the beginning, conservative approaches for healing decubitus/bedsore ulcers recommended their drying and exposure to the environment [5]. In 1962, Winter [34] presented in vivo research, which proved that a humid environment would be more suitable for bedsores healing, whose results were further confirmed in 1963 by Hinman and Maibach [35]. Recent studies have suggested the ideal wound dressing should address exudate self-cleaning, prevention of desiccation, non pro-allergenic effect, gas exchange, protection against dust and toxic contaminants, thermic insulation, and debridement protection [1, 5, 36, 37, 38]. The PCL anionic nature, enhanced by thiolates of L-Cys [26], renders L-Cys-g-PCL the advantage of preventing electrostatic interactions with negatively charged surfaces, such as mucosa [39], which rely on its negative charge on pendant sulfate groups [40]. This bears debridement protection to the patient. Moreover, an adequate moisture degree elicits a desirable biological medium for cicatrization, allowing a more efficient cellular metabolic activity and activation of growth factors [5].

During this work, the interactions between *S. aureus* and L-Cys-g-PCL were studied under conditions that simulate DUs; namely, pH and exudate. The effect of duration of grafting (1 or 8 h) and pH (7.0 and 8.9) on wettability, surface energy, swelling, and porosity, as well as on *S. aureus*-L-Cys-g-PCL foam interaction were evaluated.

3D foams were developed from PCL solutions according to the thermally induced phase separation technique, with the posterior sublimation of the solvent. These were subsequently grafted through the covalent reaction aminolysis, with L-Cys. PCL presents ester groups (-COO-) in its structure, which were hydrolyzed to carboxylic acid under slightly acidic condition (solution of 5% (w/v) L-Cys pH 6.5) and heat, through the reverse of Fischer esterification. Amines of L-Cys were covalently grafted on the carboxylic acid of PCL.

The developed application was a porous structure, averaging 54.65 μm. The porosity controls the amount of fluid of a weeping wound, and it constitutes itself an important parameter for gas exchange. Although the optimal porosity is not fully understood yet, the degree of pore interconnectivity seems more important than its size [21]. The low TIPS temperature led to low-sized, homogeneous, and organized pores and fibers with low thickness. Furthermore, they showed a large surface area and were interconnected.

Readings with EDS, Datacolor, and FT-IR indicated that L-Cys was successfully grafted to PCL foam. Results also demonstrated a higher efficiency of grafting for L-Cys-g-PCL foam with 1 h of functionalization when comparing with 8 h. L-Cys-g-PCL foam increased its volume/swelling after incubation in pH 7 and 8.9. At pH 8.9, the maximum pH detected in a chronic wound environment [41], the swelling was even more prominent. The mechanism at this alkaline pH is related to the ionization of hydroxyl and sulfhydryl groups of L-Cys with the consequent repulsion of its chains. L-Cys-g-PCL thus acted as a smart material, responsive to pH. At alkaline pH 8.9, 1 h L-Cys-g-PCL had swelling ratios of 382% after 2 h of incubation in solution, and 431% after 24 h of incubation in solution. This is important for a drug-delivery system application, where at physiological pH chains are tight and compact, whereas at alkaline pH chains swell and release the drug from inside. Furthermore, depending on the degree of cross-linking with L-Cys, it will affect the crystallinity of the foam structure, and its hydrolysis can lead more or less quickly to the erosion of the wound dressing, with the consequent release of L-Cys. Owing to covalent grafting on a PCL substrate, L-Cys acted as a by-contact antimicrobial, which prevented its leaching to the environment after 24 h (at pH 8.9) (data not shown), a condition important to minimize the development of resistance by *S. aureus*.

Surface energies were estimated according to the approach in [42–44]. L-Cys grafting turned PCL from hydrophobic to hydrophilic for both 1 and 8 h treatments. A change in the surface energy from 7.17 mN/m for untreated, to 19.14 mN/m for 1 h L-Cys-g-PCL and 19.99 mN/m for 8 h L-Cys-g-PCL was assessed by the boost in the polar part of the surface energy. With extended grafting time (8 h), although the sample became more hydrophilic than the 1 h graft, its surface energy remained unaffected. One of the factors that rule water absorbency is the hydrophilicity of the pendant group as well as degree of crosslinking. L-Cys is polar and therefore attracts water, which is also polar. This property made the herein developed foams hydrophilic. One hour of grafting did not show as prominent hydrophilic characteristics as 8 h of grafting, probably due to the higher porosity, hiding the polar groups. Moreover, for 1 h L-Cys-g-PCL, the higher XRD peak relative intensity, as shown in Fig. 2, was evidence that suggested a higher crystalline structure for this material. Melting temperatures (T_m) also decreased (Fig. 3), which implied that aminolysis was successfully achieved. Furthermore, L-Cys covalently bonded to ester made it difficult to break, which left weaker intermolecular forces easier to break. Moreover, its decreased enthalpy of fusion, 0.58 mW/mg, explains the chain breakage in two parts.

In accordance with the chemical surface analyses for 1 h L-Cys-g-PCL, microbiologic data for *S. aureus* at 0 and 24 h of incubation showed 97.93% and 99.67%, respectively. Such an inhibition is sustainable because L-Cys, when adsorbed to the foam, has the thiol groups more readily available to react with bacterial proteins and cause a bactericidal effect [26], compromising the membrane metabolic and bioenergetic machinery [27]. On the other hand, for 8 h L-Cys-g-PCL, the killing rates decreased for 77.86% and 84.40% for 0 and 24 h, respectively. This is understandable by the less efficient grafting of L-Cys. The shape and amount of *S. aureus* were evaluated, under electron microscopy, when adsorbed on 1 h L-Cys-g-PCL and 8 h L-Cys-g-PCL for 24 h. It was demonstrated that fewer bacteria adhered to grafted foams when comparing with controls, albeit 1 h L-Cys-g-PCL had the lowest and debilitated adhered *S. aureus*.

These interactions between *S. aureus* and L-Cys-g-PCL are partly in accordance with the theoretical model predicted, in that albeit the unfavorable capacity of *S. aureus* to adsorb the native PCL foam ($\Delta G_{\text{Adhesion}}^{\text{TOT}} = 7.17$), when they were exposed to the modified foam, namely 1 h L-Cys-g-PCL ($\Delta G_{\text{Adhesion}}^{\text{TOT}} = 19.14$) and 8 h L-Cys-g-PCL ($\Delta G_{\text{Adhesion}}^{\text{TOT}} = 19.99$), as the $\Delta G_{\text{Adhesion}}^{\text{TOT}}$ increased to similar values, the adhesion capacity of *S. aureus* to grafted PCL would decrease equally for both. Different porosity between 1 h L-Cys-g-PCL and 8 h L-Cys-g-PCL may explain this fact. Furthermore, bacterial phase variation mechanisms, which fluctuate with pH, may have influence on the *S. aureus* wall. It can express different extracellular structures, such as fimbriae and extracellular polymeric substances [45], which can protect *S. aureus* better from inefficient grafting or lower porosity at 8 h L-Cys-g-PCL.

After comparing with the literature [1, 5, 41, 46−50], the herein developed wound dressing is foam-shaped and composed by a polymeric material, with absorbence eased by the incorporated pores, and with potential to be semi-occlusive. This absorption capacity varied according to foam porosity, as well as grafted L-Cys. It can be used to drain wounds, and to work as a pressure relief wound dressing. Moreover, it is gas and water vapor permeable (porosity), and offers protection against shear forces. This novel material is proposed to be in contact with the wound surface, absorbing its exudate, which can contribute to the moisture environment, once its safety is proved against 3T3 fibroblasts. Although it is stated that foam wound dressings should not be used in infected wounds [5, 50], this research work shows other perspective.

This study allowed the covalent hydrophobic-to-hydrophilic conversion of a PCL substrate via aminolysis with L-Cys. This was demonstrated by the contact angle, free energy of adhesion, X-ray spectroscopy, infrared spectroscopy, X-ray diffraction analysis, differential scanning calorimetry, and scanning electron microscopy.

L-Cys showed optimal grafting to PCL foam with 1 h of functionalization under slight acidic environment and heat. In accordance with the chemical data, microbiologic results for *S. aureus* at 0 h and 24 h of incubation showed 97.93% and 99.67% of bacterial reduction, respectively, which revealed our application to be microbicidal. Furthermore, the number of bacteria adhered after 24 h was negligible and with a compromised morphology.

A new pH-responsive smart material with antioxidant, safety, and swelling properties, ability to be shaped to fill in sunken holes (pressure ulcers stage III), and strong antimicrobial properties is herein presented for potential use as a wound dressing for decubitus ulcers.

Acknowledgments

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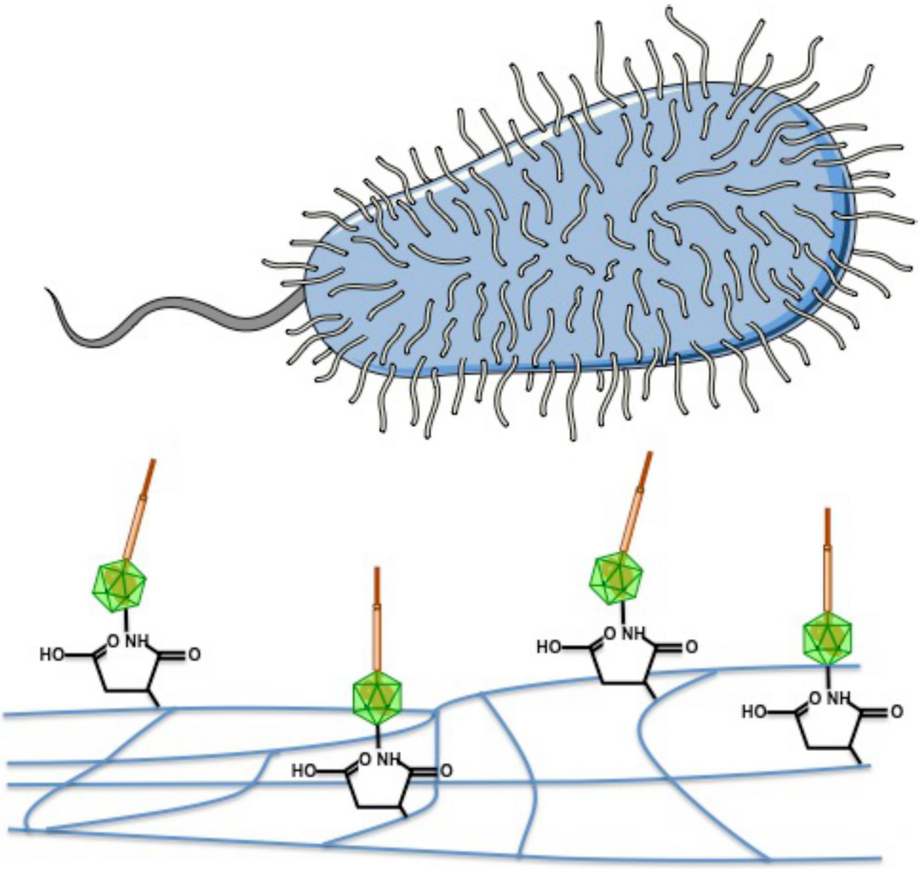
Paper VI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Immobilization of bacteriophage in wound-dressing nanostructure

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Abstract

Opportunistic bacteria that cause life-threatening infections are still a central problem associated with a healthcare setting. Bacteriophage capsid immobilization on nanostructured polymers maximizes its tail exposure and looks promising in applications toward skin-infections as alternative to antibiotics standardly used. The main goal of this work was to investigate the covalent immobilization of vB_Pae_Kakheti25 bacteriophage capsid on polycaprolactone (PCL) nanofibers (non-woven textile), as a potential effective antimicrobial, laundry resistant and non-toxic dressing for biomedical use. Surface analyses showed that the immobilization of vB_Pae_Kakheti25 bacteriophage capsid on PCL nanofibres oriented bacteriophage tails to interact with bacteria. Furthermore, antimicrobial assays showed a very effective 6 log bacterial reduction, which was equivalent to 99.9999%, after immediate and 2 hours of contact, even following 25 washing cycles (due to covalent bond). The activity of PCL-vB_Pae_Kakheti25 against *P. aeruginosa* was immediate and its reduction was complete.

Key words: Bacteriophages; Antimicrobial agents; Surface immobilization; Electrospinning; *Pseudomonas aeruginosa*

The skin of patients with inflammatory skin-diseases alongside with chronic or burn wounds and exit-sites of catheters is particularly susceptible to infection by different microorganisms. Opportunistic pathogens are the cause of skin diseases, infections, and the inability of chronic wounds to heal.[1–4] They are capable of producing virulence factors, including enzymes that promote tissue invasion and extracellular polymers, which form the biofilm that contributes to the perpetuation of skin inflammation, even in normal-appearing skin. Fortunately, the majority of our resident skin microorganisms are non-pathogenic and many of these contribute to maintaining health.[1] Accordingly, skin-disease/injury management demands an integrated approach aimed not only at diminishing infection but also at regulating the skin microbiome.[2,5]

P. aeruginosa is the most common infectious agent among *Pseudomonas* spp. As a versatile and opportunistic microorganism it can colonize the skin, soft tissue, gastrointestinal tract, armpits, eye and ear.[6–8] *P. aeruginosa* is the agent responsible for the most common infections under hospital settings, through catheter and ventilator contaminations leading to nosocomial infections, such as pneumonia, urinary tract and wound burn infections, as well as bacteremia, especially in patients with diabetes or immunodeficiency.[8] The major concerns about the control of nosocomial infections vary from the problems of drug safety associated with a high human toxicity, the long-term and large scale application of broad-spectrum antibiotic drugs, to the increased resistance to conventional therapies. These infections tend to chronicity and may fail to be treated with almost any combination of antibiotics, showing mortalities up to 61%.[9] The combination therapy to fight *P. aeruginosa* infections is very difficult to achieve, due to the compromised immune system of the majority of infected patients, and the intrinsic resistance of microorganisms to various antibiotics.[10]

Recent research has been conducted on the three groups of naturally occurring antimicrobials as novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases (BCWHs), and

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antimicrobial peptides (AMPs).[11] Among them, bacteriophages are the most highly specific toward both Gram-positive and Gram-negative bacteria and they are also highly efficient and relatively cost-effective. In contrast, AMPs have a broad-spectrum against bacteria and fungus, low level of induced resistance, but may cause toxicity at high doses in order to be efficient, and are more costly to produce.[11] BCWH has limitations toward Gram-negative bacteria, as a result of the presence of the outer membrane, and important Gram-positive pathogens like *S. aureus* are already resistant to lysozymes. To overcome the changing tide of nosocomial diseases and increasing reports of microorganism-acquired resistances, recently the United States, Canada and European countries have started to take a close interest in bacteriophage-based therapies,[12,13] in which they act without mechanism-based host toxicity.

The bacteriophage vB_Pae_Kakheti25 has a potent activity against *P. aeruginosa* and appears as an alternative approach to conventional treatments, especially as an antimicrobial agent for dressing. It is representative of siphoviral family, has dsDNA as its genome, icosahedral capsid, and long non-contractile tail. vB_Pae_Kakheti25 undergoes a lytic cycle through which it self-replicates and lyses a broad range of *P. aeruginosa* strains in order to spread copies of itself.

Polycaprolactone (PCL), an hydrophobic polyester, can be explored as a substrate for skin regeneration due to its high elasticity and slow biodegradability.[14,15] Furthermore, it is not broken down by enzymes and microorganisms.[14] These features seem worthy of dressing applications.[16]

The textile and medical industries continue to look for eco-friendly processes that may replace the currently used toxic textile chemicals and the use of antibiotics, respectively.[17–19] The demand for medicinal products alternative to antibiotics has increased considerably and our proposed application seems promising due to its versatility, low content of impurities, antistatic properties and good mechanical properties. Furthermore, as PCL is unfavorable for the development of microorganisms, its shelf life and users' health status are also ensured.

In this work, in order to eliminate the growth of *P. aeruginosa*, PCL electrospun nanofibers were threaded and then vB_Pae_Kakheti25 bacteriophages were covalently immobilized by their capsid via acid-amine reactions, forming amide linkages. The effect of anti-*P. aeruginosa* activity of PCL-vB_Pae_Kakheti25 dressing was evaluated under various parameters, so as to produce appropriate applications toward skin-infections, and aiming to further highlight the potential of phage as the "antibiotic" of the millennium by minimizing bacterial resistance and preserving skin-microbiome.

Methods

Materials

Polycaprolactone nanostructure (PCL), average Mn 45,000 (Sigma), was functionalized in a vB_Pae-Kakheti25 bacteriophage solution. PA25 (DSM 25642) clinical isolates of *Pseudomonas aeruginosa* from the Eliava culture collection were used for isolation and subsequent growth of vB_Pae-Kakheti25 bacteriophages. *P. aeruginosa* was grown on Brain Heart Infusion (BHI) agar and then on Brain Heart Infusion (BHI) broth (Sigma) at 30 °C and in shaker at 200 rpm.

Methods

Electrospinning

Nanofibers were produced by NanoSpider (Elmarco s.r.o. Liberec, Czech Republic). PCL 15% (w/v) was dissolved in a mixture of absolute ethanol/chloroform (65:35 vol.%) to prepare a homogeneous solution. Different ratios of ethanol/chloroform solvents were used in order to optimize the final nanostructure. The final concentration of PCL and ratio of solvents were set according to the homogeneity of resulting nanofibers, their easy-detachment from polypropylene-coated collecting electrode and tensile strength. The electrospinning process was done under the following experimental conditions: RH ≈65%, temperature ≈25 °C, electric voltage ≈80 kV, distance between electrodes = 8.98 cm, and electrode spin = 7 r/min (44 Hz). Figure 1 shows the representative images of these nanofibers.

Tensile strength assays

Tensile strength of electrospun PCL was evaluated with resistance-to-rupture assays. These assays were performed in a Dynamometer (Thwing-Albert Instrument Co.) according to Standard EN ISO 2062, at 20 ± 2 °C, under 60% relative humidity. Samples were strip-cut 1×5 cm, with an average thickness of 50.33 μm, grammage of 18.6 g/m^2, and placed between dynamometer tweezers. A defined pre-tension was set in the beginning, and the test ended with the rupture of samples. Seven replicates were used.

Protein structure modeling – I-TASSER method

The sequence of amino acids (UniProtKB) of the Major capsid protein referred to as H6WTZ9–1 and Major tail tube protein referred to as H6WU05-1 of vB_Pae-Kakheti25 bacteriophage were the following, respectively: MALSDLAV
YSEYAYSAFSETLRQQVDLFNTATGGAIMLQSAAHQGD
FSDVAFFAKVTGGLVRRRNAYGSGTVAEKVLKHLVDTS
VKVAAGTPPVRLDPGQFRWIQQNPEVAGAAMGQQLAV
DTMADMLNVGLGSVYSALSQVSDVVYDATANTDAAD
KLPTWNNLNNGQAKFGDQSSQIAAWIMHSTPMHKLYG
SNLTNGERLFTYGTVNVVRDPFGKLLVMTDSPNLFAAG
TPNVYHILGLVPGGVLIGQNNDFDANEETKNGDENIIRT
YQAEWSYNIGVKGFAWDKANGGKSPTDAALFTSTNWD
KYATSHKDLAGVVVKTN;

MVCEIAKIDSNITGLAFAEEECLKQLPTTPVWYGLEPN
SYSDFGGELSTVARAPIDPSRQNKKGTITDLDASGGFNA
DFTKTNLARILQGFFFADARELPSTQPLNGASVALTGVT
AIDSTYAAASGLGVFGADMLVYATGFANAANNGLKTV
VSATAAGVVVAETLIDETPPAGAKLECVGRQLAAADAN
IAVTGNVVSLIVTAGDFTTMPELFAGRWVFVGGDATAN
RFANNVGYARIKSVAAKALVFDDVTWQAVNETGTGKSI
RLFVGTVIKNEKTPALIKRRSYQIERTLGEGLNGTQCEYL
EGAVPNEFTLNVPQADKLNADLSFVACDNTYRSGDPGD
EQKAGTRVPAPGEDAYNTSSDVYRIKMAVHDAASSNPA
ALFGYVSEANVSINNNVTPNKAVGVLGAFDTSAGNFEV
GGSITAYFTTVAAVKAVRANADVGLSVISAAKNAGFVF
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EVFSYLPNLAMPD.

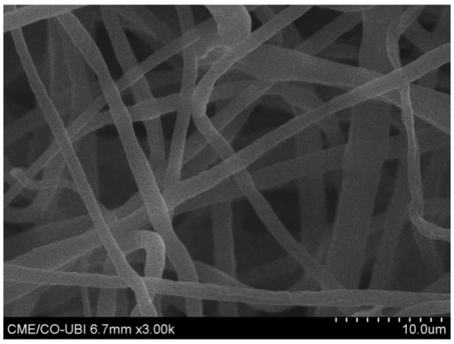

Figure 1. Polycaprolactone (PCL) 15% (w/V) electrospun fibers. Tendency of forming layers of curlicues. 3000× magnification.

For the simulation, these sequences were threaded from Protein Data Bank library, where multiple template alignments were generated. Then, the continuous fragments were excised, reassembed, clustered and this full process was repeated. I-TASSER used the most significant templates with the lowest energy.[20–23] The predicted structures were analyzed for polarity and ligand binding sites.

Ninhydrin

In order to ascertain the presence of free amines on vB_Pae-Kakheti25 capsid, i.e. the estimation of the quantity of vB_Pae-Kakheti25 that PCL nanofibers were able to crosslink, ninhydrin assays were done. Ninhydrin hydrates with amino groups, producing purpurous Ruhemann's cromophore (λmax 570 nm). This colorimetric assay was performed after drawing a calibration with known concentrations of L-cysteine. Each sample was added 2 mL of 10^8 vB_Pae-Kakheti25/ml and 1 ml of 8% Ninhydrin (acetone). ddH_2O was used for controls. Test tubes were placed in water bath at 100 °C for 10 minutes and then cooled down to room temperature before they were read at 570 nm.

PCL substrate preparation

Nanofiber functionalization. After being formed with electrospinning, non-woven nanofibrous PCL was washed for 4 hours with distilled water, at 40 °C, 110 rpm, and dried afterwards at 30 °C until used, in order to remove any surface residues and impurities. Samples were stored in the desiccator until use. For the activation of the surface, samples were submersed in acidic solution (pH 5.5) for 60 minutes at 40 °C. Soon afterwards they were washed in deionized water. After the activation process, these were submitted to the functionalization process by exhaustion with vB_Pae-Kakheti25 bacteriophages via their amine compounds. For the exhaustion functionalization process, a MOI of 0.1 vB_Pae-Kakheti25 bacteriophages was prepared. Each sample was immersed in a bioactive solution at a liquor ratio of 1:50 in the beakers of the Ahiba datacolor dyeing machine and submitted to 40 °C for 60 minutes. Finally, samples were washed in deionized water and stored in the fridge.

Washing cycles assay – resistance to laundry. Laundry operations were performed before any characterization, so as to assess PCL-vB_Pae-Kakheti25 covalent bonding resistance. The methodology was adapted from the international standard EN ISO 105-C06:2010, as follows: (i) washing in ddH_2O throughout 5 and 25 washing cycles, at 40 °C for 30 minutes each, and (ii) washing in a 4 g/L solution of AATCC 1993 Standard Reference Detergent WOB throughout 5 and 25 washing cycles at 40 °C during 30 minutes each.

Fourier transform infrared spectroscopy (FT-IR)

The chemical arrangement of PCL nanostructure and PCL-vB_Pae-Kakheti25 was analyzed after three rinses (no washing cycles), 5 and 25 washing cycles with Thermo-Nicolet is10 spectrophotometer. Samples were scanned 64 times, with a spatial frequency resolution of 4 cm^{-1}.

Antimicrobial activity assessment

PCL-vB_Pae-Kakheti25 substrates were tested according to Japanese Industrial Standard JIS L 1902:2002, designed to test all fabric samples tailored to healthcare industry. *Pseudomonas aeruginosa* was the selected agent once it is responsible for the most common infections under hospital settings, with mortalities up to 61%.[9] Briefly, the inoculum of *P. aeruginosa* was adjusted to $1–5 \times 10^6$ bacteria/mL, of which 200 µl were inoculated on 0.4 g PCL-vB_Pae-Kakheti25 circle samples. Following an incubation of 2 hours, antimicrobial activity was calculated in order to determine bacterial growth inhibition (% of Inhibition) at 2 hours:

$$\%Inhibition = \frac{C-A}{C} \times 100$$

Where C is the average value of Colony Forming Units (CFU) of controls, and A represents the average value of CFU of PCL-vB_Pae-Kakheti25.

Growth kinetics of infected bacteria

One-step growth experiments were performed in order to evaluate the latent phase and burst size of vB_Pae-Kakheti25, according to.[24]

Transmission electron microscopy (TEM)

The vB_Pae-Kakheti25 life cycle together with its host *P. aeruginosa* was monitored by TEM (HITACHI HT7700) at 0, 15, 30, 60 and 120 minutes. Furthermore, the bonding frequency of vB_Pae-Kakheti25 on PCL nanofibers was also visualized. Samples were stained with 2% uranyl acetate during 5 minutes, and framed on a thin bar copper web covered with formvar. Various magnitudes were used at an accelerating voltage of 80 KV.

Cytotoxicity Activity Assay

The cytotoxicity of PCL-vB_Pae-Kakheti25 was performed according to,[25] in two different assays: perspiration extract assay and direct contact assay. In the first assay, 2 g of PCL-vB_Pae-Kakheti25 samples were immersed for 24 h at 37 °C in PBS. The resulting perspiration extract was filtered (0.2um) and was then blended with cells, at concentrations of 4.4, 6.6, 9.9, 14.8, 22.2, and 33.3%. The second assay, direct contact assay, consisted in placing at the center of each well a PCL-vB_Pae-Kakheti25 sample disc that covered

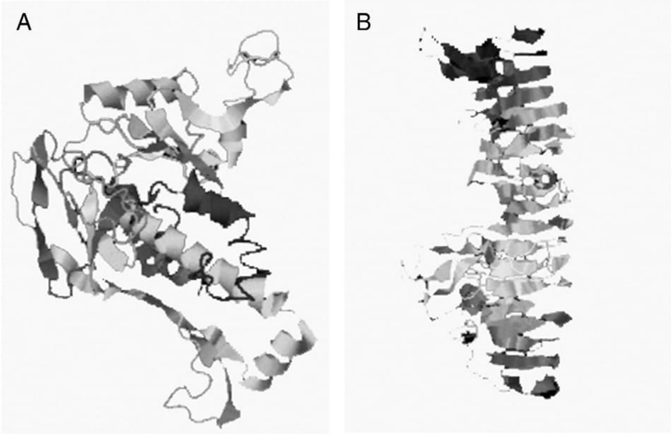

Figure 2. Major capsid protein (**A**) and Major tube protein (**B**) modeled structures generated by SPICKER program[16–19] based on pair-wise similarity. C-scores varied from −1.72 to −1.38.

1/10 of its area. Fibroblasts 3 T3 were used to seed each well at a concentration of 2×10^4 cells/well. In the final part of both assays incubation was conducted at 37 °C for 24 h, under a humidified atmosphere containing 5% CO_2. MTS viability assay was performed. Solvent, positive and negative controls were included.

Statistical analysis

Statistics software SPSS 21.0 was used to calculate one-way ANOVA and Tukey post-hoc tests for establishing multiple comparisons between all samples. P-values below 0.05 were considered statistically significant.

Results

Sample characterization

Electrospun fibers

Nanofibers were produced with an average thickness of 1.57 nm ± 0.26 Figure 1.

Tensile strength assays

Tensile tests evaluated the mechanical properties of developed electrospun samples. Elastic modulus was observed for electrospun PCL nanofibers (10.64 Mpa ± 2.99) with an elongation of 13.95% ± 2.43. Results between samples were not significantly different, which means it demonstrated reproducibility.

Protein structure modeling

The predicted secondary structure of Major capsid protein was arranged in globular domains, forming hexamers, comprised of alpha and beta structures. Major tube protein was arranged in β-helix, according to the I-TASSER algorithm.[16–19] Furthermore, the bioinformatics modeling of these structures were generated using the SPICKER program based on pair-wise structure similarity. C-scores varied from −1.72 to −1.38. In Figure 2, *A* and *B*, it is presented the structure predictions of Major capsid protein and Major tube protein, respectively. Non-polar side chains remained uncharged at physiological pH and were incapable of participating in hydrogen bonding. On the other hand, in Major tube protein, seven polar leucine-15,47,88,112,178,385, and 464 were identified. Furthermore four polar asparagine191,259,277, and 336 were detected, as well as two polar glutamine-11 and 303. All of them contained an α-amino group protonated at $–NH_3^+$ under biological conditions. These are important for cross-linking to outer sheath proteins around Major tube protein. Furthermore leucine-507, methionine-509, proline-510 and aspartate-511 constitute an acidic terminal region, important to bind and be capped by the spike protein.

The first subunit of the Major capsid protein presents polar lysine-38 and glycine-45. Furthermore it presents an RGD sequence-44,45,46; aspartate-82,98, valine-85, and glycine-90,112 in the second subunit; aspartate-144,148, leucine-132, valine-135, serine-137 in the third subunit; aspartate-157,174, serine-177,186, methionine-184,189, histidine-185 in the fourth subunit; aspartate-224, serine-225, valine-236, glycine-245, glutamate-259, asparagine-263 in the fifth subunit; glycine-284 in the sixth subunit. Under physiological conditions (pH 7.4) lysine, arginine, histidine side chains were positively charged $–NH_3^+$ and important for covalent bonding to PCL surface.

Ninhydrin

The free amines on vB_Pae-Kakheti25 capsid were quantified with ninhydrin reagent. A calibration curve ($r^2 = 0.99$) was performed with known concentrations of L-cysteine (which contains only one amino group), and finally, the concentration of amines in bacteriophage solution was determined. Each bacteriophage was found to have 2.85 picogram of free amines.

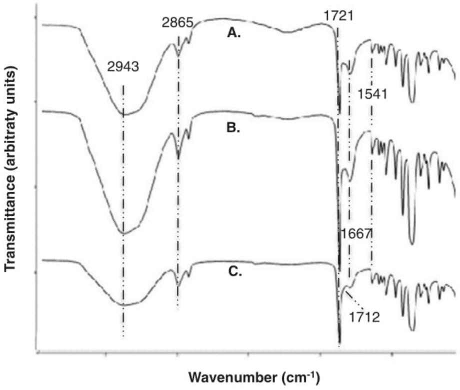

Figure 3. IR spectra of unmodified PCL (**A**) and PCL-vB_Pae_Kakheti25 after (**B**) 5 washing-cycles and (**C**) 25 washing-cycles.

Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectrum of unmodified nanofilm is shown in Figure 3, *A*. This spectrum displays the expected representative peaks of polycaprolactone, namely C-H, related with CH_2 stretching vibrations at 2943 cm^{-1} and 2865 cm^{-1}, and with carbonyl adsorption at 1721 cm^{-1}.[26]

The covalent bonds between PCL and vB_Pae-Kakheti25 on nanofibers, were also assessed by FT-IR after 5 and 25 washing cycles. IR spectra showed PCL surface with carboxylic acid after its activation at 1712 cm^{-1}, and vB_Pae-Kakheti25 grafted on nanofibers, as denoted by the increase of the peaks owed to covalent bonds formed with PCL surface, namely at 1667 cm^{-1} and 1541 cm^{-1}, assigned to Amide I and II, respectively, as well as the decrease of free carboxylic acid at 1711 cm^{-1}, as shown in Figure 3, *B* and *C*.

Antimicrobial assays

Quantitative method (suspension)

In order to evaluate the antimicrobial effectiveness of PCL-vB_Pae-Kakheti25, the adsorption test was performed to quantify the ability of nanofibers functionalized with bacteriophages to prevent bacterial growth Figure 4. The results showed that there were marked reductions in *Pseudomonas aeruginosa* at T0 h and T2 h. PCL activated nanofibers showed outstanding microbial inhibition, as shown in Figure 4. We could observe that PCL-vB_Pae-Kakheti25 eliminated 6 log of *Pseudomonas aeruginosa at* 0 and 2 hours, which was equivalent to 99.9999% reduction.

After 5 and 25 washing cycles with WOB at 40 °C, the same 6 log elimination of *Pseudomonas aeruginosa* was observed, either at 0 or at 2-hour. These results proved that covalent bonding between PCL and the bacteriophage capsid was stable and strong enough to resist to several washings.

In order to know whether the samples behaved as bacteriostatic or bactericidal, according to Japanese Industrial Standard JIS L 1902:2002, the bacteriostatic activity result can be not less than 2.0 and bactericidal activity result cannot be less than 0. Briefly, bacteriostatic activity = log10 of the average of bacterial colonies on the control samples at T24 h - log10 of the average of bacterial colonies on the functionalized samples at T24 h; and bactericidal activity = log10 of the average number of bacterial colonies on the control samples at T0 - log10 of the average of bacterial colonies on the functionalized samples at T24 h.

The calculated values from these equations yielded the following values: after three rinses, bacteriostatic activity was 0.94 and bactericidal activity was 1.63; after 5 washing cycles, bacteriostatic activity was 0 and bactericidal activity was 0.98; after 25 washing cycles, bacteriostatic activity was 0 and bactericidal activity was 0. These results state that PCL-vB_Pae-Kakheti25 has a bactericidal effect after 3 rinses, 5 and 25 washing cycles, which means that the immobilization of vB_Pae-Kakheti25 on PCL lead to death of *P. aeruginosa* and do not interfere in the development and growth of bacteria.

Growth kinetics of infected bacteria

This assay aimed to study the growth kinetics of the infected host cells, using a control (uninfected bacteria), at the dressing change temperature of 29.9 °C Figure 5.

At this temperature the infected *P. aeruginosa* showed lower absorbance values than those obtained by the non-infected *P. aeruginosa* from 20 minutes after the beginning of incubation. This indicated that vB_Pae-Kakheti25 had been using the bacterial machinery for its benefit (latent period). A decrease in bacterial growth rate after 120 minutes were also observed, which indicated the onset of cell lysis phase, resulting from bacteriophage infection.

Transmission electron microscopy (TEM)

The interaction between vB_Pae-Kakheti25 tail and *P. aeruginosa* membrane is shown in Figure 6, *A* and *B*. The vB_Pae-Kakheti25 life cycle inside *P. aeruginosa* cells was also monitored at 0, 20, 30, and 120 minutes, as shown in Figure 6, *C-F*. vB_Pae-Kakheti25 bacteriophage fiercely degraded *P. aeruginosa* membranes, especially after 20 minutes (Figure 6, *D-F*). Furthermore, the spacing of the bonding of vB_Pae-Kakheti25 on PCL after 25 washing cycles was also visualized, as shown in Figure 7. The frequency of covalent bonds of vB_Pae-Kakheti25 on PCL nanofibers averaged 118 nm, as shown in Figure 7, *A*.

Tumoral activity assay

PCL-vB_Pae-Kakheti25 did not show any cytotoxic effect over mouse fibroblast cell line BALB/c 3 T3 Figure 8. Both perspiration extract assay and direct contact assay showed values that did not ranged beyond 30% from controls. Only an alteration under or over 30% in comparison with controls would be considered cytotoxic or pro-tumorigenic, respectively.[21]

Discussion

As part of natural defenses of ecosystem, the mode of action of bacteriophages is very specific and relies on infecting exclusively their host bacteria, leaving the rest of microbiota and human tissues intact. In this investigation, the capsid of the bacteriophage vB_Pae-Kakheti25 was covalently cross-linked to

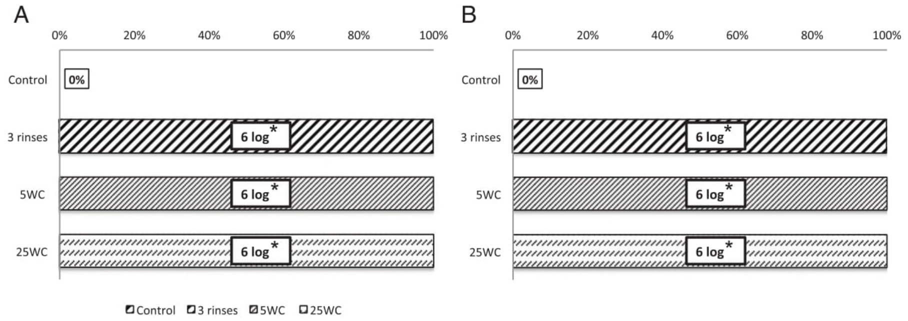

Figure 4. Percentage of microbial inhibition at 0 hours (**A**) and 24 hours (**B**) – all of these values were obtained by comparison with results of native PCL. *Statistically significant from controls ($p < 0.05$).

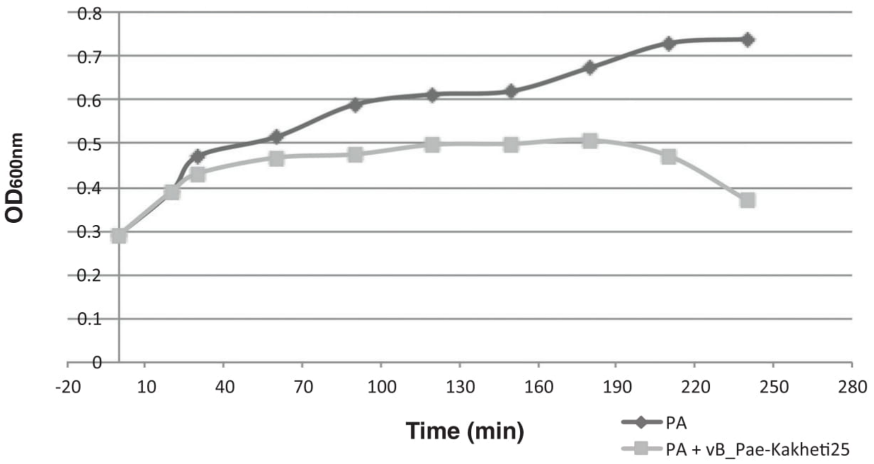

Figure 5. One-step growth kinetics of *P. aeruginosa* strain and *P. aeruginosa* infected with the bacteriophage vB_Pae-Kakheti25 at 29.9 °C.

polycaprolactone (PCL) nanofibrous dressing, in order to maximize vB_Pae-Kakheti25 tail exposure, evaluate its stability, and its antimicrobial effect over *Pseudomonas aeruginosa* bacteria. The electrospun nanofibrous structures offer larger area of contact and a smoother surface, which diminishes roughness and scratching.[27]

A nanofibrous dressing with an average thickness of 1.57 nm was produced by electrospinning. For the covalent immobilization of bacteriophage capsid onto PCL nanofibers, the first step was the formation of reactive acid groups on PCL, namely ester groups (−COO-) that were hydrolyzed to carboxylic acid groups from ester under acidic solution (pH 5.5) via the reverse of Fischer esterification. Amino groups of vB_Pae-Kakheti25 capsid – 2.85 picogram of free amines per bacteriophage – reacted as soon as they contacted the carboxylic acids of PCL, via acid-amine reactions, forming amide linkages. vB_Pae-Kakheti25 bacteriophage contains an icosahedral head structure that is composed of many copies of Major capsid protein (UniProtKB) with 325 amino acid length and lysine, arginine, histidine side chains that were positively charged under physiologic pH. Also vB_Pae-Kakheti25 presents a tail that recognizes surface receptors of *P. aeruginosa*. Mechanical properties of PCL nanofibers showed high elasticity and were not significantly altered in any treatments in this study, which means PCL structure was not degraded during the experiment.

Fourier Transform Infrared Spectroscopy (FT-IR) and Transmission Electron Microscopy (TEM) provided evidence for the proposed bacteriophage immobilization aligned in a head-to-tail orientation. FT-IR spectra confirmed that bacteriophage capsid was covalently immobilized on PCL surface, by the comparison of

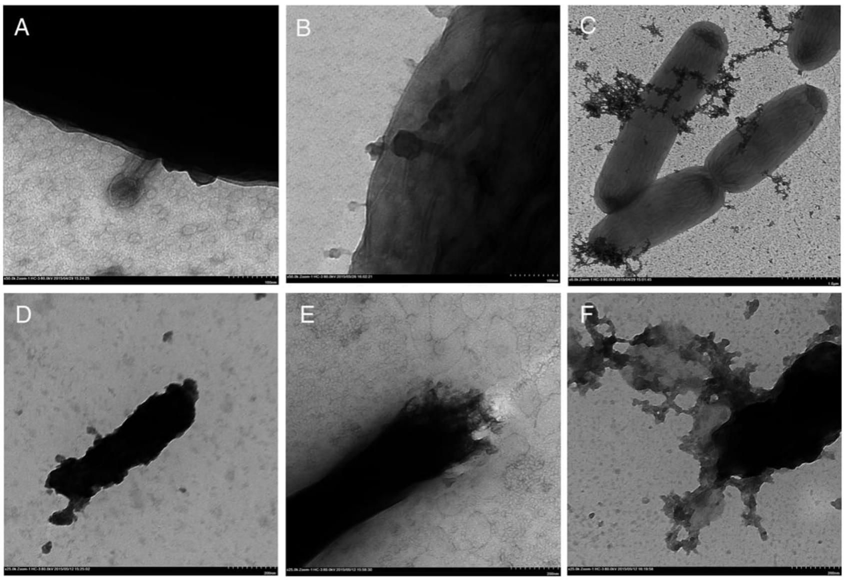

Figure 6. Adsorption of vB_Pae-Kakheti25 to *P. aeruginosa* membrane at 50000× magnification, (**A**) and (**B**). vB_Pae-Kakheti25 life cycle infecting *P. aeruginosa* monitored at 0 (**C**), 20 (**D**), 30 (**E**), and 120 minutes (**F**). 6000× and 25,000× magnifications.

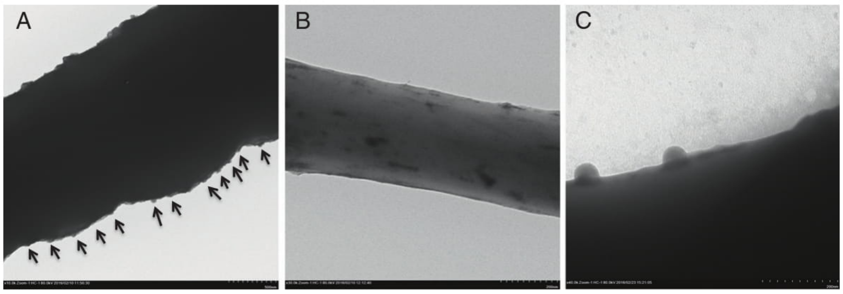

Figure 7. The bonding frequency of vB_Pae-Kakheti25 to PCL single nanofibre (**A**), 3D visualization (**B**), and vB_Pae-Kakheti25 adsorption arrangement (**C**). 10,000×, 30,000× and 40,000× magnifications.

peak height proportions (1667 cm^{-1}, 1541 cm^{-1} and 1711 cm^{-1}) between PCL and PCL grafted with vB_Pae-Kakheti25. Furthermore, spectra showed the covalent cross-link between PCL and vB_Pae-Kakheti25 to be multiple washing-resistant – 5 and 25 washing cycles – and consequently stable, because the aforementioned peaks height proportions were maintained. In accordance with these previous data, TEM images showed vB_Pae-Kakheti25 immobilized on PCL nanofibers and aligned in a head-to-tail orientation after it had been washed 25 times. Immobilized bacteriophages were also spaced ≈118 nm apart on both sides.

Antimicrobial assays of vB_Pae-Kakheti25-bearing PCL showed that it acted as soon as it contacted with *P. aeruginosa*, with antimicrobial reducing rates of 99.9999% (6 log), $p < 0.05$. In addition, PCL-vB_Pae-Kakheti25 was identically active after 25 washing cycles, either at immediate contact, or at 2-hour contact. These results were higher than the 5 log reduction observed by Huang et al. for silver-chitosan.[28] The low natural adhesion of PCL[29] due to its hydrophobic character - contact angle of 109.9° ± 5.0°[29] – may also have facilitated the contact of *P. aeruginosa* with the bacteriophage tails. H. Yang and Y. Deng[30] also showed that a

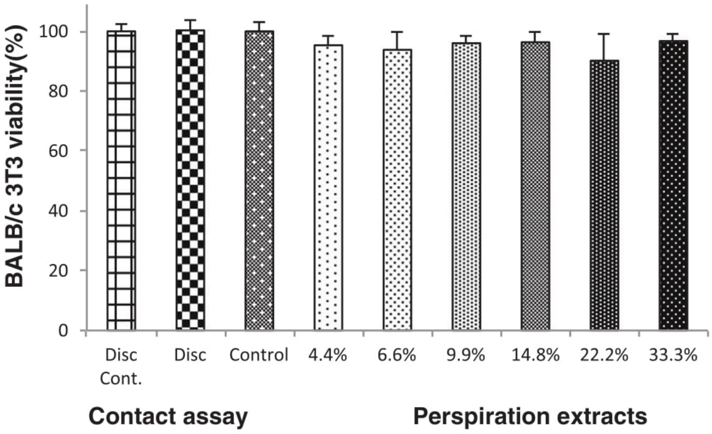

Figure 8. Perspiration extract assay and direct contact assay against mouse fibroblast cell line BALB/c 3 T3.

hydrophobic surface reduced adhesion capacity of PCL to bacteria.[30]

Although Karumidze *et al*[24] studied the effect of vB_Pae-Kakheti25 on *P. aeruginosa*, the immobilization of vB_Pae-Kakheti25 on whichever surface has not been reported yet. Since a vB_Pae-Kakheti25 multiplicity of infection of 0.1 led to a general growth inhibition of *P. aeruginosa* in BHI agar plates,[24] it would be assured that a surface functionalized with the same amount of vB_Pae-Kakheti25 would also kill *P. aeruginosa*, especially due to its maximized tail exposure and the larger area of contact of electrospun nanofibers. On the other hand, when in solution, vB_Pae_Kakheti25 bacteriophages aggregated together by generating bonds between their tails, and consequently decreasing their accessibility to interact with *P. aeruginosa*, as also proposed by Langlet et al.[31]

The growth kinetics was studied at 29.9 °C, which is the temperature of the dressing change procedure. The *P. aeruginosa* was observed to halt its exponential growth as it became infected by vB_Pae-Kakheti25 bacteriophage. This demonstrated that as soon as *P. aeruginosa* was infected, vB_Pae-Kakheti25 used its machinery for self-replication, the so-called latent period. The lysis phase began when bacteria started to have a decrease in bacterial growth rate at 120 minutes, where *P. aeruginosa* mainly undergone the release of recently-formed bacteriophages.

Our samples were adequately tested on mouse fibroblast cell line in order to ascertain whether they were cytotoxic. The perspiration extract of samples showed an average cell growth inhibition of 5.3% and the direct contact assay showed an average growth enhancement of 0.33%. A variation of growth of more than 30% in comparison with the controls is regarded as cell-toxic effect.[32] Therefore, the cytotoxicity assay for PCL-vB_Pae-Kakheti25 showed less than 30% of cellular viability reduction, making our invention a safe agent for use in contact with skin.

While there are a few commercial antimicrobial applications, they present limitations. Dermasilk® is a silk-based material with Si-QAC (Silane quaternary ammonium compounds) combining the smoothness of silk with the antimicrobial properties.[2,33] Another example is Padycare® a micromesh polyamide/lycra containing woven silver filaments.[34] Similarly, TheraBond® is a silver fabric dressing designed to optimize wound fluid management without compromising the antimicrobial properties.[2,3] In vitro studies of these garments demonstrated a significant decrease in *P. aeruginosa*. Even though there is no evidence for skin sensitization by bulk silver, a possible systemic absorption of silver particles because of a disrupt skin barrier has to be considered, while Si-QAC is reported to cause bacterial resistance and show evidence of skin sensitization.[2,33,35] For this reason, the relatively lower incidence of adverse reactions of natural products has been intensively investigated.[4,33,35] Overall, antimicrobial textiles have found uses in hygienic rather than medical field especially due to possible cytotoxic effect and sometimes low effectiveness.[4,35] Antimicrobial textiles have been designed to address activity against a broad spectrum of microorganisms[33,35,36] but there remain open questions regarding toxicity on human health, which is causing a paradigm change to move on to highly specific agents toward pathogenic bacteria. Likewise, the rising flow of strains resistant to last-resort antibiotics rekindles interest in novel approaches.[11,35–37]

Phage immobilization strategies on organic and inorganic surfaces have been recently reported[37–45]: while in some cases electrostatic binding showed phage instability and detachment,[46] covalent attachment offers a much stronger bond.[47] Still, for antimicrobial surfaces designed to utilize the inherent binding specificity and lytic activity of phage toward the bacterial host, tailed phage should be immobilized through the capsid and filamentous phage with their bacterial-capturing pole exposed.[44–46]

Therefore, in this work we surface-anchored vB_Pae-Kakheti25 bacteriophages to develop highly specific antibacterial non-woven textiles/dressings especially – but not limited to – for skin associated infections, as a skin microbiome regulator. Such textiles could also find application as outside catheter dressings to provide adequate fixation and inhibition of bacterial adhesion during catheter lifetime. To authors' knowledge this is the first investigation exploiting phages as antibacterial agents for textiles. The closest approach that one can consider related has been reported by Mao et al.,[48] where a non-lytic filamentous phage (M13) was engineered to express negatively-charged glutamates further glutaraldehyde-crosslinked upon extrusion of fibers and Kevlar coatings with the ability to attract Ag^+ in a silverization process. The engineered phage was the basis of the fiber and coating but silver was responsible for the antibacterial activity since non-lytic phage was used. Our strategy, in contrary, immobilized lytic phage onto non-woven textile as antibacterial agent.

This study showed that vB_Pae-Kakheti25 covalent immobilization on PCL nanofibers with a head-to-tail orientation added antimicrobial properties to the proposed dressing. It presented a microbial reduction of 6 log, even after 25 wash cycles, comparatively with the non-modified nanostructure after 2 h of incubation. Its activity was immediate and *P. aeruginosa* reduction was complete.

On the contrary, when in solution, vB_Pae_Kakheti25 bacteriophage aggregated and decreased its availability to interact with *P. aeruginosa*.

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Paper VII

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Polymers

Electrospinning polypropylene with an amino acid as a strategy to bind the antimicrobial peptide Cys-LC-LL-37

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ABSTRACT

Hospital isolation gowns are increasingly competitive, with brands and manufacturers contesting consumer preferences. The textile materials in contact with the skin can acquire secretions and multiresistant microorganisms, causing discomfort and health risks, respectively. A new nanofibrous substrate—polypropylene grafted with L-Cys—was developed with an increased crystallinity, providing its surface with –SH hooks necessary to efficiently cross-link the antimicrobial peptide Cys-LC-LL-37 in order to protect against nosocomial pathogens and their spread to community. Furthermore, this application does not require environmental control of humidity, and it is not susceptible to enzyme and microorganism degradation.

Introduction

Textiles are considered an attractive substrate for bacteria because of their rich nutrient content, which can function as a reservoir for bacterial infections, especially under optimal conditions of humidity and temperature. In a hospital setting, patients and clinical staff can acquire these bacteria through direct contact with contaminated textiles or their surroundings [1]. When infected in this way, they can transmit the infectious agent with ease to co-workers and patients, which is potentially dangerous if the latter are immunocompromised [2].

Synthetic fibers account for about 62.1% of the world textile market [3]. In 2015, the production of synthetic fibers roundabout 60.7 million tonnes compared to 6.1 million tonnes of cellulosic fibers. Between 2000 and 2015, the synthetic fibers production has increased [4, 5] due in large part to the enhancement of properties such as wetting and hydrophilicity [6, 7]. From 1960s, polypropylene began to conquer the markets and to become a serious competitor of natural and cellulosic fibers, due to its wrinkle-resistant properties, good mechanical strength and fast drying [8]. Polypropylene is composed by saturated linear fibers of aliphatic

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hydrocarbons, and each carbon atom possesses one methyl ramification. It has a low density of 0.90 g/cm^3 and a crystallinity between 45 and 60%, the chains of which present a helical form and group through van der Waals interactions. It has interesting properties, such as good thermobinding properties, smoothness, humidity transfer by capillarity and low cost of production. On the other side, its elasticity is inferior to polyamides. Usually, polypropylene is melted and extruded into filaments, which are then stretched, oriented and stabilized [9]. In fact, most polypropylene textiles are not fibers directly produced by extrusion, but yarns of tapes (strips or filmstrips) prepared from wider films or extruded tapes through rows with elongated holes. There are some works related with the modification of polypropylene. These modifications can be done physically during the manufacturing process, or chemically combining with other copolymers or chemicals. These methods can unfold the internal structure of the fiber, which is distinguished by its low tendency to pilling formation, and may leave modified polypropylene with a different behavior than native polypropylene during dyeing or finishing procedures [5].

The use of antimicrobial textiles is an important step in the fight against a range of hospital pathogens. They should kill a broad spectrum of harmful and antibiotic-resistant bacteria, prevent the bacterial adhesion or biofilm development and not cause skin irritation. Nosocomial infections are associated to pathogenic microorganisms that are easily spread, with Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* generally regarded as the most hazardous [10].

Both *S. aureus* and *P. aeruginosa* infections are primarily opportunistic, i.e., they seldom cause diseases in a healthy immune system, but seek to exploit any weaknesses in the body of immunocompromised patients, such as victims of burns, cancer patients or cystic fibrosis, in order to cause infections. Both strains have the ability to grow in any environment, present important virulence factors and have resistance to a large number of antibiotics [11, 12].

In recent years, because of the increasing resistance of bacteria against the antibiotics, quaternary ammonium compounds, silver, polyhexamethylene biguanides and triclosan have been tested, although with limited success. They have powerful bactericidal activity as indicated for its MIC value (minimum inhibition concentration). However, the majority have a reduced spectrum of microbial inhibition and may cause skin irritation, ecotoxicity and bacterial resistance. In addition, its incorporation in textiles substantially slows their performance and limits their accessibility to microorganisms. Moreover, these compounds lose their activity particularly during the intended use and after some washes. For this reason, large amounts of these biocides are applied to textiles to efficiently control the bacterial growth and to keep their durability [13].

To overcome these disadvantages, natural compounds mostly chitosan, hyaluronan and plant extracts are being tested as antimicrobial agents for textiles [14–17]. Also, to fulfill the customers demand for hygienic clothes, and furthermore, the growing market of medical and bioactive textiles has stimulated the research and development of new antimicrobial textiles.

Based on careful literature review, our innovative strategy to develop new antimicrobial textiles considers the utilization of natural defensive amino acids and antimicrobial peptides (AMPs) that are found in every living organism. The organisms from the elementary to most complexes, including humans, produce AMPs against microorganisms [18]. Natural antimicrobial substances have a high structural diversity and a broad spectrum of activity that includes Gram-positive and Gram-negative bacteria, fungi, and in particular cases, some viruses, with the additional advantage of not displaying cytotoxicity or ecotoxicity. Many of these substances are of peptide nature, rich in L-cysteine (L-Cys) residues [18].

The use of L-Cys and AMPs in cosmetic industry and their natural presence in the skin, hair and nails of human beings offers a guarantee of biocompatibility and non-toxicity once in prolonged contact with the body. These guarantees were already proven by our recent results regarding the biofunctionalization of cotton [19, 20], silk textiles [21, 22] and polycaprolactone foam grafted with L-Cys [23], along with a low MIC value, high durability and safety both for user and environment. In general, cationic AMPs with the best antimicrobial activity are those that have the charged and hydrophilic portions to either side with a hydrophobic portion inbetween [24]. In accordance with this, cathelicidin peptides range in length from 12 to 80 residues and may have α-helical, β-sheet or other types of tertiary structures [25]. Likewise, Cys-LC-LL-37 antimicrobial peptide has a broad spectrum, namely against Gram-positive *S. aureus* and Gram-negative *P. aeruginosa*.

This work aims to investigate the potential of natural compounds—L-Cys and Cys-LC-LL-37—as effective, durable and non-toxic antimicrobial agents with the potential for development of biomedical textiles. A new process for biofunctionalization of polypropylene textile-based material and its assessment of the biocidal activity, stability, durability and toxicity was investigated.

Experimental

Polypropylene (PP) was grafted with L-cysteine (L-Cys) (Sigma). The resulting blend was threaded on electrospinning and subsequently cross-linked with Cys-LC-LL-37 (AnaSpec). *S. aureus* was cultured on nutrient agar (Panreac) and nutrient broth (Panreac) at 37 °C 110 rpm; *P. aeruginosa* PA25 (DSM 25642) was cultured on brain heart infusion (BHI) agar and brain heart infusion (BHI) broth (Sigma) at 30 °C and 200 rpm.

Development of PP-g-SH substrates

Surface-active bulk additive grafting of PP with L-Cys

PP grafted with L-Cys (PP-g-SH) (80:20) was dissolved in trifluoroacetic acid (TFA) and dichloromethane (DCM), and stirred at 80 °C overnight. Since PP is extremely nonpolar, and dissolution follows the rule "like dissolves like," PP was dissolved in DCM, a nonpolar protic solvent with a low dielectric constant of 9.08 (< 15) [26]. TFA, with F^- as nucleophile, is also miscible with hydrocarbons and a versatile solvent for materials stable in acid [27], such as PP.

Electrospinning

Nanofibers of PP (used as a control) and of PP-g-SH (80:20) were threaded from the NanoSpider apparatus (Elmarco s.r.o. Liberec, Czech Republic). The chosen proportion 15% (w/v) PP/solvent was selected according to the homogeneity and easy detachment from the collector coating. The operating parameters were: voltage = 80 kV, droplet-collector distance = 16 cm and electrode spin = 7 r/min. The environmental parameters were: temperature = 25 °C and humidity at 20%.

Washing cycles

In order to evaluate the stability of the thiol group and if it resisted to laundry, PP-g-SH samples were subjected to 25 washing cycles each in fresh ddH_2O, according to procedure adapted from international standard EN ISO 105-C06:2010.

Characterization

Fourier transform infrared spectroscopy (FT-IR) The chemical structure as well as resulting bonds between the polypropylene and the L-Cys was analyzed using Thermo-Nicolet iS10 spectrophotometer (64 scans; spatial frequency resolution of 4 cm^{-1}).

Differential scanning calorimetry (DSC) The differences in crystallinity between the pure polypropylene and the polypropylene grafted with L-Cys were observed by differential scanning calorimetry (DSC 204 Phoenix (Netzsch)). Each sample was placed in aluminum receptacles, followed by an analysis with nitrogen atmosphere ranging from 30 to 300 °C, with a temperature gradient of 2 °C per minute. The routine procedure was performed in order to eliminate the thermal history of the analyzed samples. Melting point (T_m), enthalpy of fusion (ΔH_m) and onset temperature (T_{onset}) results were obtained. The degree of crystallinity of the pure polypropylene substrate, and the blend was calculated according to Eqs. 1 and 2, respectively:

$$\chi_{PP} = \frac{\Delta H_m}{\Delta H_m^0} \times 100\% \quad (1)$$

$$\chi_{PP-g-Cys} = \frac{\Delta H_m}{\text{Blend fraction } x \ \Delta H_m^0} \times 100\% \quad (2)$$

in which χ stays for the crystallinity, ΔH_m is the enthalpy of melting, ΔH_m^0 is the reference enthalpy of melting of 100% crystalline polypropylene.

Scanning electron microscopy (SEM) The polypropylene nanofibers, as well as the nanofibers resulting from the grafting of polypropylene with L-Cys, were observed under SEM (Hitachi S2700).

Substrate functionalization with Cys-LC-LL-37 peptide

Minimal inhibitory concentration (MIC) of Cys-LC-LL-37 activity against P. aeruginosa

The minimal inhibitory concentration of Cys-LC-LL-37 against *P. aeruginosa* was determined using the broth microdilution method, as described on M07-A6 guidelines JIS L 1902:2002, in order to define the best concentration to be used.

Cys-LC-LL-37 was dissolved in sterile ddH_2O to a final concentration of 10 µg/mL and serially diluted with sterile Mueller-Hinton broth. For each Cys-LC-LL-37 dilution, *P. aeruginosa* was inoculated as liquid culture with its turbidity adjusted to 0.5 McFarland (approximately 1×10^8 CFU/mL). After incubation for 24 h at 37 °C, antibacterial activity was observed as sediment of dead *P. aeruginosa* on the bottom of the wells. Control tubes of the medium and broth with Cys-LC-LL-37 were also incubated.

Cys-LC-LL-37 cross-linking onto PP-g-SH surface

The produced nanofibrous samples underwent dialysis (Medicell Membranes Ltd) in ddH_2O for 3 days at room temperature while gently stirred to remove any solvent and impurities. The PP-g-SH nanofibers presented thiol groups available on the surface, which were used to covalently bind Cys-LC-LL-37 antimicrobial peptide (AMP) through its thiol group, forming a disulfide bond. For this purpose, all samples and controls were functionalized by an exhaustion process with 2.5 µg/mL of Cys-LC-LL-37 at 45 °C for 60 min at 15 rpm. Finally, they were washed to remove any unbound/misbound Cys-LC-LL-37. In this way, the cationic terminal part of Cys-LC-LL-37 became exposed in order to eliminate bacteria.

Yield of the cross-linking In order to check how efficient the cross-linking was, Bradford's reagent was used to determine the concentration of Cys-LC-LL-37 in solution before and after the cross-linking. The procedure is based on the formation of a complex between the dye, Coomassie Brilliant Blue G and the amines of Cys-LC-LL-37 in solution. Briefly, 1 mL of each sample was added to a test tube containing 1 mL of Bradford's reagent, vortexed, incubated at room temperature for 5 min and measured spectrophotometrically at 595 nm.

Washing cycles

In order to evaluate the stability of the bounding of Cys-LC-LL-37 and if it resisted to laundry, PP-S-S-LC-LL-37 samples were subjected to 25 washing cycles each in fresh ddH_2O, according to procedure adapted from international standard EN ISO 105-C06:2010.

Elemental analysis

The atomic composition of the functionalized substrate was analyzed by energy-dispersive X-ray spectroscopy (EDS), a device interconnected with SEM.

Antimicrobial activity assessment

Samples were tested through E 2180-01 Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agents that is designed for testing hydrophobic materials suited for the healthcare industry. Briefly, *Staphylococcus aureus* and *Pseudomonas aeruginosa* inocula were prepared from an overnight liquid culture. Bacterial concentrations were then adjusted to 1–2 × 10^8 cells/mL (equivalent to 0.5 McFarland) and working standards prepared to a final concentration of $1 \pm 0.3 \times 10^5$ CFU/mL. Textiles were placed in Falcon tubes to release the bacterial cells from the textile samples after the 24-h incubation period. The resulting suspensions were then plated to determine the viable counts. Antimicrobial activity was calculated in order to determine bacterial growth inhibition (% of Inhibition) at 24 h:

$$\%\text{Inhibition} = \frac{C - A}{C} \times 100 \tag{3}$$

where C is the average value of colony-forming units (CFU) of controls, and A represents the average value of CFU of PP-S-S-LC-LL-37.

To determine whether the developed substrate has bacteriostatic or bactericidal activity, the following equations were solved:

$$\text{Bacteriostatic activity} = M_b - M_c \tag{4}$$

$$\text{Bactericidal activity} = M_a - M_c \tag{5}$$

where M_a is the log10 of the average number of bacterial colonies on the control samples at T0, M_b is the log10 of the average of bacterial colonies on the control samples at T24 h, and M_c is the log10 of the average of bacterial colonies on the functionalized samples at T24 h.

Statistical analysis

Samples were analyzed statistically, and comparisons that had a p value < 0.5 were considered statistically different. One-way ANOVA and Tukey post Hoc tests for multiple comparisons between all samples were performed with SPSS 21.0.

Results

Surface-active bulk additive grafting

Fourier transform infrared spectroscopy (FT-IR)

Figure 1 depicts the IR peaks relative to the polypropylene. The strong peaks of polypropylene at 2980 and 2971 cm^{-1} are associated with C–H stretching vibrations of methyl group; 2888 cm^{-1} is associated with C–H stretching vibrations of methylene group. The vibrations at 1463 and 1382 cm^{-1} are due to methylene and methyl bending, respectively. In addition, medium intensity peaks are observed at 1150 cm^{-1}, which is related to both methyl symmetric and antisymmetric deformation, whereas the one at 952 cm^{-1} is related to methyl group rocking vibration, respectively.

PP-g-SH after 25 washing cycles was also assessed by FT-IR. IR spectra confirmed L-Cys grafted on PP, as shown by the evidence of the weak intensity peak associated with -SH exposure on PP surface at 2550 cm^{-1}. The peaks observed in the high-frequency region, namely 2950 and 2910 cm^{-1}, are related to the vibration of the alkyl thiol chains. Furthermore, S-H deformation vibration was detected with the peaks 895, 839 and 805 cm^{-1}. Also the peaks at 2950 and 2879 cm^{-1} are related to both methyl asymmetric and symmetric vibrations. These bands (narrow peaks) might be attributed to the alkyl chains of PP crosslinked with the L-Cys.

The peak at 3630 cm^{-1} is assigned to structural hydroxyl groups of L-Cys, which occupy the empty positions of PP-g-SH.

The peaks at 2888 and 1463 cm^{-1} assigned to methylene vibrations increased in intensity as crystallinity develops—PP-g-SH—and were less evident with the less crystalline state of PP.

Differential scanning calorimetry (DSC)

The thermal properties of PP and PP-g-SH were investigated (Fig. 2). The melting temperatures (T_m) for control and PP-g-SH were obtained from the heating scan. PP showed a T_m of 164.8 °C, while PP-g-SH presented one of 167.42 °C. Furthermore, the crystallinity of PP-g-SH also increased—65.07%—when compared to PP – 51.39%. This higher number of connections causes an interconnected pore network and a high surface area per unit mass, which is an advantage to microbial killing. The enthalpy of fusion

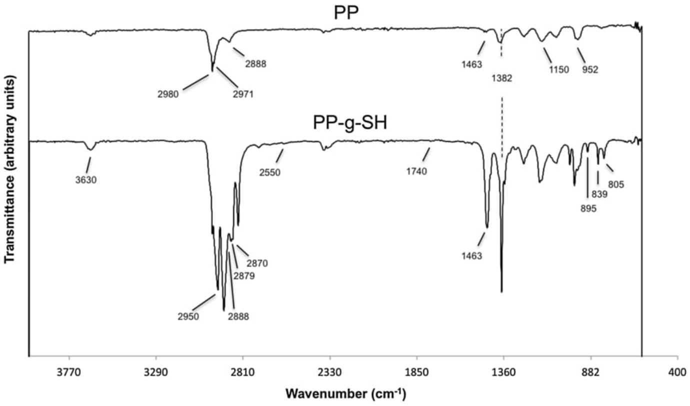

Figure 1 IR spectra of native PP and PP-g-SH.

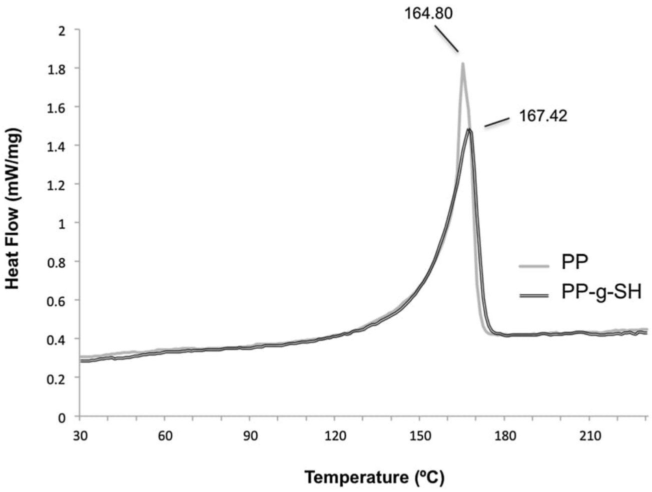

Figure 2 Differential scanning calorimetry spectra of PP and PP-g-SH.

(108.8 J/g) of PP-g-SH was higher than the enthalpy of fusion for PP (107.4 J/g).

Scanning electron microscopy (SEM)

The topography of PP and PP-g-SH nanofibers was observed by SEM microscopy, and their structural differences are shown in Fig. 3. The PP-g-SH condition revealed higher number of nanofibers per area with increased cross-linking between them, when comparing to PP nanofibers. Hence, PP-g-SH exhibited an increased surface area per mass.

Substrate functionalization

Yield of the cross-linking

The concentration of Cys-LC-LL-37 in solution before and after its cross-linking on PP-g-SH resulted in the cross-linking efficiency of 95.30%.

Minimal Inhibitory concentration (MIC) of Cys-LC-LL-37 activity against P. aeruginosa

The minimal inhibitory concentration (MIC) value for *P. aeruginosa* was 5 μg/mL.

Energy-dispersive X-ray spectroscopy (EDS)

EDS analysis allowed us to analyze the atomic elements of the PP, PP-g-SH and PP-S-S-LC-LL-37 samples. The fact that polypropylene does not present sulfur in its native structure allowed us to easily compare the results of the modifications that were made. The first modification of the polypropylene, made by surface modifying additives grafting, generated thiol groups on the surface of the nanofibers (PP-g-SH), at 0.10% wt.(percentage by weight). The coating of Cys-LC-LL-37 on thiol groups yielded PP-S-S-LC-LL-37. PP-S-S-LC-LL-37 amount of sulfur was 0.21 wt%. In conclusion, for each sulfur detected in PP-g-SH, there was one Cys-LC-LL-37 bound, which means a very high cross-linking efficiency.

Antimicrobial activity assessment

The E 2180-01 Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) in Polymeric or Hydrophobic Materials was followed to determine the viability reduction with the application of the PP, PP exhausted with Cys-LC-LL-37, PP-g-SH and PP-S-S-LC-LL-37. The antibacterial efficiency of these samples was quantitatively evaluated against *S. aureus* and *P. aeruginosa* (Fig. 4).

Following 25 washing cycles, the assay showed for *S. aureus* that after 24 h of contact with PP-S-S-LC-LL-

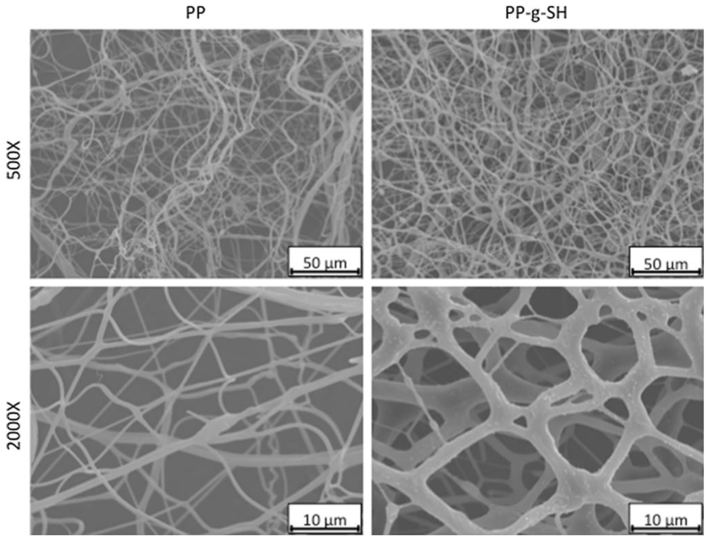

Figure 3 SEM images of 15% (w/v) PP and PP-g-SH. These nanofibers were electrospun from NanoSpider.

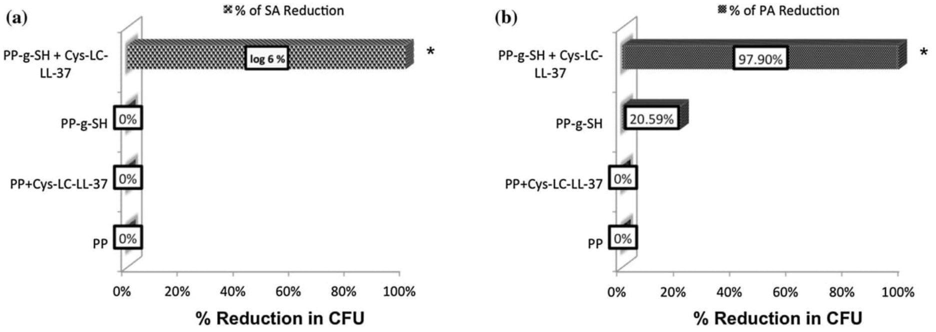

Figure 4 Percentage of microbial inhibition against *S. aureus* (**a**) and *P. aeruginosa* (**b**). Values were derived from results of native PP. * Statistically significant ($p < 0.05$).

37, there was a total reduction in their viability of 99.9999% in comparison with the PP, as shown in Fig. 4a. For *P. aeruginosa* after 24 h of contact with the same condition—PP-S-S-LC-LL-37—there was a remarkable reduction in viability of 97.41% in comparison with the PP (Fig. 4b). However, when thiol groups were exposed at the surface of PP, they were unable to inhibit the growth of *S. aureus* at all, but 20.59% of *P. aeruginosa*. As expected, the condition in which PP was exhausted with Cys-LC-LL-37 had no reactive groups to form any linkage, hence no microbial reduction was observed for both strains. It is possible to observe that the percentages of viability reduction were the highest and significant for PP-S-S-LC-LL-37, which translates the stability of the disulfide bond between PP-g-SH and Cys-LC-LL-37 throughout 25 washing cycles and the effectiveness of the antimicrobial peptide Cys-LC-LL-37.

According to Eqs. 4 and 5, for *S. aureus* – – 0.09 – and *P. aeruginosa* – 0.16 –, there was no bacteriostatic effect of PP-g-SH, but a bactericidal effect of PP-S-S-LC-LL-37, for both *S. aureus* – 3.52 – and *P. aeruginosa – 1.68*. This meets JIS L 1902, which states that the bacteriostatic activity of a sample cannot be inferior to 2 and the bactericidal value cannot be inferior to 0.

Discussion

Due to the high number of patients afflicted, our approach was rather on an application that would act toward the prevention of microorganism establishment in garment and, in this way, a consequent eradication of these microorganisms in the hospital air. It is important to underline that polypropylene fibers are the most used in hospital garments either as surgical protective clothing as patient disposable garments used in clinical settings. Therefore, this investigation is of the utmost importance since it is the first to report novel ways of having polypropylene garments with an added antimicrobial function. Our investigations led to the development of a garment with thiol hooks, which allowed the functionalization with the antimicrobial peptide Cys-LC-LL-37 through its cysteine thiol. In this way, we sought to obtain an application resistant to multiple washing cycles, namely whether the disulfide bond formed would be resistant to 25 washing cycles and if Cys-LC-LL-37 would be intact and equally bioactive after these washing cycles had been performed.

At first we looked at how far we could modify polypropylene through modification by L-Cys and produce nanofibers by electrospinning, other than by the extrusion technique that would most probably deactivate the activity of the biomolecules. The production of nanofibers was successful, both for PP (polypropylene) and PP-g-SH (PP grafted with L-Cys), yielding the latter ones more conspicuous nanofibers and in a greater number, as compared to polypropylene control. The results of FT-IR and EDS showed that PP-g-SH was exhibiting thiol groups at the surface of its nanofibers. In addition, DSC showed an increase in T_{fusion}, which meant that stronger bonds were formed as well as higher number of cross-links. As consequence of stronger bonds, an increase in crystallinity was also observed. Furthermore, this higher number of cross-linking caused an interconnected pore network and a larger surface area per unit mass, which is an improvement to microbial killing. Moreover, when PP melted, dissolved and combined with L-Cys, its decreased enthalpy of fusion (1.48 mW/mg) might be due to weak intermolecular forces and the grafting reactions between PP and L-Cys, where PP chain was split into parts to combine with L-Cys to produce PP-g-SH. The enthalpy of fusion for PP was 1.82 mW/mg.

These data led us to test, in a second approach, whether (i) the thiol hooks exposed on the modified polypropylene (PP-g-SH) were capable of exerting an antimicrobial effect themselves or, on the other hand, (ii) the coating of the antimicrobial peptide Cys-LC-LL-37 between thiols would be a better strategy for this application, in terms of antimicrobial effect. The coating of Cys-LC-LL-37 over PP-g-SH had a very high cross-linking efficiency, which accounted for 95.30%. The results provided by elemental analysis (EDS) of the aforementioned surfaces indicated the presence of the double of sulfur atoms in the samples PP-S-S-LC-LL-37, when compared to PP-g-SH. This confirms the success of the coating between PP-g-SH and Cys-LC-LL-37, since all thiol groups of PP-g-SH were mainly occupied bonding the Cys of the antimicrobial peptide. While PP-g-SH showed to exert no bacteriostatic effect (0% for *S. aureus*; 20.59% for *P. aeruginosa*), the Cys-LC-LL-37 coating on PP-g-SH, with half of the concentration of MIC obtained for both bacteria, induced a bactericidal response (99.9999% for SA; 97.90% for PA) after 25 washing cycles. We have also discovered, at the test-control level, whether mixing of PP with Cys-LC-LL-37 would lead to the formation of any bond and, as expected, it was found that Cys-LC-LL-37 did not cross-link to any nanofiber of polypropylene, either by EDS that showed no traces of sulfur – 0 wt% – or by antimicrobial assays that showed no microbial reduction. This explains why the presence of the thiol hooks is odd to establish the resistant disulfide bond when linking to Cys-LC-LL-37. We also found from EDS results and antimicrobial assays that Cys-LC-LL-37 was not released from the PP-g-SH surface, regardless of the washing cycles performed, up to 25. Furthermore, due to the fact that Cys-LC-LL-37 is charged and presents hydrophilic portions separated from the hydrophobic areas, proved advantageous over only thiolated groups. A thiolated surface with no spacer that could distance –SH from surface of nanofibers did not confer flexibility and exerted no bacteriostatic or bactericidal effect.

The release of toxic substances from a textile product with skin contact is a precondition for the formation of skin irritations. However, examination on cytotoxicity of components used separately in previous works [22, 23, 28] permitted the evaluation of the danger potential for a skin irritation. Cys-LC-LL-37 was tested against normal human dermal fibroblasts, the results of which showed no growth variation greater than 30%, which means that this antimicrobial peptide is not cytotoxic and can be used for skin contact [28]. The testing of cytotoxicity of L-Cys on connective tissue cells L 929 also showed a growth variation inferior to 30% [29]. Polypropylene showed no cytotoxicity when its extracts were blended in plasma [30], where it was shown that there was no difference on viability and cell adhesiveness of chick-embryo fibroblasts. Also, the morphology of calf-kidney fibroblasts was also normal [30]. The three agents used in this study are therefore considered to be safe agents.

Thus, considering previous research and the potential of electrospinning for the development of nanofibers for use in antimicrobial garments, we intended to develop a new garment to kill microorganisms and thereby control the safety of hospital environment. It is thus expected that a garment free of microorganisms will not promote their spread into the hospital environment.

Conclusions

This study addresses the potential of L-cysteine amino acid to graft polypropylene chain, so as to thread nanofibers through electrospinning. These nanofibers provided a novel, smoother and increased surface-to-area substrate with thiol group hooks, which could be used to bind the antimicrobial peptide Cys-LC-LL-37 through disulfide bonding.

The skin of patients with diabetes or immunodeficiency is particularly susceptible to infection by different microorganisms. The opportunistic pathogens *S. aureus* and *P. aeruginosa* are the agents responsible for some lethal infections under hospital settings. In this way, our application sought to impede the dispersal of pathogens to the air, which should minimize their settling and reduce the chances of cross infection.

The grafting of L-Cys on polypropylene did not provide any significant antibacterial activity. Instead, its free thiol groups promoted an effective means of cross-linking to Cys-LC-LL-37, which dramatically reduced *S. aureus* by 99.9999% (Log 6) and *P. aeruginosa* by 97.90%.

In this regard, a new material, safe, with strong antimicrobial properties and alternative to antibiotics is herein presented for potential use as garment under hospital settings.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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Chapter IV - General discussion and concluding remarks

General discussion and concluding remarks

The use of antimicrobial textiles, especially in close contact with the patients and in the immediate and non-immediate surroundings, may significantly reduce the risk of infections. However, they should possess broad-spectrum biocidal properties, be safe for use and highly effective against antibiotic resistant microorganisms, including those that are commonly involved in hospital-acquired infections (Kazmierczak *et al.*, 2014).

Herein it was presented chemical and/or physical strategies to crosslink antimicrobials on textile or fibrous materials, which allowed, in most cases, the bacteria to be attracted and eliminated. In order to evidence the mechanism of antimicrobials adsorption and stability after several washing cycles at the molecular scale, the utilization of textile and polymer models with a well and defined surface structure, were used, such as cotton, silk, polycaprolactone (PCL), and polypropylene (PP). Textiles, rather than thin model surfaces, were used in order to observe the antimicrobial effect in a real context, once antimicrobial compounds are able to rearrange towards the bulk of the fibers.

The first approach intended to modify cotton into a surface that would adsorb L-Cysteine (L-Cys) in a covalent and irreversible way, providing a natural coat of this amino acid on the surface so as to turn cotton antimicrobial. L-Cys, due to its thiol group, acts as antimicrobial and presents antioxidant activity (Nogueira *et al.*, 2014). The rationale of the covalent bonding behind this approach is that L-Cys be not leached over time, because if L-Cys covalent bonding is stable, cotton surface might maintain its antimicrobial properties after laundry. Cotton samples with different amounts of surface activator CDI were produced, ranging from 0% to 60%(w/V). Then, 5 and 7.5% owf (over the weight of the fabric) of L-Cys were added to the substrate. An increased intensity of EDS and FT-IR peaks corresponding to S atoms and -SH groups, respectively, indicated that the best adsorption was with 4%(w/V) CDI, the minimum amount of activator, either bound to 5% or to 7.5% of L-Cys. However, the antimicrobial results for 7.5% of L-Cys were lower than 5%, which could be explained due to the amino acid too densely packed and thereby compromising its binding to *S. aureus* and *K. pneumoniae*.

In the following work, cotton was then non-covalently functionalized with antimicrobial peptides Magainin I and LL-37 that were linked via their amines to cotton after the activation of surface with TEMPO reagent that exposed carboxylates on cotton surface. In order to go further concerning non-covalent and covalent bond strategies, a polymer hard to be modified - polypropylene - was modified with L-Cys, which strengthened its structure in nanofibers and endowed it with thiol hooks that allowed to bind the peptide Cys-LC-LL-37 via disulfide covalent bond. Cys-LC-LL-37 was found to have resisted successive wash cycles, and the bactericidal capacity of this antimicrobial peptide was unique to the elimination of the microorganisms. For both experiments, the covalent strategy gave absolute stability to laundry. Notwithstanding that the nanostructure produced may also have contributed to increase the surface area and better control Cys-LC-LL-37 orientation on the surface.

In order to functionalize other textile fibers normally used as medical textiles, L-Cys below MIC of 5% was tested in silk fibroin, through a double covalent strategy i. e., two covalent binding strategies at different sites of the structure were tested. It was possible to reduce L-Cys amount to 1% and make the application bioactive after successive wash cycles, which demonstrated that tyrosine was key factor for binding L-Cys, because of its exposed conformation in silk fibroin structure. The single covalent strategy, on the other hand, was not so efficient in microbial killing, which seemed more appropriate for a disposable application.

A PCL foam in the form of a cylinder was also developed to be applied in pressure ulcers stage III, which are ulcers with holes. L-Cys was covalently linked to PCL via aminolysis, which made hydrophobic-to-hydrophilic conversion due to L-Cys hydrophilic properties. Furthermore, this application showed swelling properties in the presence of alkaline pH of ulcers surface, due to -OH and -SH groups of L-Cys grafted on PCL, which seems essential to fill the hole of open ulcers.

The work and knowhow developed at this stage allowed to covalently immobilize the vB-Pae-Kakheti phage capsid to the surface of polycaprolactone nanofibres produced by electrospinning, so that the phage had its tail oriented outwardly of the nanofibers. Here again it has been confirmed that not only the presence of an antimicrobial but also the way it is linked makes all the difference in the antimicrobial process. Figure 2.

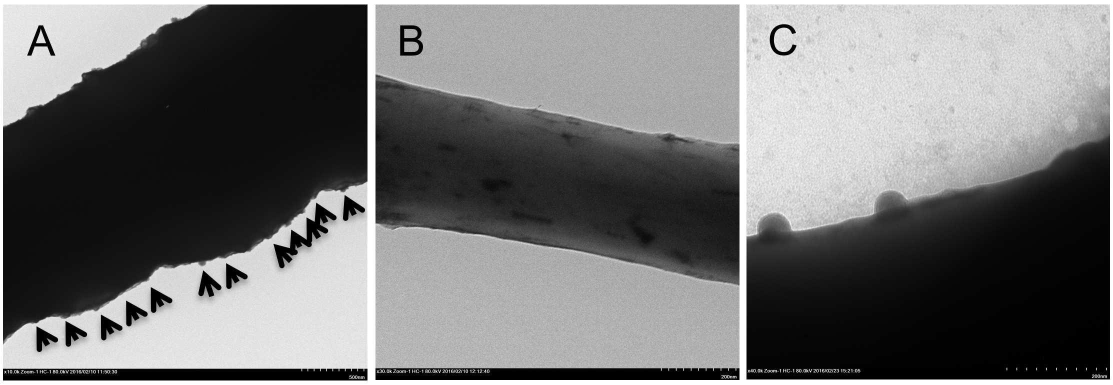

Figure 2. vB-Pae-Kakheti phage capsid covalently bound to the surface of polycaprolactone nanofibres

Antimicrobial studies were performed, according to Japanese Industrial Standard JIS L 1902:2002 and E 2180-01 Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) in Polymeric or Hydrophobic Materials, and show the association between bacteria and the immobilized antimicrobial compound at the various substrates surface tested. Collectively, all antimicrobial results (%) of all works after washing-cycles were the following:

Table 1. Antimicrobial results of gathered works

Application	*S. aureus*	*K. penumoniae*	*P. aeruginosa*
Cotton-L-Cys	83	89	-
Cotton-Magainin I	58	15	-
Cotton-LL-37	59	89	-
SF-L-Cys double cov	97.55	-	-
SF-L-Cys single cov	67.39	88.88	-
PCL-L-Cys	99.67	-	-
PP-L-Cys	0	-	20.59
PCL-phage	-	-	99.9999 (log 6)
PP-L-Cys-Cys-LC-LL-37	99.9999 (log6)	-	97.90

The antimicrobials that showed the best performance were the amino acid L-Cys, the antimicrobial peptide Cys-LC-LL-37 and bacteriophage vB_Pae-Kakheti25. Furthermore, the compounds covalently bound resisted better to washing cycles than non-covalently bound.

Adhesion studies evidenced a contrast between native textile or polymer surfaces and the coated surface. Observation of the morphology of adherent bacteria evidenced that the specific binding to antimicrobial surfaces is a required step for the pathogen to lose its characteristic shape and lyse. Figure 3.

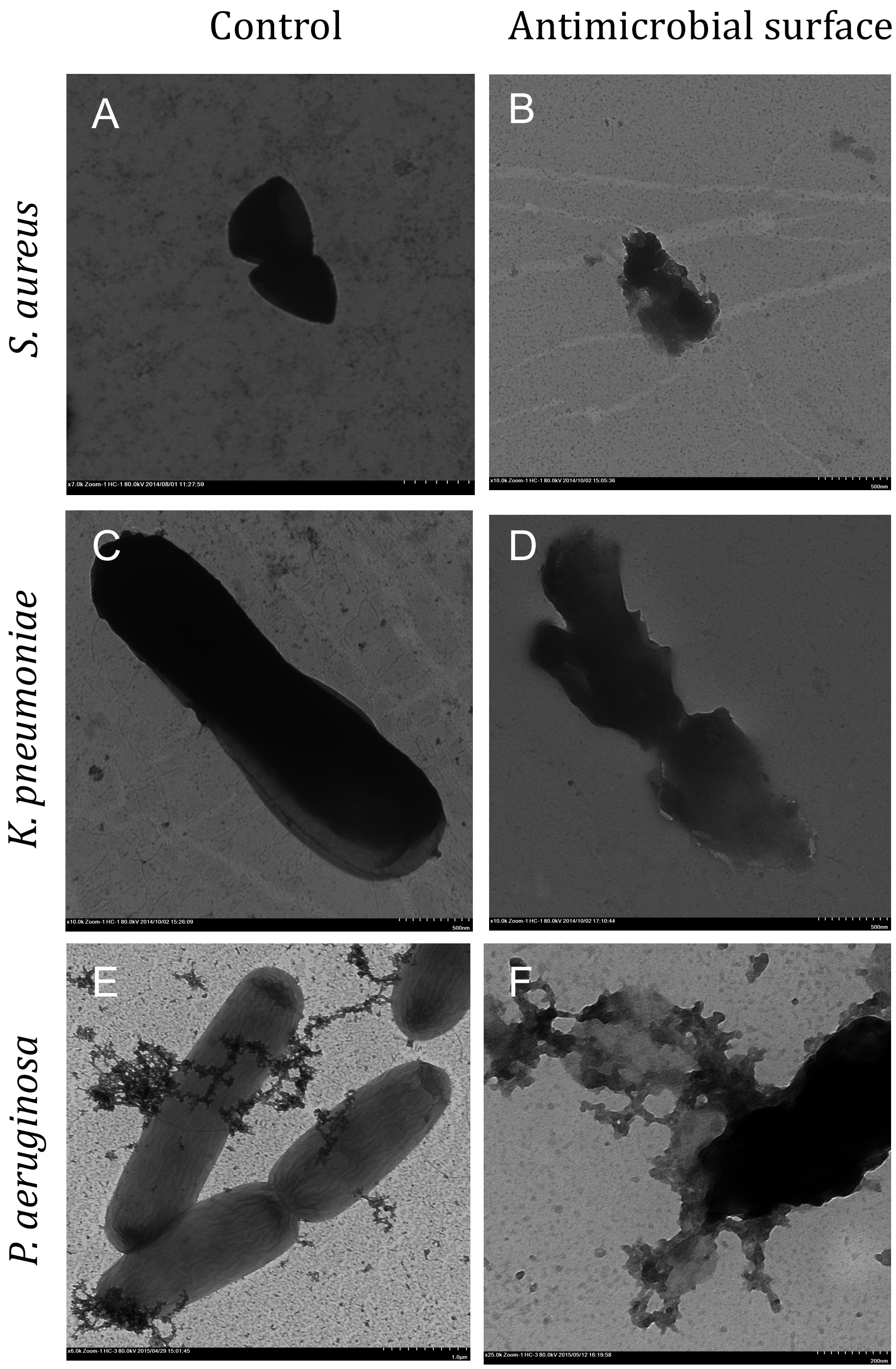

Figure 3. Morphology of healthy bacteria (left) Vs. Compromised bacteria by the antimicrobial surface (right)

All applications were considered safe after cytotoxicity tests, according to E DIN EN ISO 10993-5.

Altogether, these results highlight that the "new" antimicrobials immobilized on different surfaces with high yield are able to eliminate nosocomial bacteria. Depending on whether a long-term or short-term application is desired, covalent or non-covalent strategy can used, respectively.

Therefore, the use of these antimicrobials and respective strategies to functionalize textiles and polymers, such as cotton, silk, polycaprolactone a polypropylene, with an antimicrobial function, is a resourceful tool to be employed for the design of antimicrobial textiles to be used in clinical settings. Also, the main advantage of the ones herein presented is that it targets infection in the early stages and the most upstream possible, since it aims to prevent the skin or mucosa colonization by these bacteria.

Future Perspectives

The structural nanotopography offers new prospects for future work. It is created by the nanodeposition of material on a surface or the removal of part of it, where bacteria respond to nanotopography. In the few already published works, with some encouraging results, authors selected fly wings and dehydrated their structure. With the nanolithography technique we intend to design nanopillary structures with different combinations and spacings, and test their antimicrobial effect. We predict that the surface of the future will only be based on surface nanotopography and free of any antimicrobial compound.

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