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Layer-by-Layer deposition of bioactive polyelectrolytes with incorporation of antimicrobial agents as a new strategy to develop bioactive textiles

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Dedicatory

To my children Filipe, Ana Sofia and Maria Inês

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Resumo

Nos últimos anos, o uso de têxteis com capacidade antimicrobiana tem vindo a aumentar significativamente. Compostos sintéticos antimicrobianos utilizados em artigos têxteis são muito eficazes face a uma grande gama de microrganismos. Mas o uso de têxteis antimicrobianos de forma contínua pode levar à resistência bacteriana e sensibilização dos utilizadores, bem como causar problemas ao meio ambiente. Para minimizar estes riscos existe atualmente uma grande procura de têxteis antimicrobianos produzidos com compostos naturais não tóxicos e amigos do ambiente. A baixa incidência de efeitos adversos com origem em compostos naturais relativamente aos compostos sintéticos pode ser explorada como uma alternativa atraente e promissora para aplicações têxteis.

O método de funcionalização por camada sobre camada (Layer-by-Layer, LbL) pode fornecer novos tipos de revestimentos em materiais têxteis. Esta técnica LbL tem ganho uma grande aceitação na investigação académica e a nível industrial. Foi proposta por Decher e seus colaboradores no início dos anos 90 e desde então o seu impacto positivo pode ser comprovado através do crescente número de trabalhos publicados.

Revestimentos por multicamadas de polieletrólitos naturais bioativos tornam-se num processo novo de funcionalização de superfícies. Esta técnica é desenvolvida em meio aquoso e envolve tipicamente a adsorção alternada de polieletrólitos de cargas opostas. A possibilidade de fabricar tais camadas, graças às interações eletrostáticas, permite a funcionalização de superfícies de praticamente qualquer tamanho e forma.

Este conceito não é novo para algumas aplicações como seja a libertação de fármacos, mas é relativamente novo para aplicações têxteis. Na revisão da literatura foram encontrados alguns, mas poucos, trabalhos de investigação relativamente à aplicação do LbL em substratos têxteis naturais, como seja o caso do algodão. O uso de polímeros naturais para obter estas camadas pode auxiliar na resolução de problemas que ocorrem com os polímeros sintéticos. Relativamente aos polímeros naturais mais utilizados encontram-se o quitosano (CH) e o alginato (ALG) que são polissacarídeos bastante conhecidos por serem biocompatíveis, biodegradáveis, antimicrobianos e não tóxicos.

Neste trabalho apresentam-se os resultados sobre a viabilidade e sucesso da deposição de camadas de polieletrólitos de CH e de ALG pela técnica do LbL em fibras de algodão. O revestimento do algodão por multicamadas de CH e ALG é construído através da adsorção de CH de carga positiva e oposta à carga da superfície do algodão, seguida pela adsorção de ALG de carga negativa, ou seja oposta á carga do CH. O substrato de algodão utilizado para a deposição das várias camadas foi pré-tratado antes da deposição dos polieletrólitos, de forma a ativar a sua superfície deixando-a com cargas negativas. A deposição sucessiva das

multicamadas de polieletrolitos foi analisada por 3 técnicas diferentes. Cálculo do ângulo de contacto entre uma gota de água e a superfície da amostra, coloração com um corante catiónico e análise por ATR-FTIR (*Fourier Transform Infrared Spectroscopy* with *Attenuated Total Reflection*). Estas técnicas indicaram que houve uma deposição alternada entre o CH e o ALG e também a presença de ligações eletrostáticas entre as camadas. Ficou assim demonstrado o sucesso na deposição de CH e ALG pela técnica do LbL em substrato têxtil de origem natural, neste caso o algodão.

Com o fim de avaliar a atividade antibacteriana das amostras de algodão funcionalizadas, seguiu-se a norma Japonesa JIS L 1902:2002 para o método do halo (teste qualitativo) e método de absorção (teste quantitativo). Estes testes revelaram um efeito antibacteriano das amostras funcionalizadas, tanto para bactérias Gram-positivas (*Staphylococcus aureus*) como Gram-negativas (*Klebsiella pneumoniae*). Com estes resultados verificou-se que era possível preparar estruturas com propriedades específicas. Este método permite assim a possibilidade de desenvolver novos produtos têxteis funcionais para aplicações biomédicas, podendo também com este método do LbL obter amostras que tenham um papel no desenvolvimento de um sistema de libertação de fármacos no local pretendido.

As amostras anteriores foram ainda analisadas por microscopia eletrónica de varrimento (*Scanning Electron Microscopy*, SEM). Esta análise teve como objetivo visualizar o grau do dano sofrido na estrutura das bactérias testadas por ação do CH e ALG. A fase seguinte consistiu em otimizar um método para incorporação de L-cisteína (L-cys), que é um agente antimicrobiano, entre as camadas de CH e ALG depositadas em amostras de algodão pelo método do LbL. Entre os diversos métodos utilizados para incorporar a L-cys, o que melhores resultados produziu foi aquele onde se fez uso da propriedade do ALG em formar gel na presença de cálcio. Verificamos que a L-cys pode ser incorporada diretamente entre as camadas de CH e ALG sem que ocorra qualquer ligação covalente entre a L-cys e os polieletrólitos de CH e ALG. Desta forma o agente bioativo (L-cys) ficou imobilizado sem perder as suas características bioativas e tem como grande vantagem a possibilidade de podermos selecionar outros tipos de agentes bioativos sem a necessidade de nova otimização do método de incorporação. Nestas novas amostras foram analisadas as propriedades antibacterianas para o *Staphylococcus aureus* e para *Klebsiella pneumoniae* segundo a norma já referida anteriormente, e os resultados mostraram um aumento no efeito antibacteriano devido à presença da L-cys.

Por último, a L-cys foi substituída por péptidos antimicrobianos (antimicrobial peptides, AMPs), já que são a nova geração de antimicrobianos. Foram utilizados 4 AMPs de características diferentes. A profundidade em que cada AMPs se encontra incorporado entre as camadas foi determinada por análise de energia dispersiva de raios X (*Energy Dispersive X ray*, EDS). Para estas últimas amostras foram feitos os testes antibacterianos e analisada a citotoxicidade para o valor das concentrações usadas. Foram também analisadas as curvas de libertação para o exterior dos AMPs incorporados no algodão funcionalizado. Com os resultados obtidos confirma-

se que esta nova funcionalização de algodão revestido com camadas de CH e ALG pela técnica do LbL e com incorporação de AMPs, conduz a bons resultados antimicrobianos e de citotoxicidade, podendo assim estas amostras ser utilizadas na área da saúde, especificamente como compressas.

Palavras-chave

Layer-by-Layer, Agentes antimicrobianos, Têxteis bioactivos, Aplicações biomédicas, compressas.

Abstract

Polyelectrolyte multilayer coatings have become a new and general way to functionalize a variety of materials. Particularly, the Layer-by-Layer (LbL) method is a technique developed for the coating of solid surfaces. The LbL technique presents a unique mean to construct surface coatings that can conform to a variety of biomaterial surfaces and serve as matrices enabling controlled delivery of bioactive molecules from surface. As the deposition process is achieved in aqueous medium, incorporation of active agents is possible since the coatings obtained by LbL are less densely packed and this is advantageous for diffusion through the coating.

The coating is constructed by the alternate adsorption of oppositely charged polyelectrolytes at the surface of the material, easily obtained when it is dipped in polyelectrolyte solutions. A deposition cycle creates a layer, and these cycles can be repeated as often as needed.

This study aims to obtain novel bioactive textiles with potential application as wound-dressings. The biopolymers chosen for the functionalization of cotton (substrate), were chitosan (CH) and alginate (ALG). The multilayer coating of cotton with CH and ALG is constructed by the adsorption of CH and ALG with opposite charge on the surface of cotton substrates. The successive deposition of multilayers of CH and ALG was analyzed by three different techniques. Contact angle between a water droplet and the surface of the sample, cationic dye staining method and analysis by ATR-FTIR (*Fourier Transform Infrared spectroscopy with Attenuated Total Reflection*). These techniques showed that there was alternating deposition between CH and ALG and the presence of electrostatic bonds between the layers. In order to evaluate the antibacterial activity of the functionalized cotton, the Japanese standard JIS L 1902:2002 for the halo method (qualitative assay), and the absorption method (quantitative test) were assessed. These tests revealed an antibacterial effect on the functionalized cotton for both Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria *(Klebsiella pneumoniae*).

In addition a method was optimized for incorporating L-cysteine (L-cys) between the layers of CH and ALG deposited on cotton samples by the LbL, in order to obtain a better antimicrobial effect. Several strategies were used and the best results were obtained by the method where the ALG turns into a gel in the presence of calcium, since L-cys can be incorporated directly between the layers of CH and ALG without any covalent bond. Thus, the bioactive L-cys agent was immobilized without losing its bioactive characteristics. These new samples were analyzed for the antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* according with the previously used standard, and the results showed an increase in the antibacterial effect due to the presence of L-cys.

This new coating method has the great advantage to able to select other types of bioactive agents without needing further optimization. In this way, L-Cys was replaced by antimicrobial peptides (AMPs). The reason for the use of AMPs is related with the continuous use of antibiotics

which resulted in multiresistant bacterial strains all over the world. Consequently, there is an urgent need to search for alternatives for antibiotics. The AMPs are the new generation of antimicrobials. Four AMPs of different features were used. The depth in which each AMPs is incorporated between the layers was determined by energy dispersive analysis of X-rays (Energy Dispersive X-ray EDS). Results showed, that all AMPs used have a higher antimicrobial effect when compared with previous samples (with and without L-Cys) for both microorganisms and are non-cytotoxic to normal human dermal fibroblasts at the tested Concentrations. This confirms that this new functionalization approach of cotton coated with layers of CH and ALG by the LbL technique with incorporated AMPs leads to good antibacterial and cytotoxicity results, which make them suitable to be used as wound dressings.

Keywords

Layer-by-Layer, Antimicrobial agentes, Bioactive textiles, Biomedical applications, wound dressing

Thesis Overview

This thesis is structured in four main chapters

The **first chapter** includes an introduction divided in two sections. One section explains the importance of antimicrobial textiles, the most used antimicrobial agents (synthetic and natural compounds) and a review of antimicrobial activity in textile materials. The second section is presented as a publisher paper review form – Layer-by-Layer assembly as a promising technique for the biofunctionalization of cellulosic fibres with emergent antimicrobial agents. (**PAPER I).**

The second chapter presents the main purpose and the specific goals that were established for the development of this research work.

In the **third chapter**, the results obtained during this work are presented and discussed in the form of original research papers organized as follows:

PAPER II – Layer-by-Layer Deposition of Antibacterial Polyelectrolytes on Cotton Fibres

PAPER III – Assessment of bacteria-textile interactions using Scanning Electron Microscopy: A study on LbL chitosan/alginate coated cotton.

PAPER IV – Layer-by-Layer deposition of antimicrobial polymers on cellulosic fibers: a new strategy to develop bioactive textiles.

PAPER V – New Biomaterial Based on Cotton with Incorporated Biomolecules.

PAPER VI – Incorporation of antimicrobial peptides on functionalized cotton gauzes for medical applications**.**

In the fourth chapter is made a general discussion of the results obtained over all paper, concluding remarks about this work and some future work are suggested to complement this study.

Index

List of Scientific publications

Papers included in the thesis resulting from this Doctoral work

PAPER I - Layer-by-Layer assembly as a promising technique for the biofunctionalization of cellulosic fibres with emergent antimicrobial agents.
Ana P. Gomes, João Mano, João Queiroz, Isabel C. Gouveia (Submitted for publication in Advances in Polymer Science)

PAPER II - Layer-by-Layer Deposition of Antibacterial Polyelectrolytes on Cotton Fibres
Ana P. Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
Journal of Polymer Environment. 2012. 20:1084-1094

PAPER III - Assessment of bacteria-textile interactions using Scanning Electron Microscopy: A study on LbL chitosan/alginate coated cotton Ana P. Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
Microscopy: Science, Technology, Applications and Education
Microscopy book series. Number 4 Volume 1, pp 286-292
A. Méndez – Vilas and J. Diaz (Eds.) – FORMATEX 2010

PAPER IV - Layer-by-Layer deposition of antimicrobial polymers on cellulosic fibers: a new strategy to develop bioactive textiles
Ana P. Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
Polymers advanced technologies. 2013, 24 1005-1010

PAPER V - New Biomaterial Based on Cotton with Incorporated Biomolecules
Ana P. Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
Journal of Applied Polymer Science. 2014, APP. 40519

PAPER VI - Incorporation of antimicrobial peptides on functionalized cotton gauzes for medical applications.
Ana P. Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
(Submitted for publication in Carbohydrate Polymers, reference CARBOPOL-D-14-02437)

List of Scientific communications

Oral scientific communications of this Doctoral work

Nanobiotechnology for Textiles: Layer-by-Layer deposition of antimicrobial nanolayers on natural fibres
Ana Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
NanoSpain, Malaga, 23-26 March 2010

Assessment of bactéria – textile interactions using Scanning Electron Microscopy: A study on Layer-by-Layer chitosan/alginate coated cotton
Ana Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
Simpósio da Unidade de Investigação de Materiais Texteis e Papeleiros. Universidade da Beira Interior, 2010

Layer-by-Layer deposition of natural antimicrobial compounds in natural fibres.
Ana Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
International Conference on Engineering. Universidade da Beira Interior, 2011

Designing new antibacterial textiles by Layer-by-Layer assembling of biomolecules
Ana Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia
European Materials Research Society, E-MRS, 2012

Chapter 1

1. Introduction

1.1 Antimicrobial Textiles

Textiles play an important role in the daily lives of humans. Textile substrates, especially of natural origin, such as cotton, can easily be colonized by microorganisms because they retain oxygen, water, and nutrients required for their growth. The presence of microorganisms in fabrics affects not only the textile properties but also the wearer. These may result in offensive odors, color degradation, cross-infection or transmission of diseases, allergic responses and deterioration of textiles [1]. To combat these adversities it is highly desirable to impart antimicrobial properties to the textile materials. As a consequence, the number of biofunctional textiles with an antimicrobial activity has increased considerably over the last few years.

Antimicrobial textiles were first created to prevent damage to textiles under adverse environmental conditions during their storage or use. In fact, antimicrobial textiles were first used during World War II [2]. Actually, their application is extended to technical cloths, underwear, sportswear, home furnishing and protective clothing in areas with high risk of pathogens infection (hospitals, schools and hotels) [3]. Moreover, the antimicrobial fabrics are nowadays being used as medical devices for prevention, as surgical lab coats, or therapy, as wound dressings [3].

It is extremely important that protective clothing and hospital linen meet the demands for antimicrobial protection. Materials for use in surgery must ensure adequate protection against microorganisms, biological fluids and aerosols, i.e. impermeability for microorganisms in wet and dry atmospheres, and also for air-borne microorganisms. Disease transmission prevention is important for intracorporeal or implantable devices within the human body (vascular grafts and sutures) and for extracorporeal devices such as catheters and hollow fibres for dialyzers [4]. Furthermore, wound dressings also need to prevent infection and promote faster wound healing. Therefore, controlling the undesirable effects of microorganisms on textiles is becoming an important issue, especially within the medical textile industry. Thus, medical products will perhaps be the largest application of antimicrobial textiles [3, 5].

Another very important aspect is related with the abuse of antibiotics that has resulted in the continual emergence of resistant strains of bacteria, further complicating the clearance of infection in cutaneous wounds [6]. Textile materials are one of the main factors for disease transmission and also the need to enhance the quality of people's life (medical staff, patients, and visitors) have stimulated intensive research and development of antimicrobial textiles [20]. Thus, it is crucial to impart antimicrobial activity to textile materials in order to protect the

user from microorganisms contamination. Another aspect of antimicrobial functionalization of textiles is to add a therapeutic value to the material, intended for example, for wound healing [7].

Considering the environment, new antimicrobial textiles must be based on biodegradable molecules, due to the interest to reduce adverse effects on the environment. Among the many polymeric materials available, cellulose fibres are particularly attractive, being naturally occurring, and easy to functionalize [8].

1.2 Antimicrobial agents for textiles

1.2.1 Synthetic compounds

A number of different antimicrobial agents have been employed to impart antimicrobial activity to textile materials. These antimicrobial agents include inorganic salts, organometallics, iodophors, phenols and thiophenols, antibiotics, heterocyclies with anionic groups, nitro compounds, ureas, formaldehyde derivatives, amines [9], silver salts or silver nanoparticles [10], quaternary ammonium salts, triclosan, dyes and regenerable N-halamine compounds [9, 11]. The possible toxic effects produced by some of these agents on human beings are listed in table 1.

Table 1 – Possible toxic effects of some commercially available synthetic antimicrobial agents on human being (adapted from Shahid et al., 2013 [12]).

Synthetic agent	Toxic effect	Ref.
Quaternary ammonium compounds	Respiratory irritation, nausea, skin and eye irritation	[13]
Silver	Argyria, contact dermatitis, mucous membrane irritation	[14]
Zinc pyrithione	Developmental and neurotoxicity	[15]
Synthetic azo dyes	Carcinogenic	[16]
Triclosan	Endocrine disrupter, skin and eye irritation	[17]

Most of these agents are toxic to humans and are not environmental friendly [1, 18]. In addition, another big concern is that some of these agents are being increasingly resisted by microbial pathogens [9]. Therefore the role of antimicrobial textile has now become increasingly

demanding and has strengthened the interest in alternative ecofriendly and biodegradable antimicrobial agents.

1.2.2 Natural compounds

To minimize the risks listed in table 1 associated with the application of antimicrobial agents (synthetic compounds), there is a great demand for antimicrobial textiles based on non-toxic and ecofriendly bioactive compounds [19]. Due to the relative lower incidence of adverse reactions of natural products in comparison with synthetic pharmaceuticals, they can be exploited as an attractive ecofriendly alternative for textile applications [20].

Recently, the use of natural biopolymers has been preferred for textile modification, since they have several advantages such as abundant availability, biocompatibility, and biodegradability, and therefore ecological safety [21]. In textiles the incorporation of natural polysaccharides is a new concept which has been introduced in recent years. Table 2 show a brief list of the sources and important characteristics of some natural biopolymers explored on the textile substrates.

Table 2 – Characteristics of some biopolymers used in antimicrobial finishing of textiles, (adapted from Shahid et al., 2013 [12]).

Biopolymer	Source	Characteristics
Chitosan	Crustaceans and fungi	Biocompatible, biodegradable, antimicrobial activity, antistatic activity, non-toxic, chelating property, deodorizing property, film forming ability, chemical reactivity, polyelectrolyte nature, dyeing improvement ability, cost-effectiveness, thickening property, wound healing activity
Cyclodextrin	Starch	Ecofriendly nature, inclusion complex forming ability, insecticidal delivery, slow release of fragrances, solubilizing ability, ease of production, cost-effectiveness, chelating activity, drug carrier ability
Sericin	Silk worm (*Bombyxmori)*	Biocompatible, biodegradable, UV resistant, oxidative resistant, moisture retention capacity, antibacterial, gelling property, adhesion ability

Biopolymer	Source	Characteristics
Alginate	Brown sea weeds	High moisture absorbing capacity, biocompatibility, wound healing ability, gelling property, antibacterial activity

1.3 Evaluation of antimicrobial activity

The antimicrobial agents, synthetic or natural, kill or inhibit the growth of microorganisms. Among these agents are bacteriostatic that inhibit bacterial growth, without killing them. The agents that kill the bacteria are named bactericidal.

In recent years, the industry has grown rapidly and developed to form a new global industry, with Japan, the United States, Europe, China and other countries gradually establishing their own antibacterial standards and guidelines. There are a wide range of methods available to examine the interaction of microorganisms with textiles. Several testing methods are published on the qualitative and quantitative evaluation of antimicrobial activity of textiles. Qualitative test methods are used widely for evaluation of bacteriostatic activity and include procedures such as measurement of the zone of inhibition for evaluation of samples treated with antimicrobials. Quantitative test methods are used to evaluate the bactericidal activity of textile materials by measuring the reduction in bacterial numbers when contacted by test samples under defined conditions. Generally, a typical Gram positive organism, such as *Staphylococcus aureus* and a Gram negative organisms such as *Klebsiella pneumoniae,* are used in the test.

The major in use qualitative tests for evaluation of antimicrobial activity of textile materials are [2]:

- AATCC 147-1998 – antibacterial activity assessment of textile materials.
- JIS L 1902-Halo method - testing method for antibacterial activity of textiles, qualitative test.
- ISO 20645 – textile fabrics – determination of the antibacterial activity – agar plate test.

Quantitative tests for evaluation of antimicrobial activity of textile materials more know are [2, 22]:

- ASTM E2149-01- standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions.
- AATCC 100-1999 - antibacterial finishes on textile materials.
- JIS L 1902-Absorption method - testing method for antibacterial activity of textiles, quantitative test.
- ISO 20743 – textiles- determination of antibacterial activity of antibacterial finished products.

The methods described in AATCC-100, AATCC-147 and JIS L 1902 appears to be the most commonly employed. In the effort to move toward global standardization, the Japanese Standard JIS L 1902 has been revised and harmonized with the European Standard and International Standard EN ISO 20743. This standardization reflects the opinions of multiple countries and will help to promote the development of textile products with antibacterial functions that can be used around the world. In the review of the literature it was found that for the qualitative tests antibacterial AATCC 147 and JIS L 1902-Halo method, no differences were observed between them. Concerning the two quantitative methods, AATCC 100 and JIS L 1902-Absorption method, the results showed that the JIS L 1902 method is more sensitive to the amount of antimicrobial agent than the AATCC 100 test [2, 23].

Therefore in this thesis the antibacterial activity of cotton samples was evaluated using the antibacterial test JIS L 1902:2002, which is recommended by the renowned German test of Hohenstein institute.

1.3.1 Antibacterial test JIS L 1902:2002

The advantages concerning the antibacterial test JIS L 1902:2002 are as follows: the method parameters are more carefully spelled out than an alternative antimicrobial fabric method AATCC 100; this quantitative method is generally reproducible; this method tests are for both bacteriostatic (growth-inhibiting) and bactericidal (bacteria-killing) properties on a given antimicrobial fabric; microbial concentrations are standardized and bacteria are provided with nutrients during the incubation period, which provides them with ample opportunity to grow if the test fabrics are not sufficiently antimicrobial; the method stipulates triplicate experimentation, which helps estimate the precision of the individual tests and increases overall experimental accuracy and the method includes a “pass/fail” criterion for the calculated levels of antimicrobial activity observed in test samples, making determinations of antimicrobial activity less discretionary.

JIS L 1902 was developed in Japan for testing silver-based antimicrobials. It primarily differs from AATCC 100 in that the nutrient level in the inoculums broth is diluted to 1:20. JIS L 1902 also is explicit about calculating results for treated products versus those for untreated controls

and calls for testing in triplicate. The low nutrient level for JIS L 1902 biases testing to provide more positive results for antimicrobials such as silver and cationic antimicrobials, which can be neutralized by proteins in the nutrient [24].

In the qualitative method, samples of textiles are placed onto agar plates, which have been inoculated with bacteria and are then incubated under humid conditions at 37ºC for 24 – 48 hours. The intention is that with intimate contact between the textile, the bacteria and the growth medium will result in the inhibition of growth either immediately adjacent to the textile or in an area around the textile. These methods are generally acknowledged as being non quantitative although they could potentially be employed as assays of certain antimicrobial products in the same manner that such techniques are used for some antibiotics. Although these techniques are considered to be unsuitable for quantifying the effect of the antimicrobial effects of treated textiles [2].

In addition to the qualitative tests, it can be provide quantitative data on the effect of treated textiles on bacteria. In this case replicate samples (6 of the control and 3 of the treated) are inoculated with individual bacterial species (*Staphylococcus aureus* and *Klebsiella pneumoniae)* suspended in a heavily diluted nutrient medium. The samples are incubated under humid conditions at 37ºC for a specified contact time. Activity is assessed by comparing the size of the initial population in the control with that present following incubation. No neutraliser is employed during cell recovery [2]. This method needs much time to be realized and the procedure is very complex. When the number of samples increases, the complexity also increases.

Quantitative bacterial testing can be used for all antimicrobials. Comparisons can be made between different antimicrobial treatments as well as various treatment levels on the same textile. These methods better simulate real-world conditions than other methods. There are also disadvantages to quantitative tests, the disadvantages are, these tests are long, complex, and expensive, requiring a large number of manipulations to the sample and organisms.

1.4 The potential use for antimicrobial textiles

1.4.1 Wound dressings

Wound dressings should prevent bacteria or toxic materials from entering through the wound site. The skin plays an important role in human body and prevents from being infected by microbes. Skin generally needs to be covered with a dressing immediately after it is damaged. Conventional wound dressings such as bandages, gauze and foam dressing just cover the surface of the wound and absorb tissue exudates. However they cannot provide an appropriate environment for tissue repair and regeneration, as they easily adhere to wound and damage

the new epithelial tissue leading to bleeding. Advanced dressings, including biological and synthetic scaffolds, can provide a physical barrier against secondary infection, as well as a compatible physiological environment [25]. Literature has predominantly investigated metallic nanoparticles, mostly silver [26], as promising antibacterial agent. Silver nanoparticles have historically been the leading candidate because they have a very low minimum inhibitory concentration for most bacteria [26], indicative of their strong antibacterial activity. However, the use of silver is problematic because silver resistance has already been observed in *Pseudomonas aeruginosa* and the continued use of silver as an antimicrobial agent will likely lead to increased silver resistance in other microorganisms. Additionally, it has been demonstrated that metallic nanoparticles have multiple organ toxicity [27].

Wound dressings based on alginate (ALG) is well known in literature and retain exudate away from the wound bed, thus preventing harmful proteases from disrupting healing, whilst maintaining a moist environment for improved wound healing. ALG interact with the wound by donating calcium ions to the bed in exchange for sodium ions present in wound exudate, facilitating blood coagulation, thus assisting haemostasis [28].

A major problem is the tendency of dressings to adhere to the wound surface since when the dressing is removed, considerable damage is inflicted on the newly formed epithelium. The gel forming property of ALG helps in removing the dressing without much trauma and reduces the pain experienced by the patient during the change of dressing. It also provides a moist environment that leads to rapid granulation and re-epithelization.

Chitosan (CH) is also known in the wound management field for its haemostatic properties. Further, it also possesses other biological activities and affects macrophage function that helps in faster wound healing [29]. Several different mechanisms for microbial inhibition by CH have been proposed. The most accepted one is the interaction of the positively charged CH with the negatively charged residues at the cell surface of many fungi and bacteria, which causes extensive cell surface modifications and alters cell wall permeability [29, 30]. As a result, CH inhibits the normal metabolism of microorganisms and finally leads to the death of these cells.

Systems simultaneously composed of CH and ALG offer the advantages of both materials and can be tailored for several biomedical applications, such as wound dressing and drug delivery systems. According to Paul and Sharma (2004), skin injuries treated with CH-ALG membranes show a substantial decrease in the healing period and minimum scar formation when compared with the use of conventional covers [31].

1.4.2 Wound dressing for drug delivery

Wound infection is the major difficulty in the field of wound care management, because such infection can cause to form exudate, delay the wound healing and facilitate improper collagen deposition [32]. Polymer networks have shown the promise as a way of incorporating antimicrobial agent into polymer such as the antibiotic streptomycin sulphate [25] or tetracycline [30] where the polymer networks act as a carrier for the antibiotics delivery system.

Wound dressings for drug delivery systems containing drugs and growth factors are being researched so that they can be used to improve for difficult wound treatments. Wound dressings that include biological factors, such as growth factors and drug helping wound healing, have been developed in various forms, including aqueous solutions, creams, and ointments [33]. Modern dressings, such as hydrocolloid, hydrogel, ALG, polyurethane foam/film, and silicon gel, are used to deliver the biological factors to the wound sites [34].

These dressings can deliver too therapeutic agents such as antibiotics, vitamins and mineral supplements to the wound site and help in improving wound healing. The polymers that are employed for drug delivery include poly(vinyl pyrrolidone) [35], poly(vinyl alcohol) [36], collagen [37, 38], CH [39, 40] and ALG [31, 41]. In modern wound care practice, drugs such as gentamicin [42], minocycline [43], tetracycline [30, 44, 45], streptomycin [46], silver sulfadiazine [41, 47] are generally used to treat wounds.

Improved wound dressing that provide an inherent antimicrobial effect by eluting germicidal compounds have been developed to respond to problems associated with conventional topical treatments with ointments and creams, usually incorporating silver ions as the active agent. Wang et al. (1985) and Boosalis et al. (1987) have demonstrated significant absorption of silver from large burn wounds treated topically with silver sulfadiazine cream, which may increase the risk of cytotoxicity of the treated tissues [47-49], as already mentioned above that silver ions are highly toxic and may delay burn wound healing if applied indiscriminately to healing tissue areas.

A variety of wound dressings that incorporate active agents are available on the market; they include iodine (Iodosorb by Smith & Nephew), chlorohexidime (Biopatch by J&J), and silver ions (Acticoat by Smith & Nephew, Actisorb by J&J, and Aquacel by ConvaTec.) [50].

Final remarks

Accordingly, the research work presented in this thesis revealed the success of biofunctionalization of cotton with antimicrobial agents of CH and ALG. A durable antimicrobial effect over *Staphylococcus aureus* and *Klebsiella pneumoniae* was obtained without

cytotoxicity. In addition, due to the widespread resistance of bacteria to antibiotics, antimicrobial peptides were incorporated into biofunctionalized cotton, as samples candidates for future therapeutic use as wound dressings.

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Paper I

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Layer-by-Layer assembly as a promising technique for the biofunctionalization of cellulosic fibres with emergent antimicrobial agents.

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Layer-by-Layer assembly as a promising technique for the biofunctionalization of cellulosic fibres with emergent antimicrobial agents.

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Abstract

Polyelectrolyte multilayer coatings have become a new and general way to functionalize a variety of materials. Particularly the Layer-by-Layer (LbL) method is a technique that allows coating solid surfaces, giving them several functionalities, allowing as well the controlled release of bioactive agents starting from the surface. Presently there is a large number of applications of the LbL technique in various areas, however still little explored in the textile area. In this mini-review we present an overview of LbL on textiles, either synthetic or natural fibres, more specifically on cotton materials to obtain a new bioactive textile with potential application in medical field. We also review the recent progress in the embedding active agents into multilayers obtained from LbL as a novel way to have a "reservoir" where bioactive agents can be loaded between the multilayers for subsequent release.

Keywords: Layer by Layer; Cotton; Bioactive agents; Bioactive Textiles;

1. Introduction

1.1 Antimicrobial textiles

The number of functionalized textiles with antimicrobial activity has increased considerably over the last few years. Antimicrobial textiles were first created to prevent damage to textiles under adverse environmental conditions during their storage or use. Textiles are widely used in day-to-day life and there has been a growing need to develop finishes for textiles materials that can offer improved protection to the users from microbes (bacteria, fungi), which can cause numerous problems. Hence there is a pressing need to develop functionalized textiles that are resistant to microbes and these textile substrates can find various applications in the health-care area. Synthetic antimicrobial compounds used in textile articles are very effective against a wide range of microorganisms. But, the continuous use of antimicrobial compounds can lead to bacterial resistance and desensitization of users and cause environmental problems [1-4].

To minimize these risks, there are currently a high demand for antimicrobial textiles produced with non-toxic natural compounds and environmentally friendly. The low incidence of adverse effects on natural compounds in relation to synthetic drugs can be explored as an attractive and promising alternative for textile applications [5-7].

Cotton is the textile substrate more used in the health sector, and is known for its versatility, natural comfort, softness, breathability, and ability to absorb moisture [8], being mainly composed of cellulose fibres [3, 4]. It's used to make all kinds of clothes, for industrial purposes, as well as in biomedical applications. So due their properties, cotton becomes a potential to be used as wound dressing. Today cotton gauze is still the most commonly used textile for wound dressing in hospitals, however new products have emerged that help wound healing and protects from the entry of bacteria. For exudative wounds, there are a range of absorptive products including various hydrophilic foam dressings, hydrogels and alginates, which can absorb up to 20 times their weight [9]. Also, the cotton is an excellent surface for growth and development of microorganisms, making it an attractive material for the biofunctionalization with antimicrobial agents.

1.2 Current functionalization processes for textile materials

Coating is an important technique to add value to technical textiles and is a way to their functionalization. It is a process in which a polymer layer is applied directly to one or both surfaces of the fabric. Nanotechnology has received special attention by the textile industry through the application of nanotechnology in textiles to attain multifunctional [10] or special

functions, such as antimicrobial coating with potential use in reducing the risk of microorganism transmission. There are several processes for the application of coating to the textile material depending upon the requirement of the end product. The most significant processes are: sol-gel technique, which is a wet process that is broadly employed in the textile field using a simple pad or dip coating; magnetron sputter coating, which is one of the physical vapour deposition methods, and plasma, which is a suitable technique for modifying the structure and topography of the surface as well as deposing of composites onto the surfaces [11]. These methods have a number of disadvantages, such as: the use of expensive solvents and equipment, several steps need to be followed which makes them relatively complex, under certain conditions it may be necessary to use high temperature and are dependent on the surface topography. With all these disadvantages, new strategies were attempted, in particular the LbL technique that is very attractive due to its simplicity and efficiency. LbL technique is nowadays used to provide coating in textiles surface and a wide range of functionalities have been imparted [12]. The prerequisite for the successful of LbL coating is the presence of a minimal surface charge in the substrate. However the LbL deposition process has not been extensively implemented in textile, particularly in textiles from natural fibres, maybe due to the unique characteristics of natural fibres including the chemical heterogeneity of their surfaces which complicates the application of such coatings.

2. LbL Assembly Technique

LbL is a simple and versatile method which can provide new types of coatings for textile materials. It was proposed by Decher and his collaborators in the early 90s [13] and since then its positive impact can be demonstrated by the growing number of published papers. This technique has been described as being able to cover many kinds of surfaces when they are charged [14].

The LbL assembly is a technique of depositing multilayers with controlled architecture and composition performed in aqueous solutions. The electrostatic interaction is the main driving force within the neighbouring layers of polyelectrolyte multilayers. Generally, LbL assembly proceeds as follows: (1) a charged substrate is immersed in a solution of an oppositely charged colloid to adsorb the first monolayer, then (2) a washing cycle follows to remove unbound material and, finally (3) the coated substrate is submerged to deposit a second layer and the multilayered structure is formed [15]. A deposition cycle creates a bilayer, and these cycles can be repeated as often as needed. Therefore, cross-linking is often applied to convert LbL multilayers to surface hydrogel [16]. The number of deposition cycles and the types of polyelectrolytes used in the construction allows full control of the thickness and roughness of the multilayered film [17]. Usually, multi-layered films based on electrostatic interaction tend

to be affected by the environmental conditions, such as, pH, concentration of polyelectrolytes, nature of solvents, ionic strength and pH of solutions [14, 18, 19].

Depositing materials can be selected from a large variety, including small organic molecules, polymers, natural proteins, inorganic clusters, clay particles or colloids. It open´s new possibilities never tested before since it able to combine such a diversity of components in single devices whose architecture and features are controlled. Surface functionality can be controlled directly by choosing appropriate polyelectrolytes. Surface modification results in a multitude of new properties that were previously not associated with the native material (substrate). These changes include modifications of the electrical, optical, magnetic, physicochemical and biological properties of the materials. These multilayer coatings of bioactive natural polyelectrolytes become a new process for biofunctionalizing surfaces. By this technique it is also possible to control the required multilayer thickness, location and sequence of the layers.

One advantage of LbL technique is that the process is inexpensive, relatively fast and simple, it does not require sophisticated equipment and precise stoichiometry, nor does rely on complicated chemical reactions to deposit successive layers [20]. Another advantage to the LbL deposition is the independence of size or shape of the substrate. It means that an LbL assembly can be realized not only on planar substrates, but also on substrates with different shapes. Caruso et al., (1998) have demonstrated LbL deposition on a spherical template. After template dissolution, microcapsules were obtained [21]. So, theoretically the substrate can have any size, shape, topography, or topology, and no stoichiometric control is necessary to maintain surface functionality nor propagation of defects [22].

Another advantage of the LbL technique is that pH can be used as a parameter to adjust the strength of inter-layer bonding and over the required multilayer thickness. The only disadvantage of this technique is the prerequisite for successful LbL coating, consisting of the presence of a minimal surface charge. However, charge can be induced to still facilitate the LbL technique [23].

All knowledge about the LbL was transferred to the field of drug delivery systems allowing the creation of sophisticated delivery systems, such as the production of capsules where the drug is encapsulated inside, in order to be released gradually. A major challenge in drug delivery is to produce controlled, sustained or triggered release systems for small encapsulated drug molecules. These processes can be found in a review article developed by Wohl and Engbersen (2012) [24].

Other research groups have employed the LbL technique to create multilayer coatings of synthetic and natural polyelectrolytes with application in the biomedical field [25]. This multilayers have the characteristic of low packing density, allowing easier diffusion of a bioactive agent through these multilayers. The bioactive agents are incorporated in most cases by embedding through the multilayers, being a recent area that has attracted the attention of

researchers, due to the advantage of controlling the incorporation and release of the bioactive agent.

The release of bioactive agents can involve their diffusion from the multilayers. Various parameters such as pH, ionic strength, temperature, light or chemical or electrochemical stimuli have been used to tune release and/or retention of bioactive agents within multilayers. This parameter for release is able to provide the dose of drugs on demand with reduced toxicity and increased efficacy [16].

Using the concept of diffusion from the multilayers, several authors immobilized more than 10 different water soluble proteins into multilayers ensuring the inhibition of protein denaturation [26-28], preservation of the functional characteristics of some compounds showed good results in the incorporation of proteins and drugs between the multilayers by diffusion with subsequent release [29-33].

Studies have emerged, in which bioactive proteins, peptides, hormones, growth factors or drugs could be directly integrated in the LbL architecture without any covalent bonding with a polyelectrolyte, maintaining their native structures and their activities [29, 34-41]. The strategy described in all these papers can be valuable for various drug/bioactive agents. This result opens the route for substrates functionalization by multilayers with embedded bioactive agents, therefore the multilayers can act as a reservoir for bioactive agents and these can be gradually released and controlled.

LbL technique is not new for many applications as shown in a recent review article that analyses exhaustively the potential uses of LbL method in biomedical engineering [42], but it is relative new for textile applications. In recent years, researchers have used the LbL process to modify the surface of textile fabrics to impart or improve upon numerous surface properties including UV protection [43, 44], hydrophobicity/hydrophilicity [45, 46], flame retardancy [47-49] and antimicrobial activity [50-52].

In the present mini-review, our aim is to assess the feasibility of obtaining functionalized cotton samples with antimicrobial properties achieved by the method of LbL with bioactive agents between the layers, and subsequent controlled release. This approach is supported by the work of Caridade et al. (2013) where they studied the production of thick membranes by LbL of chitosan and alginate and the membrane permeability to the bioactive agent [53].

3. LbL in textile materials

The LbL process has been widely used to create multilayer films on various substrates. However, it has not been extensively employed in textile fibres. Textile fibres has some unique challenges for LbL assembly including the chemical heterogeneity of their surfaces as well as their irregular

shapes [54]. Since LbL is a new and innovative method of functionalization of materials, there are presently some works applied to textile fibres, which can be divided in two categories, synthetic and natural fibres.

3.1 LbL in textile materials of synthetic fibres

Synthetic fibres form an important part of textile industry. There are many different kinds of synthetic fibres, but the most used are polyester, polyamide (nylon), polyvinyl alcohol and polypropylene. A great disadvantage of some synthetic fibres is their low hydrophilicity. This affects the processing of LbL because the fibre surface is not easily wetted. Table 1 shows a summary of the state of the art in the last decade referring the use of LbL in textile materials of made of synthetic fibres.

Table 1 shows that the polypropylene is the synthetic textile substrate more used followed by polyester and polyethylene terephthalate (PET). The deposition of several layers gives to the textile several features, but only recent studies have appeared where the objective is to obtain synthetic textiles with antimicrobial properties.

Table 1 – State of the art - summary (LbL application in textile materials of synthetic fibres)

Author (year)	**Substrate**	**Polyelectrolytes**	**Notes**
Polowinski, S. (2005) [55]	Polypropylene	PAH/PAA	The dyeing technique allows the type of external layers deposited in succession to be identified. LbL method deposits layers of polymeric complexes, not only onto polypropylene, but also onto other textile materials with a smooth surface
Dubas et al. (2006) [56]	Nylon	PDADMAC/anionic scarlet dye	The LbL of PDADMAC/anionic scarlet dye has a high dependence with the number of layers, salt concentration, and concentration of chemicals but almost independent on the dipping time.
Polowinski, S. (2007) [57]	Polypropylene Polyester	PAH/PAA	LbL method was used to deposit thin polymeric layers on textile fabrics. A necessary condition for using this method was a smooth surface on the fibres of the fabric.
Jantas et al. (2007) [58]	Polyester	PAA/PVP	The surface of fibres in the fabric becomes smoother after depositing PAA/PVP nanolayers.
Polowinski, S. (2007) [59]	Polypropylene	PAA/PDAMA/PAH Nanoparticles: Au, Pt, Ag	LbL method is a convenient way of depositing colloidal particles of silver, gold or platinum on textiles
Stawski et al. (2009) [60]	Polypropylene	PAH/PAA	Deposition of succeeding polyelectrolyte layers fail to provide a complete coverage of the modified surface.

Park et al. (2009) [61]	Nylon 6	Alginic acid sodium salt and chitosan	The morphology of polyelectrolyte multilayer coated nylon 6 fibres was uniform and smooth. The surface morphology, stiffness, and hydrophilicity of polyelectrolyte multilayer coated nylon 6 fibres can be controlled by regulating the number of polyelectrolyte nanocoating.
Carosio et al. (2011) [48]	PET	Silica nanoparticles	PET fabrics were coated with silica nanoparticles. This study demonstrates the ability to impart flame retardant behaviour using a water-based, environmentally-friendly protective coating.
Martin et al. (2013) [62]	Non-woven PET	MB/chitosan/polyCD (cyclodextrin polyelectrolyte)	The aim of this work was to develop an antibacterial multilayer coating activated with MB. The authors prepared two types of samples, one with MB-free and another with MB-loaded.
Martin et al. (2013) [63]	Non-woven PET	Chitosan/polyCTR-beta CD (beta cyclodextrin polymer)	In this work it was developed the formation of a multi-layered coating onto PET textile support in order to obtain reservoir and sustained release properties towards bioactive molecules.

PAH-poly(allylamine hydrochloride)PAA-poly(acrylic acid); PDADMAC-poly(diallyldimethylammonium chloride); PVP-poly(vinyl pyrrolidone); PDAMA-poly(diallylamine-co-maleic acid); PDDA-poly(dimethyldiammonium chloride); PET-polyethylene terephthalate;PSS-poly(sodium styrene sulfonate);MB-methylene blu

3.2 LbL in cellulose-based textiles

In the field of natural fibres, cotton is one of the most commonly used for textiles and is very applicable for medical usage, specially wound dressings due to its high liquid absorbency and hygienic nature. Cotton is characterized by his heterogeneity, which will cause problems in conventional coatings, in this way is a good possibility to use the LbL technique. Currently there is some work published with LbL method applied on cotton. In this section we present the most important applications of functionalized cotton obtained by LbL technique. A special note for antimicrobial cotton obtained by LbL is referred at the end of this section.

Hyde et al. (2005) were pioneers in application of LbL on cotton substrates. They found that the cationization process produces a cotton surface capable of supporting polyelectrolyte films via LbL deposition. They observed that the LbL deposition process is more dependent on the nature of the polyelectrolytes rather than on the nature of the original substrate and the analysis reveals conformal and uniform coating of the cotton fibres [64]. This indicates that for the same substrate there are various functionalities depending of the polyelectrolyte deposited. The LbL is a versatile method, in which it is only required to define which property it is intended and then select the appropriate polyelectrolyte. In the LbL, the dependency on the type of substrate is lower, allowing the LbL to be applied smoothly onto cotton, After this work, other works have emerged in which various methods of analysis of samples obtained by LbL are presented [65]. Studies were also carried out where the influences of variation of physical parameters, such as pH, concentration, ionic strength of polyelectrolytes and cationization level of substrate [54, 66]. The deposition process was not significantly influenced by the degree of cotton cationization, but the other physical parameters have influence on the success of LbL.

In the last decade, some studies where the technique of LbL onto cotton to give it certain properties and applications, were published. One of these properties is the protection against UV in cotton fabrics [43, 44, 67, 68]. High UV protection factors were obtained and a good resistance to washing, revealing a stability of the layers obtained by LbL in cotton. In samples in which it is necessary a durability of the coating, the LbL is recommended. Another important property that textiles must have in some applications is the hydrophobicity [45, 69]. In this case LbL is an easy method for fabricating hydrophobic or hydrophilic cotton fabrics only by coatings.

More recently, several authors used the LbL technique to provide a coating cotton with specific polymers in order to enhance their flame retardant properties. The results of all studies show that flame retardant coatings can be readily applied to textile fabrics for commercial and industrial applications. As a consequence the treated fabrics have shown a strong reduction of the flammability and combustion [47-49, 70].

Providing antimicrobial property for textiles is an effective way to prevent disease transmission with applications in the consumer and healthcare markets. Many textiles are treated to afford protections against bacteria, fungi and other related microorganisms. During the past few years, there have been some studies aimed at functional antimicrobial modification via LbL assembly process onto cotton fibres. Cotton textiles were obtained with antimicrobial properties and potential applications for medical textiles with N-halamine polyelectrolytes deposited on cotton via LbL [71, 72].

Another approach for obtaining antimicrobial cotton is related to the use of CH nanoparticles [50, 51], copper based nanoparticles [73], and ZnO nanoparticles [68]. In these works, the nanoparticles were coated on cotton to form a nanocoating using LbL.

The LbL technique also offers new opportunities for the preparation of functionalized biomaterial coatings and the possibility of incorporating bioactive molecules between the layers [74-76]. Peptides, proteins and active agents adsorbed or embedded in multilayer films have been shown to retain their biological activities [41], whereas a covalent attachment to the active agents can reduce or even destroy their biochemical activity [76]. So, with the LbL technique, active agents can be directly integrated in the architecture without any covalent bonding.

Cotton fibre is the basis of many wound dressings and wound dressings containing antibiotics have been developed for the inhibition of wound infection [77-79]. But the continuous use of antibiotics has resulted in multiresistant bacterial strains all over the world. Consequently there is an urgent need to search for alternatives for antibiotics.

4. Antimicrobials of the future – antimicrobial peptides

Antibacterial resistance is a natural biological phenomenon that occurs in microorganisms and is potentiated by indiscriminate use of antibacterial agents. If the microorganism becomes resistant to a particular antibacterial agent, when an infection occurs, the effect of the antibacterial agent will be reduced or null. Therefore it is urgent to discover and use new antibacterial agents. Recently, a large group of low molecular weight natural compounds that exhibit antimicrobial activity have been isolated from animals and plants, thus resulting in new generation of antibacterial agents, the antimicrobial peptides (AMPs).

AMPs are promising agents due to some characteristics such as, they are natural compounds [80, 81], have a broad spectrum of action [82, 83], exhibit high activity even with low concentrations [83, 84], have a low tendency to develop resistance due to its different mechanism of action [80, 81, 85-87], have a quick and efficient action against bacterial agents

[83, 87] and their size is generally small and have low mammalian toxicity [83, 87]. The ability of these AMPs to kill multidrug-resistant microorganisms has gained considerable attention and clinical interest. Another approach to wound healing with AMPs is related to the function of AMPs in removing the destructive proteases from the wound that cause considerable destruction of growth factors and connective tissue proteins during the prolonged inflammatory phase of a chronic wound [88]. The review paper by Gouveia, 2010, is the first work in referring that AMPs are promising candidates as antimicrobial agents for textiles [89], so AMPs can be incorporated into textiles to develop non-toxic antimicrobial textiles.

AMPs produced in bacteria, insects, plants, invertebrates and vertebrates, are an important component of the natural defences of most living organisms. AMPs exhibit potent killing of a broad range of microorganisms, including Gram-negative and Gram-positive bacteria, fungi and viruses [86, 90, 91].

4.1 AMPs – characterization and classification

Currently AMPs database (http://aps.unmc.edu/AP) contains over than 2400 AMPs. Their characterization is complicated due to the great diversity, thus the characterization is easier based on their secondary structure. AMPs are mainly grouped into four classes: ß-sheet, α-helical, loop, and extended peptides [92]. There are AMPs with positive charge [93], other with negative charge [94], amphipathic molecules (which possess both hydrophobic and hydrophilic regions), some have a sequence with less than 10 amino acids, but others near 100 amino acids [95]. In general, the AMPs are described as small molecules constituted by 12 to 50 amino acids with a cationic charge between +4 and +6, due to the presence of the amino acids lysine and arginine [87, 96] and as anionic, that generally have a net charge in the range of -1 to -7, due to the presence of glutamic and aspartic acids. AMPs are mainly cationic and interact with membranes in a general mechanism that involves interactions between charged residues of peptides and anionic components of the membrane surface.

4.2 Mechanisms of action

The AMPs in bacteria can lead to the disruption of the membrane resulting in its lysis, or alternatively lead to pore formation, allowing efflux of essential ion and nutrients. In this case, the AMPs are transported into the cell and will cause inhibition of DNA and RNA synthesis, inhibition of ribosomal function and protein synthesis, and targeting of mitochondria [97]. Many models of antimicrobial action on the level of membrane have been proposed. Models that have greater acceptance in the scientific community are: carpet model, toroidal pore and the barrel-stave [84].

AMPs also possess anti-viral properties, inhibiting viral fusion and egress, thus preventing infection and viral spread via direct interactions with the viral membranous and host cell surface molecules [97]. These properties combined with the broad range of activity and the short contact time required to induce killing, have led to the consideration of AMPs as excellent candidates for novel therapeutic agents.

4.3 Applications of AMPs

The AMPs have many applications and are not limited in the development of new drugs, because there are also other medical, environmental and industrial applications. Within the potential medical applications of AMPs we can refer to:

- Prevention and treatment of eye diseases and antimicrobial coating for contact lenses [98]
- Through the antimicrobial coating on polymeric materials, such as implants and catheters for prevention of bacterial colonization and biofilm formation on the surfaces of the implants [84]
- Through functionalization of textile materials, for example, application in heart valves, socks for diabetic feet, gauze of chronic wounds [84]
- Wound healing, treating fibrosis, acne, Crohn's disease [86]

Recently our research group found AMPs applications in textiles, particularly in cotton gauzes for wound dressings [91]. Incorporation is the most popular method for preparing immobilized AMPs onto a variety of surfaces and still retain their ability to kill bacteria [99, 100]. The main advantage of using AMPs comes from their presence in nature, thus they should be well tolerated by our body and low concentrations required. In addition, the synergistic biocide mechanism of action is more effective against multi-resistant bacteria.

5. Concluding remarks and future prospect

Successful deposition of multilayers onto textile fibre surfaces via the LbL technique can open the door to the development of functional textiles for a broad range of applications. In addition, the LbL technique in textile is entirely new and is a simple and effective method with strong possibility of implementation in the industry.

Bioactive agents can be directly embedded between the layers of polyelectrolytes without any covalent bonding, which able the development of a new strategy to obtain antimicrobial cotton based on the incorporation of AMPs into polyelectrolyte multilayer films built by LbL technique over cotton.

These coatings onto cotton textiles are new and potentially useful as antibacterial fabrics in a wide variety of biomedical applications. In addition, LbL deposition allows the easy fabrication of multimaterial films, in which different layers carry different functionalities or repeat the same functionality several times to control the quality or the quantity of active agents. This mini-review lay the groundwork for scale-up and in near future open new avenues towards the development of non-toxic and safe biomedical textiles. One promising application of these functionalized cotton would be its use as external wound dressings.

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Chapter 2

Aims of the thesis

The number of biofunctional textiles with an antimicrobial activity has increased considerably over the last few years. Medical products will perhaps be the largest application of antimicrobial textiles. So, biofunctionalized cotton with chitosan and alginate and with incorporated antimicrobial peptides are serious candidates for future therapeutic use as wound dressings.

The main goal of this thesis is to develop a structure capable of incorporating antimicrobial agents without cytotoxicity with potential use as a wound dressing.

To achive this principal purpose, the work was be developed concerning the following specific aims:

- Explore the feasibility of depositing layers of chitosan and alginate by LbL technique in cotton fibres.
- Design new processess for the biofunctionalization of cotton with antimicrobial agents, in particular, L-cysteine and antimicrobial peptides to obtain novel bioactive textile with potential application as wound dressings.

Chapter 3

Paper II

.

Layer-by-Layer Deposition of Antibacterial polyelectrolytes on Cotton Fibres

Ana P. Gomes, João F. Mano, João A. Queiroz, Isabel C. Gouveia

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ORIGINAL PAPER

Layer-by-Layer Deposition of Antibacterial Polyelectrolytes on Cotton Fibres

Ana P. Gomes · João F. Mano · João A. Queiroz · Isabel C. Gouveia

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Abstract The introduction of molecules with biological properties on textile materials is essential for a number of biotechnological applications. With the purpose of testing new processes applied to textiles, in this study, we present the first results on the feasibility of using the Layer-by-Layer (LbL) deposition process in natural fibers such as cotton, with natural polyelectrolytes like chitosan (CH) and alginic acid sodium salt (ALG), the durability of CH/ALG multilayer on cotton were evaluated. The increase of negative charges to the substrate cotton was made with NaBr and TEMPO, to ensure the success of the process of LbL. Three characterization methods to assess electrostatic LbL deposition were performed: the contact angle between a liquid (water) and the sample surface, in order to characterize the wettability of the samples with the different layers of CH and ALG; dyeing of the CH/ALG assembled cotton fabric with cationic methylene blue that shows regular changes in terms of color depth (*K*/*S* value), which indicate that the surface were alternately deposited with CH and ALG layers and, finally, the analysis by infrared spectroscopy using Fourier Transform with Attenuated Total Reflection (ATR-FTIR), to assess the changes in the interaction between CH and ALG deposited on cotton samples.

Keywords Layer-by-layer · Contact angle · ATR-FTIR · Chitosan · Alginate

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Introduction

The challenges facing the textile finishing industry have intensified during the last decade. Current awareness of the negative environmental impact of chemical processing of textiles, combined with increased strict legislation on industrial effluents, has led to the search for advanced, non-polluting processes for coating textiles. Coating on textiles is a new way to give functionalities and properties on textile surfaces without compromising on fabric properties and they open a whole new vista of value-addition possibilities in the textile sector. The coating can be used to give wrinkle resistance, improve color or light fastness, flame retardancy, water or oil repellency and antibacterial properties [1]. Newer methods of coating textiles become possible to improve the functionality and durability of the coating to a higher level compared to the conventional

coating techniques. These techniques include immobilization of enzymes, LbL assemblies, nano coatings and use of plasma for deposition of functional molecules. All these techniques are distinct from conventional finishes in that they impart special functionalities to textile surfaces by bringing about modifications at micro or nano level, without affecting the bulk properties. These processes add functionality with minimum effect on the strength, feel, handle or breathability of textiles [2]. Some of these techniques have been tested and validated at lab scale, but most are still in research stages. Most of the conventional coating techniques either affect the fabric flexibility, comfort and permeability or deteriorate the mechanical properties of the treated fabric [3, 4].

Functional coating methods provide a flexible alternative to conventional finishing methods in that they are independent of fabric type, require low quantities of additives and allow combinations of different functionalities in a simple way [2]. Polyelectrolyte multilayer (PEM) coatings have become a new and general way to functionalize surfaces [5]. This technique has been described as being theoretically able to cover many kinds of surfaces when they are charged [6]. The mechanisms allowing for film coating essentially involve electrostatic interactions, but the assembly of such multilayer structures have also been show on non-ionic or apolar substrates [7]. The film is constructed by the alternate adsorption of oppositely charged polyelectrolytes at the surface of the material, easily obtained when the material is dipped in polyelectrolyte solutions. The driving force for the film construction is related with the excess charge (alternatively positive and negative) which appears after each new polyelectrolyte adsorption [8, 9]. A deposition cycle creates a bilayer, and these cycles can be repeated as often as needed. The number of deposition cycles and the types of polyelectrolytes used in the construction allow for the control of thickness and roughness of the multilayered film [10]. A broad range of applications for these films has been considered, going from drug delivery to specific bio-applications based on surface modifications. For example, the multilayer film technique has been used to create microcapsules, defined as micro and nano-containers for storage, transport, and release of active macromolecules [11]. Martins et al. analyzed the potential and achievements of LbL technique as a promising approach to functionalize biomaterials surface in a controllable and facile manner. They found that the build-up of CH/ALG system presented a linear growth, meaning that no polymer has the ability to diffuse "in" and "out" of the film after each deposition step [9].

A new method for the modification of textile fibers was introduced by Hyde et al. [12], they demonstrated that a polymer thin film could be deposited directly onto cotton fabrics by following the widely studied LbL deposition method known as PEM. The LbL method involves essentially electrostatic interactions. Using transmission electron microscopy (TEM) they have shown the uniform coating provided by the LbL onto the cotton fabric [12]. It seems possible that by following the same LbL deposition method, a wide range of molecules, nanoparticles, and other functionalized polyelectrolyte could be deposited on textile fabric leading towards the development of new applications for technical textile applications. So the LbL self assembly method may provide new coatings or films that can be constructed by the alternate adsorption of oppositely charged polyelectrolytes at the surface of the material, easily obtained when the material is dipped in polyelectrolyte solutions. LbL is a simple and inexpensive method for preparation of controlled layered structures and it is applicable to a variety of materials. It has the advantages of simplicity, low cost, ability to incorporate different bio molecules and molecular control. LbL film structures are less densely packed and this is advantageous for diffusion through the films [13]. But certain details of the process are still not clearly understood. The LbL deposition process has not been extensively implemented in textile and natural fibers, as they possess unique challenges including the chemical heterogeneity of their surfaces as well as their irregular shapes.

Numerous studies involving different polymer substrates and several synthetic polyelectrolytes have been published. But, there are very few scientific articles concerning the deposition of alternate polyelectrolyte on natural textile supports. During a deep revision, we found few reports concerning the LbL method involving cotton fibres.

Hyde et al. [14], they evaluated three different levels of cotton cationization. Variations in the cationization degree were achieved by manipulating the ratio of 3-cloro-2-hydroxy propyl trimethyl ammonium to NaOH. The deposition of the polyelectrolytes was monitored using XPS and CHNS elemental analysis. The experimental results they obtained, indicated that the deposition process was not significantly influenced by the degree of cotton cationization.The build-up of further polyelectrolyte layers was found to be less sensitive to variations in the cationic character of the substrate once a critical number of alternating layers was deposited [14].

Wang et al. [15], they have utilized two different methods for the characterization for LbL deposition of two polyelectrolytes poly (sodium styrene sulfonate) (PSS) and poly (dimethyldiammonium chloride) (PDDA) on cotton fabrics, a dyeing method and a UV absorption method. Two types of dyes, anionic Direct Red 80 and cationic Methylene Blue, were utilized to dye the self-assembled cotton in order to reveal the change of surface electric property after LbL deposition of polyelectrolytes on cotton.

The UV absorption method could monitor the growth of polyelectrolytes on cotton substrates in terms of UV absorbance at characteristic absorption wavelengths [15].

Wang et al. [16], they have studied a new approach for UV protection of cotton fabrics based on LbL self-assembly. Three fluorescent brightening agents and polycation PDDA were used on cationized cotton fabrics through direct LbL deposition technique. The assembled cotton fabrics could obtain excellent rating of UV protection when the fluorescent brightening agents and PDDA were built up on the cotton substrates. Good durability to washing revealed the stability of multilayers films on the cationized cotton, which is important for actual application of textiles [16].

Ugur et al. [17], they obtained ZnO nanoparticle-based multilayer nanocomposite films were fabricated on cationized woven cotton fabrics via LbL deposition process. In this study they concluded that the process by LbL provides a novel and simple method for nano-ZnO nanocomposite film deposition on cotton fabrics and their application onto cotton fabrics to gain antibacterial and UV protection functions [17].

Ali et al. [3] developed a study to evaluate the effect of different process parameters on the amount of polyelectrolyte adsorbed on a cotton textile substrate via sequential adsorption of negatively charged PSS and positively charged poly allylamine hydrochloride (PAH) using LbL [3].

Recently Ali et al. [18] deposited CH nanocoating onto a cotton textile substrate using a LbL technique. PSS, was used as the anionic polyelectrolyte, and CH was used as the cationic polyelectrolyte. As a result, they obtained a uniform surface deposition of bilayers, as observed by scanning electron microscopy (SEM) and confirmed that during the LbL deposition, the layers did not block the fabric pores (unlike conventional coatings) [18].

As the commonly used polyelectrolytes such as PSS, poly (acrylic acid) (PAA), PDDA, poly(allylamine hydrochloride) (PAH) and polyethyleneimine (PEI) have no special functions transferred to textiles [12, 15, 16, 19–22] non-polyelectrolytes with negative charges such as nanoparticles and dyestuffs were integrated into the LbL self-assembled multilayers together with polyelectrolytes to modify the surface giving antibacterial properties and dyeability, respectively [16]. It should be noted that this new technique is not limited to polyelectrolytes. Some organic molecules with positive or negative charges have also been integrated into multilayers via LbL deposition. This opens the possibility of developing functional textiles. Therefore, here we report the first results regarding the feasibility of LbL deposition of nanolayers of natural bioactive polyelectrolytes, to give antibacterial properties to natural fibers. The purpose of this experimental work is to determine the feasibility of using the LbL deposition process in natural fibers with natural polyelectrolytes. To impart higher negative charge to the substrate cotton, CH and ALG were successfully layered over cotton fibers using LbL technique.

The introduction of negative charges onto cotton samples is made with sodium bromide, NaBr and 2,2,6,6-tetramethylpiperidinyl-1-oxy free radical (TEMPO) [23–26]. The cotton is composed for the most part of cellulose, cellulose is a natural polymer composed of β-D-glucopyranose units that are linked together by (1 → 4)- glycosidic bonds [23]. A cellulose molecular chain, depending on the source of the cellulose, consists of 300–15,000 D-glucose units. The unit has three hydroxyl groups on C2, C3 and C6, respectively, and the hydroxyl group of C6 is much more reactive than that of C2 and C3. TEMPO is a stable nitroxide radical, which can catalytically oxidize primary and secondary alcohols under aqueous condition with high selectivity and efficiency. In this study we follow the oxidation method that uses a mixture of sodium hypochlorite, sodium bromide and TEMPO. With such reagents, the oxidation is selective as it oxidizes exclusively the primary hydroxyl groups while leaving untouched the secondary ones [23, 24, 26, 27]. There are several works where TEMPO-mediated oxidation was applied to cellulose fibers under various conditions with good results [23, 27–29].

The number of biofunctional textiles with antibacterial activity has increased considerably over the last few years [30–32]. Application is now extended to biomedical products, which is perhaps the largest application of antibacterial textiles [33, 34]. There is a wide range of methods available to examine the interaction of microorganisms with textiles. In order to evaluate the activity of antibacterial textiles there are several standard methods available. The most common can be divided into two categories: (1) qualitative methods: AATCC 147:1998 and JIS L 1902:2002—Halo method and (2) quantitative methods: AATCC 100:1999 and JIS L 1902:2002—Absorption method. In the qualitative method, textile samples are placed onto agar plates, which have been inoculated with bacteria and are then incubated under moist conditions at 37 °C for 24–48 h. The intention is that intimate contact between the textile, the bacteria, and the growth medium will result in the inhibition of growth either immediately adjacent to the textile or in an area around the textile. A new approach to evaluate the effectiveness of the antibacterial activity of textile fibers as well as bacteria adhesion on textiles by using SEM is described in a previous paper [35]. The SEM analysis revealed great potential on the evaluation and effectiveness of antibacterial activity of textiles. Also, the bacterial adhesion and the morphology of bacteria after exposure to antibacterial agents, was determined using the same.

Experimental

Layer by Layer Coating of Cotton

Cotton fabrics obtained from James H. Heal & Co. Ltd was used as substrate, cotton samples were used with dimensions of 2 cm × 2 cm. TEMPO, NaBr, Sodium Hypochlorite (NaOCl) 5 %, CH (low molecular weight), Acetic Acid (CH_3COOH), ALG, Sodium Chloride (NaCl), Sodium Hydroxide (NaOH) and Hydrochloric acid (HCl) were purchased from Sigma-Aldrich. All chemicals were of analytical grade and used as received.

Antibacterial polyelectrolyte CH (1 mg/mL) and ALG (1 mg/mL) solutions were prepared by dissolving CH and ALG in 0.1 M CH_3COOH and 0.5 M NaCl solutions, respectively. The pH values were adjusted to 5 using 0.1 M HCl and 1 N NaOH solutions. The pH was selected to 5, to be approximately intermediate between the pK_a of CH (6.3) and ALG, pK_a of 3.38 and 3.65 [36]. ALG and CH are two oppositely charged natural polyelectrolyte materials and very sensitive toward changes in external factor such as pH. At pH 5 the carboxylate group of ALG mainly exists in the form of COO^- and the amino group of CH mainly exists in the form of NH_3^+. In this case the presence of both COO^- and NH_3^+ along polymer backbone could enhance the electrostatic interaction of the network structure [37]. This value of pH 5 was used in several works in the process of LbL with CH and ALG [38–43].

To apply LbL technique, two samples of the substrate (cotton) were charged by immersing cotton samples in (TEMPO + NaBr + NaClO 5 %, pH = 10.5) solution under moderate stirring for 30 and 120 min, respectively, followed by a rinse with deionized water, as described elsewhere [26, 28]. Then, CH and ALG polyelectrolytes multilayer films were deposited over cotton by the LbL assembly whereas CH was used as polycation and ALG as polyanion. For each layer deposition, the cotton substrate was immersed into the corresponding solution at room temperature and for 5 min, followed by rinsing with deionized water to remove excessive polyelectrolyte. Since cotton samples were charged negatively, the CH was deposited as the first layer. Samples were prepared with five layers (CH/ALG/CH/ALG/CH), six layers (CH/ALG/CH/ALG/CH/ALG), nine layers (CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH) and ten layers (CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH/ALG), respectively in control samples functionalized by LbL (designated by B5, B6, B9 and B10), cotton samples treated during 30 min in TEMPO and then functionalized by LbL (designated by CT5, CT6, CT9 and CT10) and finally cotton samples treated during 120 min in TEMPO and then functionalized by LbL (designated by 2CT5, 2CT6, 2CT9 and 2CT10). After the last deposition, the sample was dried in a desiccator at room temperature overnight. Control samples were also prepared using the same method by LbL, without pretreatment with TEMPO.

The functionalized samples were washed and tested the durability of the CH/ALG multilayers. The durability to washing of samples functionalized by LbL was determined following the NP 1710, textiles—wash fastness test (Portuguese Standard). The Fig. 1 show the SEM images of the cotton (control), the CH/ALG/CH/ALG/CH/ALG (six layers) sample and the sample that were washed after LbL deposition. The functionalized samples show a large and heterogeneous deposition of polyelectrolytes, Fig. 1b. In contrast, the washed samples show a less but more uniform polyelectrolytes deposition Fig. 1c.

The results indicate that the cotton assembled with CH/ALG multilayers had good durability to washing. Good durability to washing revealed the stability of multilayer films on the cotton surface, which is important in various applications of textiles, is not important for application in disposable materials.

Control Tests

Contact Angle

The measurement of the contact angle between water and sample surface is one of the easiest ways to characterize the

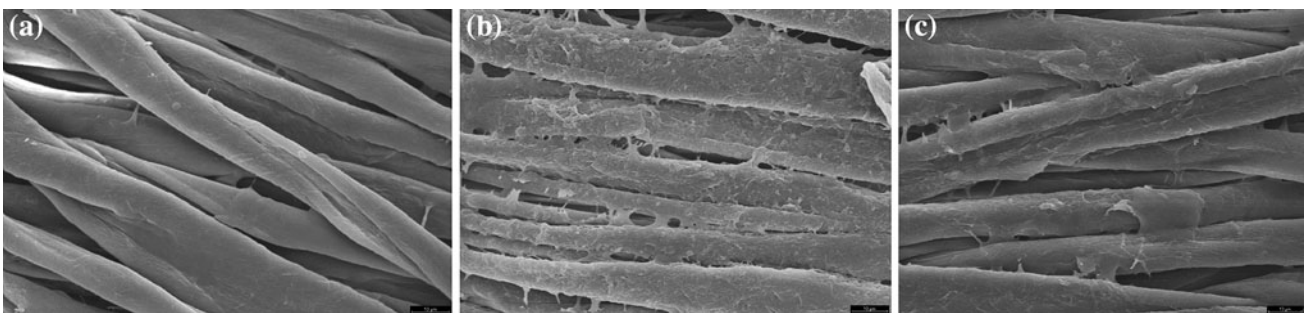

Fig. 1 SEM images. (**a**) Cotton sample, (**b**) CT6 and (**c**) CT6 after washed

wettability of the material [44]. A hydrophilic or hydrophobic surface is defined by these contact angle values. For values higher than 90° the surface is hydrophobic and less than 90° it is hydrophilic [45].

The sessile drop contact angle method [46] was used to measure the contact angles of a water drop on the functionalized cotton samples with a contact angle system Dataphysics model OCAH 200. Contact angles were determined by placing 5 µl drops of deionized water. The tip of the needle was removed, and then an image was recorded. Average values were obtained from multiple contact angle values (at least six) on each sample using an optical system connected to a video display. The measures presented were made at 100, 1,000 and 10,000 ms, according to standard TAPPI T 558 pm-95 "Surface wettability and absorbency of sheeted materials using an automated contact angle tester".

Control Method for Evaluation of Negatively Charged Layers

The present study attempts to evaluate the effect of different process parameters on the amount of polyelectrolyte adsorbed on a cotton samples via sequential adsorption of positively charged CH and negatively charged ALG using LbL self—assembly process. The amount of polyelectrolyte adsorption on cotton fabric was evaluated by measuring the color value (K/S) of methylene blue absorbed cotton surface [15, 16, 19, 20, 47]. Methylene blue exhibit adsorption is proportional to the amount of anionic groups on fibers [48]. In cotton there is abundance of carbohydrate hydroxyl groups, methods of characterization of these groups are in constant development. Several analytical techniques are being applied and new ones introduced and tested, in this paper we use the adsorption of methylene blue. This dye was extensively studied as an indicator of the amount of anionic groups on fibers [15, 16, 19, 20, 47, 48] A dyeing method in terms of K/S values has been proven a simple and quick means to characterize the change of surface polarities of LbL self—assembled textile substrates based on the attraction or repulsion between the cationic dye and polyelectrolytes. The relative color depth of the dyed fabrics expressed as K/S, was measured by the light reflectance technique using the Kubelka–Munk equation [15].

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

Kubelka–Munk theory describes optical characteristics (e.g. reflectance, transmittance and absorbance) by a variety of light scattering media including paints, textiles and paper [49, 50]. The reflectance (R) of the dyed fabrics was measured at the maximum absorbance wavelength on a Datacolor Spectraflash SF300. The ratio of the absorption coefficient (K) to the scattering coefficient (S) varies with the total light reflected according to above expression. To determine K, it is necessary to first compute S, which involves measurements either of the transmittance of the thickness, or of the reflectance of the thickness against backgrounds of known reflectance. If it could be assumed that S was reasonably constant under a given set of experimental conditions, then K/S values should be proportional to the amount of dye and could be used in much the same way that absorbance values were used in the analysis of samples by transmitted light, higher dye content leads to an increase in absorbance. Therefore, the K/S values were expected to be proportional to the amount of dye [51] The values obtained of K/S are commonly used to represent the amount of dye fixation or dye content of a given textile fibers [47].

Cotton specimens with different numbers of layers were dyed using 7.5 % owf cationic dye (methylene blue) [16]. The dyeing was performed in petri dishes without stirring at temperature of 40° C for 15 min. After immersion in the dye solution, the samples were soaked in deionized water and air dried.

ATR-FTIR Analysis

The study was made using Fourier Transform Infrared Spectroscopy in Attenuated Total Reflection mode (ATR-FTIR) with a Vertex 70 spectrophotometer. The transmittance was converted into absorbance for display.

ATR-FTIR reveals information about the molecular structure of chemical compounds and is useful for the characterization of biopolymers. The carbonyl vibrations of a carboxylate and a carboxylic acid group occur at different wave numbers, as does the N–H vibrations of amines and protonated amines [43]. These analyses were made in order to investigate the success of the LbL technique applied to samples of cotton, through the existence or absence of functional groups with specific vibrations.

Results and Discussion

Contact Angle

To identify and distinguish the wettability of each layer in the CH/ALG multilayer film, samples were prepared as described in Table 1. Since the last assembled layer has the most significant effect on the surface property, according to the outermost layer is CH or ALG [52]. The surface wettability is very sensitive to the surface compositions of the outermost layer. The sessile drop contact angle obtained on various surfaces was shown in Table 1. This table presents

Table 1 Contact angle

Sample n°	Description of sample	100 ms	1,000 ms	10,000 ms
1	Cotton	47.52 ± 11.72	–	–
2 (B5)	Control sample—with CH/ALG/CH/ALG/CH	86.13 ± 13.93	55.56 ± 18.33	–
3 (B6)	Control sample—with CH/ALG/CH/ALG/CH/ALG	–	–	–
4 (B9)	Control sample—with CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH	42.85 ± 10.14	–	–
5 (B10)	Control sample—with CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH/ALG	–	–	–
6 (CT)	Anionic cotton (30 min TEMPO), CT	23.31 ± 4.89	–	–
7 (CT5)	CT—with CH/ALG/CH/ALG/CH	114.6 ± 4.46	106.17 ± 12.93	–
8 (CT6)	CT—with CH/ALG/CH/ALG/CH/ALG	67.67 ± 13.10	–	–
9 (CT9)	CT—with CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH	114.63 ± 10.13	101.53 ± 17.27	–
10 (CT10)	CT—with CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH/ALG	82.65 ± 12.07	38.6 ± 2.97	–
11 (2CT)	Anionic cotton (120 min TEMPO), 2CT	22.25 ± 14.50	–	–
12 (2CT5)	2CT- with CH/ALG/CH/ALG/CH	122.58 ± 7.76	123.08 ± 3.99	116.95 ± 10.53
13 (2CT6)	2CT- with CH/ALG/CH/ALG/CH/ALG	54.98 ± 6.20	17.35 ± 4.17	–
14 (2CT9)	2CT- with CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH	112.57 ± 9.50	115.62 ± 6.97	113.23 ± 7.55
15 (2CT10)	2CT—with CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH/ALG	58.4 ± 8.17	–	–

the contact angle of cotton (sample n° 1), control samples functionalized by LbL (samples n° 2–5, designated by: B5, B6, B9, B10), anionic cotton treated during 30 min in TEMPO (sample n° 6, CT), previous anionic cotton functionalized by LbL (samples n° 7–10, designated by: CT5, CT6, CT9, CT10), anionic cotton treated during 120 min in TEMPO (sample n° 11, 2CT) and previous anionic cotton functionalized by LbL (samples n° 12–15, designated by: 2CT5, 2CT6, 2CT9, 2CT10). Results show that for the control samples (samples B5, B6, B9, B10) untreated with TEMPO, the contact angle values are much lower than those of the other samples. Cotton fibers contain abundant hydroxyl groups, making the fiber surface highly hydrophilic. Although cotton fibers are known to be charged slightly negatively because of the ionization of some hydroxyl groups [53], the TEMPO-oxidation method is selective as it oxidizes exclusively the primary hydroxyl group [24], so the TEMPO-oxidation makes the cotton fibers charge enough for the electrostatic assembly. For cotton, the initial contact angle was around 47.52°, for sample CT and 2CT the contact angle suffered a decrease because of the hydrophilic character of cotton when immersed in TEMPO, due to the formation of a greater number of negative charges on the sample surface. In general, the water contact angle decreases as the extent of surface charges increases and the surface becomes more hydrophilic. For all samples functionalized by LbL, the greatest reduction in contact angle was observed for the samples CT6, CT10, 2CT6 and 2CT10, confirming the presence of permanent negative charges in the polymer chains (ALG) as a generator of a hydrophilic matrix. For the samples CT5, CT9, 2CT5 and 2CT9, where the last layer is CH, higher contact angles were achieved, showing the hydrophobic character of CH. This is in accordance with previous studies, where the CH layer deposition led to a contact angle increase [52, 54, 55]. The different values of contact angle according to the outermost layer is CH or ALG which are similar to those found in literature, pure CH membranes were more hydrophobic than ALG membranes [54–60].

Figure 2 shows the contact angle (measured at 100 ms) of the multilayer films with layer number, from 0 (cotton sample), 5, 6, 9 and 10, for samples treated 30 min and 120 min in TEMPO. For all the conditions, the contact angle exhibits the zigzag feature with the layer number, indicating the alternate assembly of CH and ALG on the surface. These observations are due to the surface

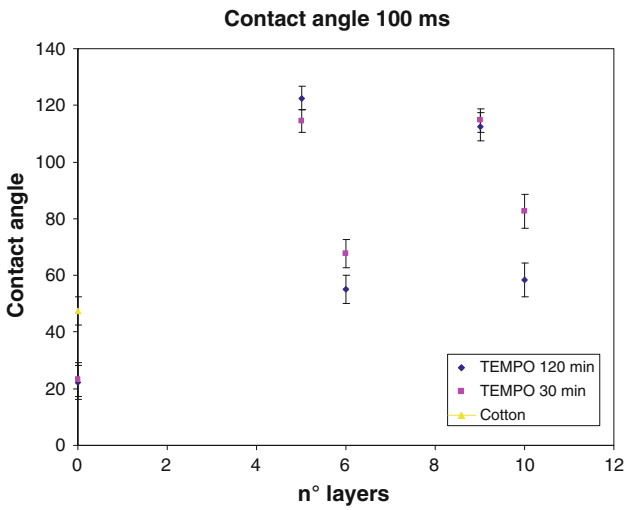

Fig. 2 Contact angle of CH/ALG multilayer films with 5, 6, 9 and 10 numbers of layers. The sample without LbL functionalized is layer zero

composition as a result of the contact angle of CH that is bigger than that of the ALG. Typically, the ALG layer induced an decrease of contact angle whereas CH layer deposition led to a contact angle increase. The wettability of a surface depends on the nature of the outermost layer and not on the initial substrate film [61].

Control Method for Evaluation of Negatively Charged Layers

The electrical properties of the cotton surface would change alternately between positively charged and negatively charged after LbL electrostatic assembly of polyelectrolytes. So, it is valuable to determine the change in surface polarity of assembled cotton to demonstrate the stepwise buildup of CH/ALG multilayers. The cationic methylene blue was used to dye the self-assembled cotton in order to reveal the change of cotton surface. The increase in absorbance at 600 nm of the samples was monitored using a reflectance spectrophotometer. We can use the increase of the *K*/*S* value as a characteristic of the LbL deposition of the dye on fibers. The growth of multilayers on the cotton surface based on LbL self-assembly was assessed in terms of the UV change absorbance of assembled substrates.

As shown in Fig. 3 the regular changes in color depth of assembled cotton samples indicate that the surfaces were alternately deposited with every CH and ALG layers. As the outermost layer alternated between CH and ALG, the *K*/*S* values present a regular oscillations, revealing that the surface is covered by CH or by ALG which is enough to change the surface polarities. The high *K*/*S* values shown in Fig. 3 indicate a high level of adhesion of the ALG on the sample. Moreover, the fact that the data measured at four different locations, including both sides of the fabric, was nearly the same demonstrates that the surface is uniformly coated. When CH first layer is deposited on activated cotton, a part of the $NH3^+$ groups in CH were bound with anionic groups of cotton through ionic bonds, the remained would make the cotton surface present net positive charges. The newly formed positively charged surface rejects the absorption of cationic direct dye because of the repulsion between the same charges, resulting in a decrease of color depth. However, when ALG was subsequently assembled on the CH coated cotton surface, the electric properties of cotton surface were reversed. The newly formed cotton surface with net negative charges would attract the cationic dye, causing the increase of *K*/*S* values. The composition of the polyelectrolytes deposited on the surface of assembled cotton directly relates to the linear increases in UV absorbance. Therefore, the growth of these multilayers could be recorded by monitoring the UV spectra of assembled cotton specimens. The behavior observed in Fig. 3, where the *K*/*S* value exhibits a zigzag feature through even/odd layer numbers, indicating the alternate assembly of CH and ALG on the surface, is consistent with other results found in the literature [15, 16].

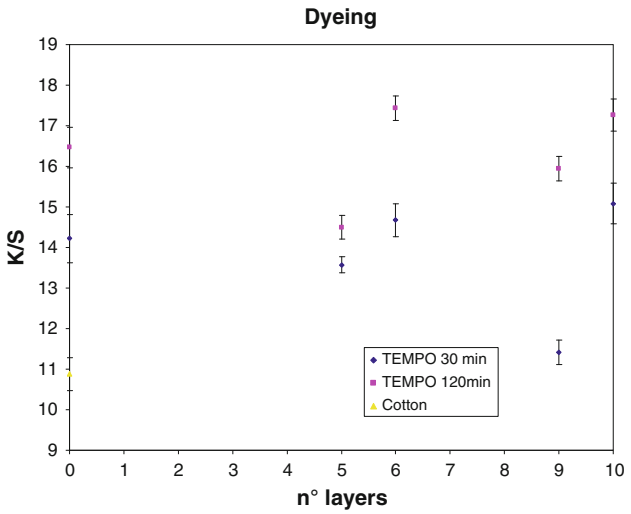

Fig. 3 Tracing of the color depth (*K*/*S*) versus the number of monolayers in CH/ALG assembled multilayers on cotton dyed with methylene blue. The first layer is CH and the surface layer alternates between CH and ALG

The electrical properties of the cotton surface changed alternately between positively charged and negatively charged after LbL electrostatic assembly of polyelectrolytes. Therefore, the change in surface polarity of assembled cotton demonstrates the stepwise fabrication of CH/ALG multilayers. As the outermost layer alternated between CH and ALG the *K*/*S* values presented regular oscillations, revealing that the surface coverage of CH by ALG and vice versa is enough to change the surface polarities.

We initially used the method for evaluation of negatively charged in samples of cotton and cotton pre-treated with TEMPO. In Fig. 4b we found that the sample has a more intense color than in Fig. 4a. This is due to the cotton sample pre-treated with TEMPO get negative charges, so there is a greater absorption to the surface of the cationic dye methylene blue. The complexity of textile surface makes the monitoring of the multilayers become difficult, because the textile substrates in fabric form have a non-planar surface. Considering the change of surface electric property of the cotton specimen during self-assembly procedure, which had been demonstrated by determining the color depth of cotton surface, it can be concluded that CH were produced on the cotton substrate via bonding with oppositely charged ALG.

ATR-FTIR

In the current study, an LbL assembly was produced on a cotton fabric to explore the interaction between CH and

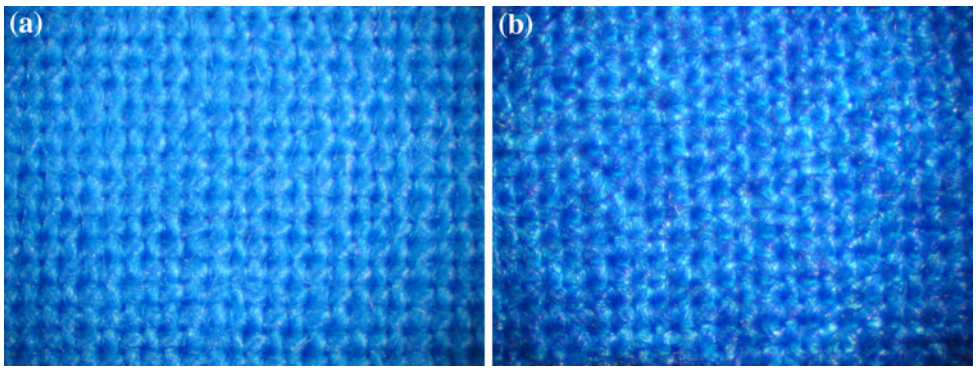

Fig. 4 Images for evaluation of negatively charged layers. **a** Cotton sample with methylene blue; **b** cotton sample pre-treated with TEMPO

ALG. In order to evaluate the ability of the functionalization process with TEMPO to produce a surface capable of supporting polyelectrolyte films via LbL deposition, ATR-FTIR spectrum of the received cotton, Fig. 5a, functionalized cotton samples when immersed 30 min in TEMPO (CT sample), Fig. 5b and functionalized cotton samples when immersed 120 min in TEMPO (2CT sample), Fig. 5c, were assessed. When cotton was immersed into TEMPO, Fig. 5b, c and took new spectrum of the functionalized cotton, the spectrum was almost identical to the original. Figure 5, shows characteristic cellulose peaks around 1,000–1,200 cm^{-1}, which are the main components of cotton [62–64]. As shown in Fig. 5, in the current study, the absorbance intensity of the characteristic peak at around 1,600 cm^{-1} varied. The band is absent in the cotton sample (Fig. 5a), but can be detected in CT and 2CT samples (Fig. 5b, c). A maximum, but not significant value for this band is reached in the spectrum (Fig. 5c) for the 2CT sample, corresponding to the maximum oxidation conditions. Thus, we can conclude that the TEMPO-mediated oxidation conditions were selectively converted to carboxylate ion ionized form (COO^-), imparting a negative surface charge to the cotton, as discussed previously [62]. The TEMPO-mediated oxidation treatment was proven to be able of modifying a surface of samples cotton to produce a surface capable of supporting multilayer films.

Figure 6 shows the ATR-FTIR spectra of cotton and cotton assembled with CH/ALG multilayers. In this figure six regions can be distinguished: first at 770–830 cm^{-1}, 1,180–1,300 cm^{-1}, 1,330–1,450 cm^{-1}, 1,600–1,800 cm^{-1}, 2,800–2,980 cm^{-1} and finally at 3,100–3,550 cm^{-1}. Figure 6 showed characteristic cellulose peaks around 1,000–1,200 cm^{-1} [64]. Other characteristic bands related to the chemical structure of cellulose were hydrogen—bonded OH stretching around 3,100–3,550 cm^{-1}, the C–H stretching around 2,800 cm^{-1} and the asymmetrical COO^- stretching around 1,600 cm^{-1} [53, 62–64]. If the carboxylate existed in ionized form (COO^-), it would show two peaks at 1,600 and 1,400 cm^{-1} for the asymmetric and the symmetric stretching of COO^- ion, respectively [62]. In carboxylate ion, if it is protonated, it would become –COOH in which double bond (C=O) and single bond (C–OH) would exist. The C=O stretching would show at around 1,750 cm^{-1} and C–OH stretching at 1,200 cm^{-1} [62, 64]. This was confirmed by the spectrum of samples shown in Fig. 6. Based on several studies [41–43, 65] the characteristic peaks of CH were detected in a region around 1,700–1,500 cm^{-1} corresponding to amino group. The

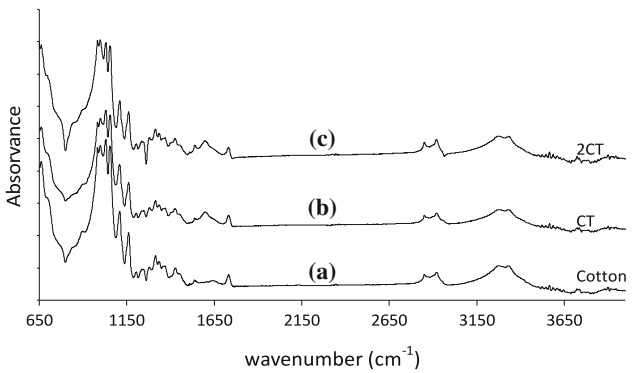

Fig. 5 ATR-FTIR spectra of cotton and of cotton TEMPO- oxidized with different immersion times. (*a*) Cotton sample, (*b*) immersed 30 min in TEMPO, (*c*) immersed 120 min in TEMPO

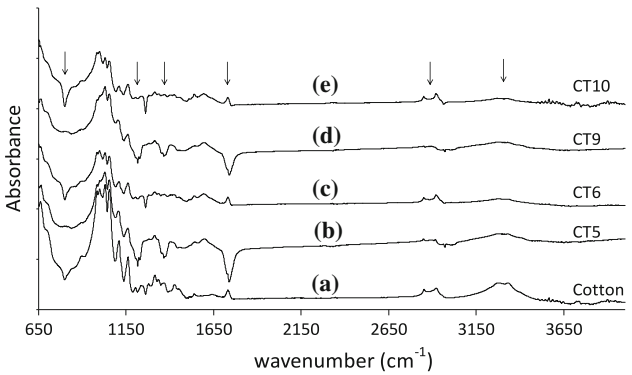

Fig. 6 ATR-FTIR spectra of cotton (*a*) and samples CT5 (*b*), CT6 (*c*), CT9 (*d*) and CT10 (*e*). *Arrows* indicates the six regions can be distinguished

ALG spectrum shows characteristic band of carboxylate (COO^-) band at 1,600 and 1,400 cm^{-1}. The observation of theses peaks in Fig. 6, indicated that the degree of ionic interaction between the negatively charged carboxylic ion group of ALG and the positively charged amino group of CH. The study of Alves et al. and Lawrie et al. [41, 43] present values of the characteristic stretching bands in ATR-FTIR spectrum which correspond to CH and ALG related to the chemical structure. When CH is the outermost layer in an LbL assembly (Fig. 6b, d), CT5 and CT9 samples, the amine groups extending into solution during fabrication will become deprotonated upon washing and drying (around 1,400 cm^{-1}).

However, when ALG is the outermost layer (Fig. 6c, e), CT6 and CT10 samples, the amine groups of the underlying CH layer will be protonated to a larger degree due to interaction with the deprotonated carboxylate groups of ALG. It was therefore expected that a higher amount of protonated amines will be present when ALG is the outmost layer [41, 43]. This behavior is observed in the peak around 1,400 and 1,200 cm^{-1} in Fig. 6. It can be seen in Fig. 6 that after the assembly of 5 bilayers, the absorption band around 3,300 cm^{-1}, corresponding to the hydroxyl groups of cellulose, became less evident, this result also confirms the presence of CH/ALG multilayers on cotton. The same study was done for samples 2CT5, 2CT6, 2CT9 and 2CT10, and it was found that the analysis of spectra was identical to that described previously. The fourth region in the FTIR spectrum at 1,680–1,800 cm^{-1}, Fig. 7b, d the spectrum for samples B5 and B9 have the same behavior that samples CT5, 2CT5, CT9 and 2CT9, but for samples B6 and B10 the behavior is the inverse of the samples CT6, 2CT6, CT10 and 2CT10. On the other hand, for this last group of samples, on the second region (1,180–1,300 cm^{-1}) and third region (1,330–1,450 cm^{-1}) the band disappeared. The behavior of ATR-FTIR spectrum of control samples is exactly the same in the various layers, this does not happen in samples CT5, CT6, CT9 and CT10, comparatively to spectra of Fig. 6. This indicates that the LbL process was not successful in these samples, because these samples were not activated with TEMPO-oxidation. Two different levels of TEMPO-oxidation (30 and 120 min) were used to functionalize cotton samples substrate in order to investigate the role of the supporting surface in the buildup of the multilayer. According to the results, we found that the activation of the substrate with TEMPO-oxidation is necessary to ensure the success of the LbL technique applied to cotton. Concerning the level of oxidation, there are no relevant differences in results for of the immersion times of 30 and 120 min in TEMPO-oxidation. Be noted that some characteristic peaks of absorption could not almost be identified from assembled cotton, even after a (CH/ALG) multilayer was fabricated on cotton. This might be due to the fact that ATR-FTIR is not sensitive enough to the small amount of the deposited materials. In addition, partial overlapping of characteristic absorption peaks between the functionalized cotton and the polyelectrolytes might also result in the above phenomenon [15].

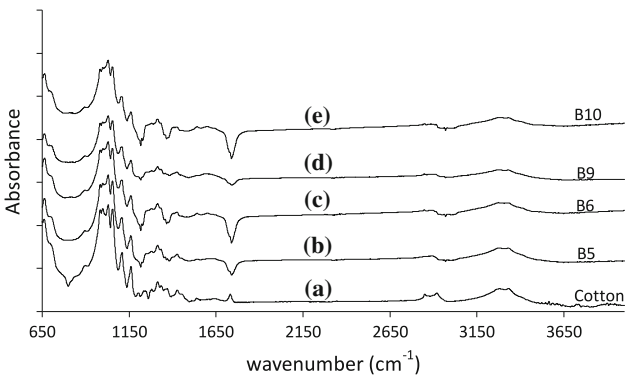

Fig. 7 ATR-FTIR spectra of cotton (*a*) and control samples (*b–e*)

The samples used in this study have been tested on the antibacterial properties on a previous study [35]. Antibacterial activity of functionalized cotton samples was determined in terms of inhibition zone formed around the sample and analyzed by SEM. Control samples showed low antibacterial activity and bacterial growth on the surface of control samples analyzed by SEM indicated the presence of colonies. LbL functionalized cotton sample presented a clear area around it with no bacterial growth (zone of inhibition). Analyzing these samples by SEM, we observed damaged bacteria under and around them.

Conclusions

The LbL deposition process was used to deposit alternate layers of CH and ALG on cotton substrates. Treatment of the cotton samples with TEMPO was proven to be an effective procedure to create a substrate able to support multilayer films. This result was confirmed by various methods of analysis used in this work. The activation of the substrate with TEMPO-oxidation is necessary to ensure the success of the LbL technique applied to cotton.

The surface wettability is very sensitive to the surface compositions of the outermost layer. Using the contact angle method it was found that differently functionalized samples presented different values. Samples where the last layer was CH had a higher value of contact angle compared to samples having ALG on the last layer. This result is consistent with the considerations found in the literature, classifying CH has having hydrophobic character and ALG has having hydrophilic character. This fact demonstrated the formation of alternating layers of CH and ALG, indicating that the process of LbL was successfully applied in cotton samples.

Dyeing of the CH/ALG assembled cotton samples with cationic methylene blue shows regular and observable "odd–even" changes in terms of color depth (*K*/*S* value), indicating the variation of surface composition of the cotton substrates due to the alternate deposition of CH and ALG on them.

ATR-FTIR provided direct and indirect evidence of the efficacy of the deposition process. It was possible to follow the formation of the multilayers of CH/ALG on cotton samples, analyzing the chemical changes in each layer. These experimental results validate the feasibility of using the LbL self-assembled deposition of natural polyelectrolytes on cotton substrates as a novel processing method for textile functionalization.

The LbL of CH/ALG on cotton textile has potent antibacterial activity toward both Gram-positive and Gram-negative bacteria. These coating cotton textiles are potentially useful as antibacterial fabrics in a wide variety of biomedical and general use applications.

Overall, the results showed a promising eco-friendly and simple technique to give functionalized textiles with antibacterial properties using natural polyelectrolytes with antibacterial agents. This method can open new avenues towards the development of non-toxic and safe biomedical textiles.

Excellent durability to washing of the CH/ALG multilayers was obtained, which indicates good adhesion between the multilayer coatings and the cotton surfaces.

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Paper III

.

Assessment of bacteria-textile interactions using Scanning Electron Microscopy: A study on LbL chitosan/alginate coated cotton

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Assessment of bacteria-textile interactions using Scanning Electron Microscopy: A study on LbL chitosan/alginate coated cotton.

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In order to evaluate the activity of antimicrobial textiles there are several standard methods available. The most common can be divided in two categories: (1) qualitative methods: AATCC 147:1998, and JIS L 1902:2002 – Halo method and (2) quantitative methods: AATCC 100:1999 and JIS L 1902:2002 – Absorption method. However, these standard methods are time consuming and require appropriate facilities with respect to microbiology. Despite their ability to restrict bacteria growth, the textile samples could be colonized with bacteria that may develop a suitable environment for future colonization. In this case, the standard methods normally used to measure antimicrobial activity of textiles are rendered ineffective concerning the assessment of bacterial adhesion.

As a result, this paper presents a new approach in evaluating the effectiveness of the antibacterial activity of textile fibers as well as bacteria adhesion on textiles by using Scanning Electron Microscopy (SEM). Antibacterial efficacy of cotton substrates functionalized via Layer-by-Layer (LbL) deposition of antibacterial polyelectrolytes (chitosan, CH and alginate, ALG) was assessed using Gram-positive and Gram-negative bacteria, *Staphylococcus aureus* and *Klebsiella pneumoniae* respectively.

The experimental results showed that the functionalized cotton samples exhibit different but ambiguous antibacterial properties, as confirmed by the different appearance of a very thin halo around the samples (JIS L 1902:2002). In addition, SEM analysis of the surfaces of the functionalized cotton samples able to identify bacterial populations. In this way, the antibacterial efficacy of the CH/ALG multilayer's was evaluated by assessing the reduction in bacteria attachment and growth on the cotton substrates.

SEM analysis is able to show the effectiveness of the several functionalized samples in preventing bacterial adhesion besides bacteria growth.

Keywords SEM; LbL; antibacterial textiles; antibacterial assays for textiles

1. Introduction

The number of biofunctional textiles with antimicrobial activity has increased considerably over the last few years [1-3]. Application is now extended to biomedical products, which is perhaps the largest application of antimicrobial textiles [4, 5]. Antimicrobial textiles were first created to prevent damage to textiles under adverse environmental conditions during their storage or use. In fact, antimicrobial textiles were first used during World War II [6].

Textiles are widely used in day-to-day life and there has been a growing need to develop finishes for textiles materials that can offer improved protection, to the users, from microbes (bacteria, fungi), which can cause numerous problems. Hence there is a pressing need to develop functionalized textiles that are resistant to microbes as the textile substrates find various applications such as masks, hospital textiles, and surgical gowns as well as the conventional apparel usage.

The number of different antimicrobial agents suitable for textile application on the market has increased drastically in recent years. Several different types of antimicrobial agents, such as oxidizing agents, coagulants, diphenyl ether (bis-phenyl) derivatives, heavy metals and metallic compounds, chitosan and quaternary ammonium compounds are used in the textile industry to confer antimicrobial properties [7]. The selection of the antimicrobial agent depends on the mechanism of antimicrobial activity (bacteria and fungi), toxicity, application method and cost.

Polysaccharide biopolymers including alginate (ALG) and chitosan (CH) have been the focus of an expanding number of studies reporting their potential use in biomedical research applications such as cell encapsulation, drug delivery, and tissue engineering, then multilayered films containing both polysaccharides could be useful in the coating of substrates for different biomedical applications [8, 9].

Moreover, there is a wide range of methods available to examine the interaction of microorganisms with textiles. In order to evaluate the activity of antimicrobial textiles there are several standard methods available. The most common can be divided into two categories: (1) qualitative methods: AATCC 147:1998 and JIS L 1902:2002 – Halo method and (2) quantitative methods: AATCC 100:1999 and JIS L 1902:2002 – Absorption method. In the qualitative method, textile samples are placed onto agar plates, which have been inoculated with bacteria and are then incubated under

moist conditions at 37ºC for 24 – 48 hours. The intention is that intimate contact between the textile, the bacteria, and the growth medium will result in the inhibition of growth either immediately adjacent to the textile or in an area around the textile. These methods are generally acknowledged as being non-quantitative, although they could potentially be employed as assays of certain antimicrobial products in the same manner that such techniques are used for some antibiotics. However these techniques are considered unsuitable for quantifying the effect of the antimicrobial effect of treated textiles [6].

In addition to the qualitative tests, quantitative data can be provided on the effect of treated textiles on bacteria. In this case replicate samples (6 of the control and 3 of the treated) are inoculated with individual bacterial species (*Staphylococcus aureus* and *Klebsiella pneumoniae)* suspended in a heavily diluted nutrient medium. The samples are incubated under humid conditions at 37ºC for a specified contact time. Activity is assessed by comparing the size of the initial population in the control with that present following incubation. No neutralizer is employed during cell recovery [6]. This method requires a lengthy period of time to be realized and the procedure is very complex. When the number of samples increases, the complexity also increases. Moreover you cannot assess the adherence of bacteria to a sample and analyze their distribution on the surface of the textile. Consequently, a new approach to evaluate the effectiveness of the antimicrobial activity of textile fibers as well as bacteria adhesion on textiles by using a Scanning Electron Microscopy (SEM) is described in this paper.

SEM is a powerful instrument that permits the observation and characterization of heterogeneous organic and inorganic materials and surfaces. The area to be examined or the micro volume to be analyzed is irradiated with a finely focused electron beam, which may be static or swept in a raster across the surface of the specimen. In the SEM, the signals of greatest interest are the secondary and backscattered electrons, since these vary according to differences in surface topography as the electron beam sweeps across the specimen. The secondary electron emission is confined to a volume near the beams impact area, permitting images to be obtained at relatively high resolution

SEM offers a relatively simple method of studying the surface morphology of samples at high magnification under optimal conditions. Another important feature of SEM is the three dimensional appearance of the specimen image, a direct result of the large depth of field, as well as to the shadow relief effect of the secondary and backscattered electron contrast. The greater depth of field of SEM provides much more information about the specimen. The basic components of the SEM are the lens system, electron gun, electron collector, visual and recording cathode ray tubes, and the electronics associated with them [10].

In addition, one of the potentials of the SEM, which remains largely unexplored, is the evaluation of the effectiveness of the antimicrobial activity of textiles, as well as bacteria adhesion on textiles as well as the morphological consequences of exposure of bacteria to antimicrobial agents.

Numerous studies that use SEM to characterize different microorganisms and different antimicrobial agents have been published [11-17]. Most studies are related with SEM observations of the different morphological changes caused in the microorganism by antibiotic; however there aren't any scientific articles concerning the SEM surface observation of functionalized textiles aiming to assess antimicrobial activity. Nor are there direct observations on clear zone surrounding the sample (antimicrobial test) and analysis of microbial inhibition under the sample.

Therefore, here we report the potential of SEM in the assessment of antibacterial activity, by analyzing the morphological changes in *Staphylococcus aureus* and *Klebsiella pneumonia*e induced by Layer by Layer (LbL) functionalized cotton fabrics.

2. Experimental

2.1 Layer by Layer coating of cotton

To apply the LbL technique cotton substrates should be charged. Anionic cotton was prepared by using 2,2,6,6,-tetramethylpiperidinyl-1-oxy free radical (TEMPO) and NaBr [18]. Chitosan (CH, 1 mg/mL), antimicrobial polyelectrolyte, and Alginic acid sodium salt (ALG, 1 mg/mL) solutions were prepared by dissolving CH and ALG in 0.1 M CH_3COOH and 0.5M NaCl solutions, respectively. The pH values were adjusted to 5 using 0,1M HCl and 1 N NaOH solutions. cotton samples were charged negatively by immersing cotton samples in TEMPO solution under moderate stirring, followed by a rinse with deionized water, as described elsewhere [19,20]. Then, CH and ALG polyelectrolytes multilayer films were deposited over cotton by the LbL assembly where CH was used as polycation and ALG as polyanion. The layer sequence can be designed to be $(CH/ALG)_n$. For each layer deposition, the cotton substrate was immersed into the corresponding solution at room temperature for 5 minutes, followed by rinsing with deionized water to remove the excess of polyelectrolyte. Since the cotton samples were charged negatively, the CH was deposited as the first layer. After the last deposition, samples were dried in a desiccator at room temperature overnight. All chemicals were obtained from Sigma-Aldrich, were of analytical grade, and were used as received.

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2.2 Assessment of antibacterial activity by the JIS L 1902:2002 method

The culture medium Brain Heart Infusion (BIH) used for the cultivation of the bacterial strains deployed in this work, were prepared according to the instructions of the manufacturer. Culture media were dissolved directly after being weighed in deionized distilled water, and then sterilized by autoclaving for 15 min at 121 ºC. Agar was used to solidify the media before autoclaving. The strains of *Staphylococcus aureus* and *Klebsiella pneumonia*e used were obtained from the American Type Culture Collection, ATCC 25923 and ATCC 13883 respectively. The cell density of bacterial suspensions was determined by measuring the optical density (OD) of appropriately diluted samples using a spectrophotometer at a wavelength of 640 nm [OD_{640}] The number of viable cells in a bacterial suspension was estimated and diluted in physiological saline (0.9% wt/vol, NaCl). 100 µL aliquots of the appropriate dilutions were plated onto the surface of agar plates with a Mueller-Hinton medium, and after approximately 10 min the functionalized cotton samples were placed on top of agar plates and incubated for 24h at 37 ºC.

2.3 Assessment of antibacterial activity with Scanning Electron Microscopy

After completing the previous procedure, samples were removed from agar plates and fixed with 3% glutaraldehyde at 4ºC overnight. Dehydration of the samples was then conducted by a series of 10, 25, 50, 70, 100 % ethanol solutions. Using a Critical Point Dryer the samples were dried further (CPD, Emitech). These samples were mounted on aluminum stubs and then coated with gold using a Sputter Coater (Emitech). Finally the samples were examined using a Hitachi (S 2700) SEM. Samples of culture medium Mueller – Hinton (agar plates) were also prepared to observe in the SEM, by the same method described previously.

3. Results and Discussion

3.1 Antibacterial activity

The antibacterial efficacy of the CH/ALG multilayer's was evaluated by assessing the reduction in bacterial attachment and growth on the functionalized cotton substrates.

SEM analysis revealed that on the CH/ALG functionalized cotton, the densities of attached bacteria decreased when compared with that of the control. It appears that the effectiveness in preventing bacterial adhesion is high regardless of whether CH/ALG is deposited as the outermost layer. This is in accordance with the expected. The use of CH as an anti-biofilm coating for medical applications has recently been suggested, as coating surfaces with CH are highly effective at restricting or preventing the formation *Staphylococcus aureus* and *Klebsiella pneumoniae* biofilms [21].

Likewise, the results of the JIS L 1902:2002 – qualitative method were used to assess the antibacterial activity of textile specimens.

In fig. 1 a positive control (sample known to have antimicrobial activity) presents a halo around the sample. Halo size provides some indication of the potency of the antimicrobial activity of textile samples.

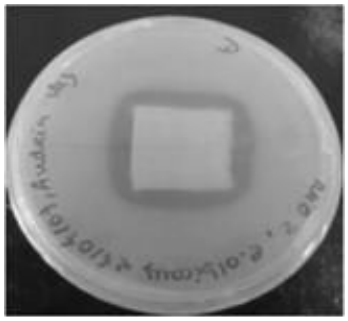

Fig.1 Image of a halo formation of a positive control (analysed using the standard method JIS L 1902:2002 - Halo method, against *Staphylococcus aureus*).

In contrast, the results of the functionalized cotton (fig. 2) showed an ambiguous inhibitory effect against *Staphylococcus aureus* and *Klebsiella pneumoniae*, although in terms of the surrounding clearing zone the different samples did not show the same inhibitory effect against the tested microorganisms. In fig. 2 (b), (c), (e) and (f), the functionalized samples showed a small but clear inhibitory zone for *Staphylococcus aureus* and a smaller halo for the *Klebsiella pneumoniae*. However, control sample against *Klebsiella pneumoniae* also presented a very thin inhibition line in the edges of the fabric. Due to these ambiguous results concerning a clear halo formation and in order to give more conclusive results the samples were removed from the Petri dishes used for testing JIS L 1902:2002 – Halo method, allowing the analysis of bacteria inhibition under the sample. This can be observed in fig. 3. It is interesting to note, that for both bacteria, *Staphylococcus aureus* and *Klebsiella pneumoniae*, the control sample (without LbL) showed little microbial growth inhibition. In contrast, the inhibition was more significant in the presence of CH/ALG.

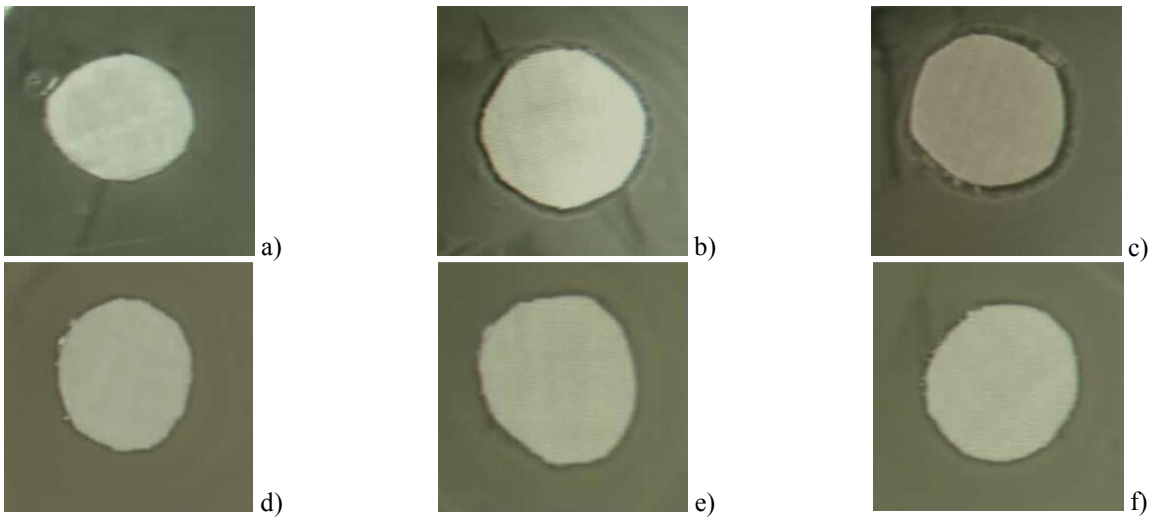

Fig. 2 Images of samples tested according with the standard method JIS L 1902:2002-Halo method. a), b), c), inhibition zone against *Staphylococcus aureus* for control sample, sample $(CH/ALG)_3$ and sample $(CH/ALG)_5$ respectively. d), e), f), inhibition zone against *Klebsiella pneumoniae* for control sample, sample $(CH/ALG)_3$ and sample $(CH/ALG)_5$ respectively.

In addition, the results also showed that the antibacterial effect of CH/ALG occurred without migration of the active agents. As CH/ALG layers are in a solid form, only microorganisms in direct contact with the active sites of CH/ALG are inhibited because CH/ALG layers are incapable of diffusing through the adjacent agar medium, as discussed elsewhere [22]. As a result, and as described previously, the possible mechanisms for antibacterial activity are: (1) the CH/ALG on the surface of the cell can form a polymer membrane, which prevents nutrients from entering the cell. (2) CH/ALG entered the cell through pervasion. Since CH could absorb the electronegative substance in the cell and flocculate them, it disturbs the physiological activities of the bacteria [23].

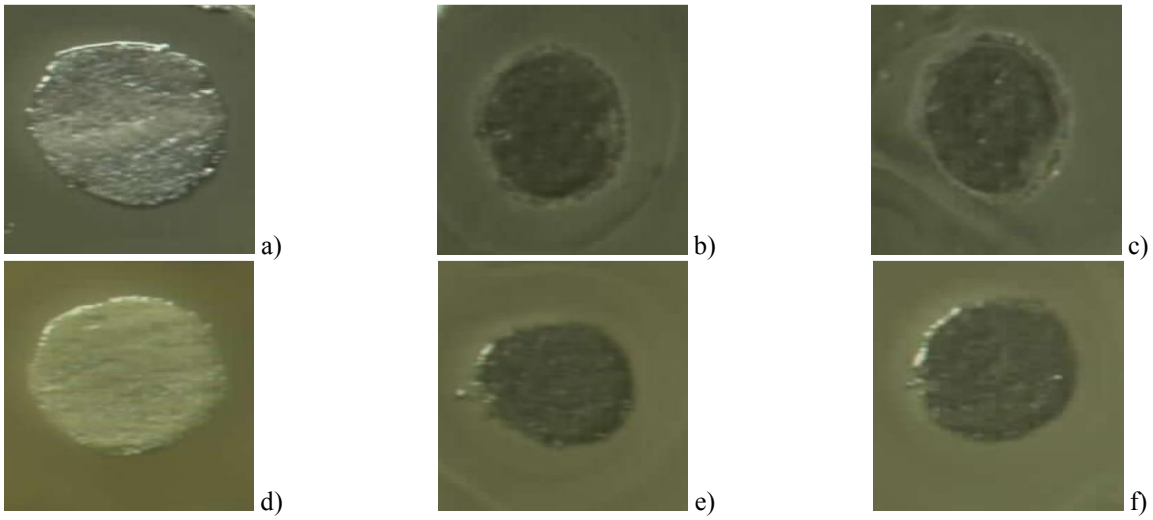

Fig. 3 Analysis of bacteria inhibition under the sample. a), b), c) control sample, sample $(CH/ALG)_3$ and sample $(CH/ALG)_5$ respectively, against *Staphylococcus aureus*. d), e), f), control sample, sample $(CH/ALG)_3$ and sample $(CH/ALG)_5$ respectively against *Klebsiella pneumoniae*.

3.2 Scanning Electron Microscopy

SEM analysis was used to examine the minor changes in cell morphology of the strains *Staphylococcus aureus* and *Klebsiella pneumoniae*. Figs. 4 and 5 represent the evolution of bacterial damage caused by CH/ALG multilayers. In the presence of CH/ALG the bacteria suffered alterations in their morphology, as it can be seen in figs. 4 e), f) and 5 e), f). In contrary, in the absence of CH/ALG, the bacteria cell morphology was apparently normal (figs. 4 d) and 5 d)).The morphological changes observed in bacterial cells after exposure to CH/ALG are similar to those found in literature [11-17]. In all these works, the authors found significant morphology changes and disorders in the bacterial surface structures when in contact with antimicrobial agents due to bacterial death. Comparing the images presented in those works with the images undertaken in this investigation, the appearance of bacteria *Staphylococcus aureus* exposed to

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CH/ALG is very similar. Typically, all SEM micrographs show distorted cells with small surface depressions, bleb formations, and cell fragments (fig 4 f)). In extreme conditions, total collapse of bacteria when exposed to antimicrobial agents can also be found.

In the case of *Klebsiella pneumoniae* healthy cells have an oval-like shape with a regular and smooth surface. Damaged cells induced by antimicrobial agent show irregular and bleb-like protrusions on their surfaces. The cells remain swollen or shrunken after biocide action (fig. 5 f)).

Generally, bacteria exposed to antimicrobial agents show significant morphological changes, presenting variations in size and shape, many being smaller than usual, others being abnormally large.

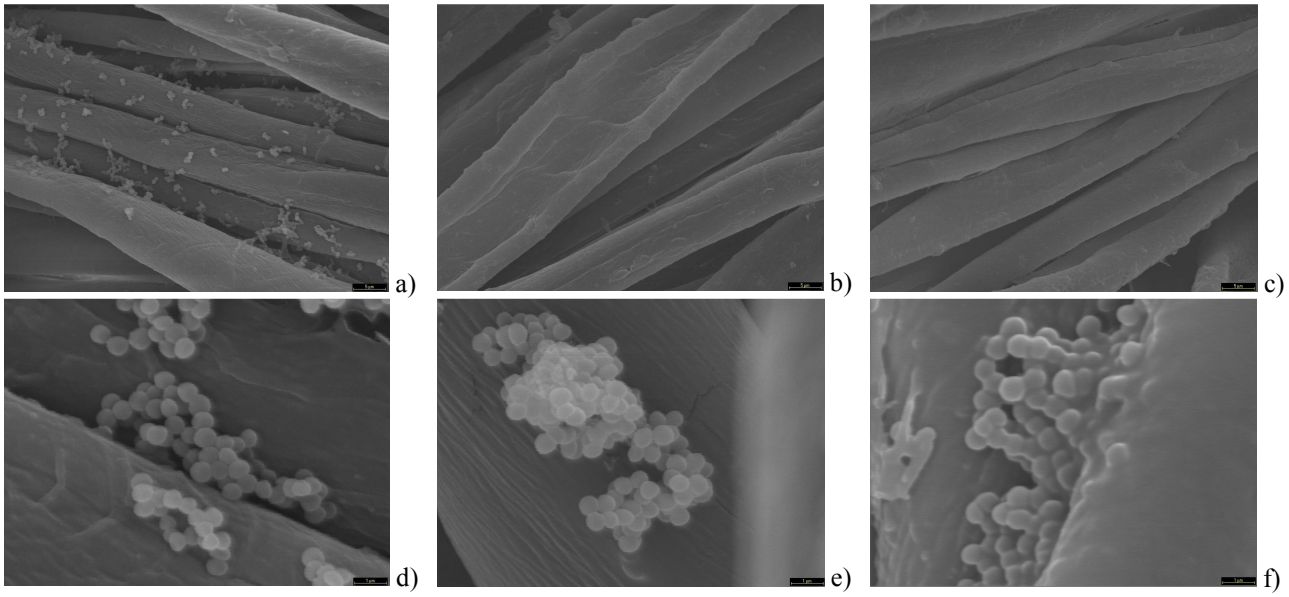

Fig. 4 SEM images of samples tested against *Staphylococcus aureus*, a) and d) control samples; b) and e) $(CH/ALG)_3$; c) and f) $(CH/ALG)_5$. Images a), b) and c) show the zone under functionalized cotton, and images d), e) and f) show the upper zone of the functionalized cotton samples. Magnifications are x2000 for a), b) and c) and x10000 for d), e) and f).

The SEM analysis of the strains under investigation clearly put in evidence the antibacterial activity of the functionalized cotton. SEM analysis was very valuable in the assessment of this antibacterial activity whereas standard tests (JIS L 1902:2002) gave ambiguous results.

However, more information can be addressed using SEM analysis. In this way, samples prepared from culture medium Mueller – Hinton, after the removal of functionalized cotton, were also analyzed under SEM. The results showed a high density and uniformity of growth of *Staphylococcus aureus* and *Klebsiella pneumoniae* by all culture mediums removed from a zone without any contact with the functionalized cotton samples and far from the halo formation (fig. 6 a) and d)). In addition, the area that was previously covered by the functionalized cotton specimen, revealed a complete inhibition of *Staphylococcus aureus* and *Klebsiella pneumoniae*, as shown in fig. 6 (c) and f)). It is also possible to observe moderate and increased growth inhibition in the zone in the edges of the functionalized cotton (fig. 6 b) and e)).

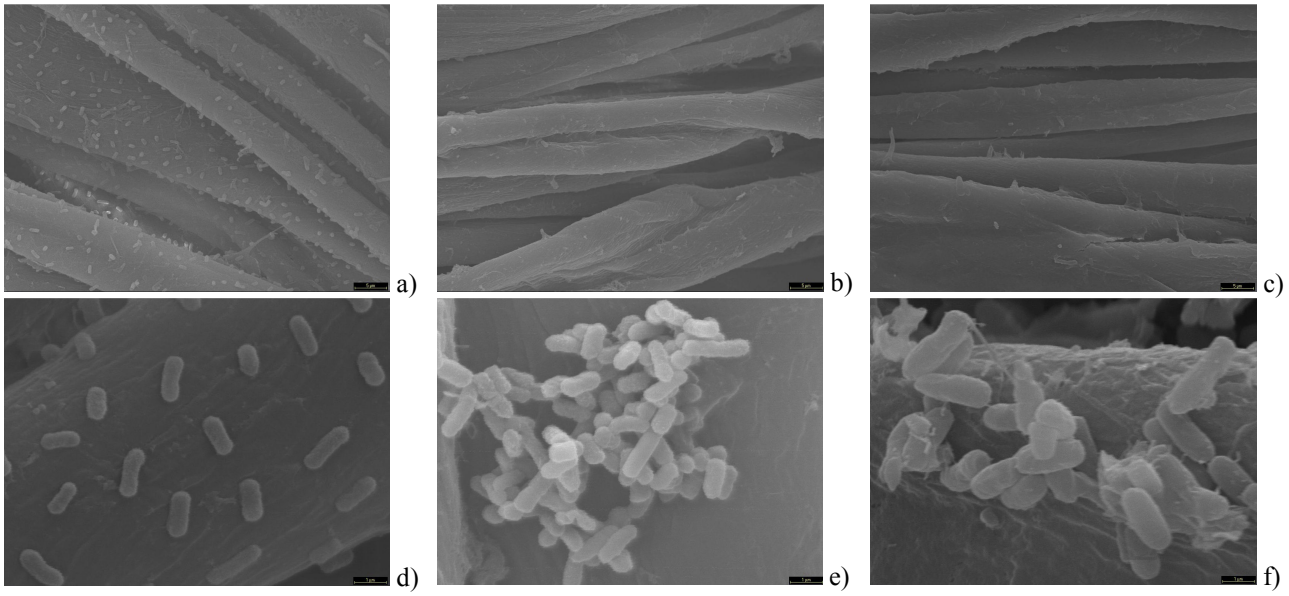

Fig. 5 SEM images of samples tested against *Klebsiella pneumonia*, a) and d) control sample; b) and e) $(CH/ALG)_3$; c) and f) $(CH/ALG)_5$. Images a), b) and c) show the zone under functionalized cotton, and images d), e) and f) show the upper zone of the functionalized cotton samples. Magnifications are x2000 for a), b) and c) and x10000 for d), e) and f).

According to the review of literature, structural changes in the cells of antibiotic resistant bacterial strains are more pronounced under the effect of antibiotics in bacteriostatic doses than under the effect of CH. This does not diminish the significance of CH as an antibacterial agent, as bacteria do not develop CH resistance. This is particularly important in the treatment of infections caused by bacterial strains with multiple antibiotic resistance, in wounds, and in burn infections, when the adhesive effect of CH is fully pronounced.

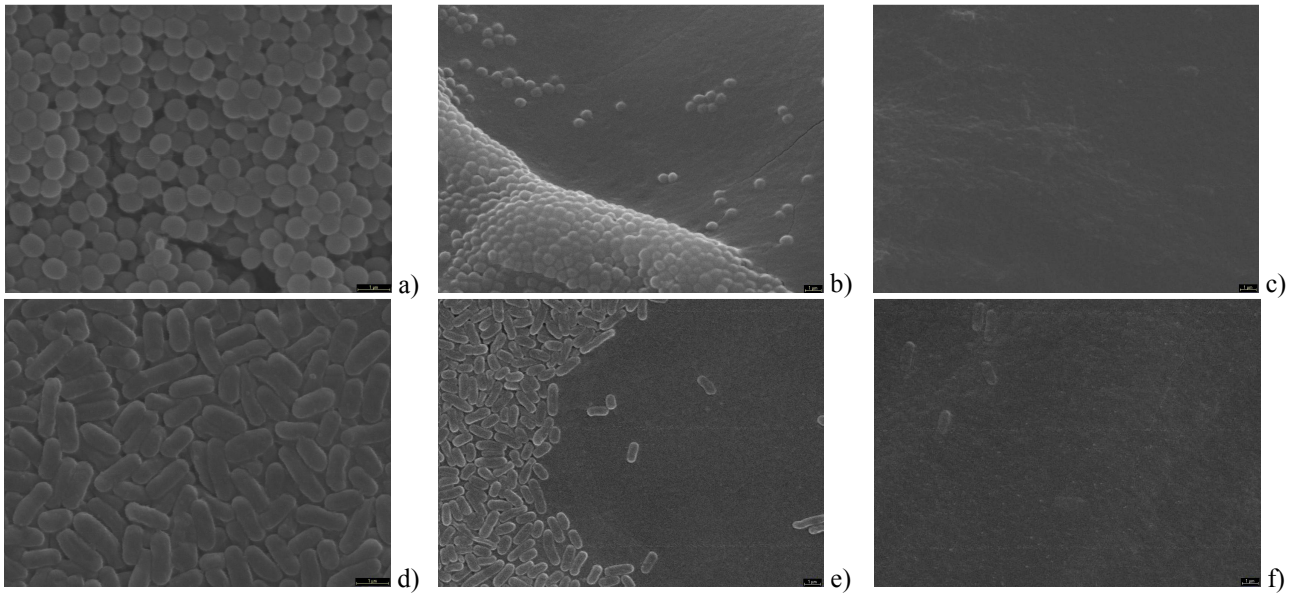

Fig. 6 SEM images of culture medium Mueller-Hinton. a) density and uniformity of growth against *Staphylococcus aureus* by culture medium (x10000), b) zone of inhibition (x5000) and c) zone under the functionalized cotton (x5000). d) density and uniformity of growth against *Klebsiella pneumoniae* by culture medium (x10000), b) zone of inhibition (x5000) and c) zone under the functionalized cotton (x5000).

4. Conclusions

The antibacterial qualitative tests fulfilled a need for a relatively quick and easy way to determine antibacterial activity of textiles. In the standard method JIS L 1902:2002 – Halo method, the samples clearly show antibacterial activity when a halo is formed. However, even if no halo appears, the samples may have antibacterial activity by direct contact when there is also no bacteria growth under the samples.

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The results reported in this investigation showed the value of the SEM in studying the effect of CH/ALG on *Staphylococcus aureus* and *Klebsiella pneumoniae.* Information about the mode of action is not obtained, as in the case of the qualitative and quantitative standards to assess antibacterial activity of textiles, but direct observation of morphological changes following antibacterial action can be observed. These changes cannot be evaluated by those standards and are not adequately seen in the light microscope, while the high magnifications and three-dimensional effect obtained with the SEM make it ideal for this purpose.

SEM analysis is able to show the effectiveness of the several functionalized samples in preventing bacterial adhesion besides bacteria growth.

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Paper IV

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Layer-by-Layer deposition of antimicrobial polymers on cellulosic fibers: a new strategy to develop bioactive textiles

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Layer-by-layer deposition of antimicrobial polymers on cellulosic fibers: a new strategy to develop bioactive textiles

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In recent years, there has been an increase of infectious diseases caused by different microorganisms and the development of antibiotic resistance. In this way, the search for new and efficient antibacterial materials is imperative. The main polysaccharides currently used in the biomedical and pharmaceutical domains are chitin and its derivative chitosan (CH) and alginates (ALG). In this study, a simple technique of Layer by Layer (LbL) of applying polycation CH and polyanion ALG was used to prepare CH/ALG multilayers on cotton samples via the electrostatic assembly with success. The CH/ALG cotton samples (functionalized) were investigated for their antibacterial properties towards *Staphylococcus aureus* and *Klebsiella pneumonia* using the international standard method JIS L 1902:2002. The antibacterial activity of the functionalized samples was tested in terms of bacteriostatic and bactericidal activity, and results showed that the samples exhibited a bacteriostatic effect on the two bacteria tested, as expected. In addition, samples with five layers (CH/ALG/CH/ALG/CH) were more effective in inhibiting bacterial growth. This new coating for cellulosic fibers is a new strategy and may open new avenues for the development of antimicrobial polymers with potential application in health-care field. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: chitosan; alginate; cotton; LbL; JIS L 1902

INTRODUCTION

The number of different antimicrobial agents suitable for textile application on the market has increased drastically in recent years. Several different types of antimicrobial agents, such as oxidizing agents, coagulants, diphenyl ether (bis-phenyl) derivatives, heavy metals and metallic compounds and quaternary ammonium compounds are used in the textile industry to confer antimicrobial properties.[1] The selection of the antimicrobial agent depends on the mechanism of antimicrobial activity (bacteria and fungi), toxicity, application method and cost.[1] Nowadays, the textile manufactures prefer eco-friendly chemicals to impart antimicrobial finishing on textiles.

Polysaccharide biopolymers including ALG and CH have been the focus of an expanding number of studies reporting their potential use in biomedical research applications such as cell encapsulation, drug delivery and tissue engineering. Therefore, multilayered films containing both biopolymers could be useful in the coating of substrates for different biomedical applications, in particular in the development of new wound dressings.[2,3]

CH, a natural biopolymer, has a combination of many unique properties such as biodegradability, non-toxicity, cationic nature, antitumor, immunostimulatory[4] and antimicrobial activity.[5,6] CH is also known in the wound management field for its hemostatic properties.[7]

ALG is a natural biopolymer, and it is non-toxic, biocompatible, biodegradable, less expensive and freely available.[8,9] ALG is known to exhibit minimum cytotoxic effects and reduced hemolysis when in contact with blood.[8]

Electrostatic LbL assembly is a versatile method of fabricating multilayer films and coating from materials in solution, notably, oppositely charged polyelectrolytes in solution. Polyelectrolytes are polymers with ionizable groups along the chain, classified into anionic and cationic according to their functional group. An advantage of LbL is that the film is fabricated directly on the surface of interest. The method is based on the successive deposition of oppositely charged polymers onto solid surfaces.[10,11] A deposition cycle creates a bilayer, and these cycles can be repeated as often as needed according to previous work.[12] The number of deposition cycles and the types of polyelectrolytes used in the construction allow for the control of thickness and roughness of the multilayered film.[13] Figure 1 can illustrate this method.

Numerous studies involving different polymer substrates and several synthetic polyelectrolytes have been published. But,

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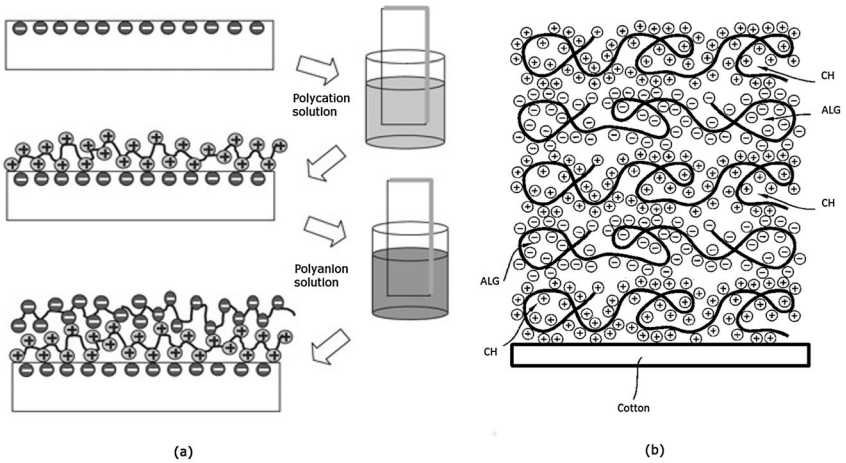

Figure 1. (a) The sequence of layer-by-layer electrostatic in negatively charged substrate, dipping into polycationic solution (CH), polycation layer deposited, dipping into polyanion solution (ALG), and the polyanion layer deposited. (b) Design of structure of the sample functionalized, adapted from Nabok, 2005.[14]

there are very few studies concerning the deposition of alternate polyelectrolyte on natural textile supports. Hyde et al. (2005) reported a pioneering application of self-assembly technique on cotton substrates by LbL deposition of poly (styrene sulfonate) (PSS) and poly (allylamine hydrochloride) on cotton fibers.[15] Dubas et al. (2006) have immobilized antimicrobial silver nanoparticles on nylon and silk fibers by LbL deposition method.[16]

More recently, lamphaojeen et al. (2012), in this study, immobilized ZnO nanoparticles on cotton fabrics using PSS, through the LbL technique. As a result, they obtained a high UV protection factor and inhibition of the growth of *Staphylococcus aureus*.[17]

Based on literature review, the aim of this study was to design a new process for the bio-functionalization of cellulosic fibers with an antimicrobial effect with potential application as a new substrate for wound dressings. For this purpose, natural antimicrobial ingredients (CH and ALG) were deposited on cotton samples by LbL and investigated as a new potential antibacterial coating for cellulose-based textiles with a broad range of application in hospital, medical and hygienic products where antimicrobial activity is of the utmost importance

EXPERIMENTAL

LbL coating of cotton

To apply the LbL technique, anionic cotton was prepared by using 2,2,6,6,-tetramethylpiperidinyl-1-oxy free radical (TEMPO) and NaBr, in order to give higher surface charge for polyelectrolyte deposition, as described elsewhere.[18] Cotton samples were charged negatively by immersing cotton samples in TEMPO solution under moderate stirring, followed by a rinse with deionized water, according with literature.[19,20] Chitosan of low molecular weight and 80% degree of deacetylation (CH, 1 mg/ml), a natural antimicrobial polyelectrolyte, and Alginic acid sodium salt (ALG, 1 mg/ml) solutions were prepared by dissolving CH and ALG in 0.1 M CH_3COOH and 0.5 M NaCl solutions, respectively. The pH values were adjusted to 5 using 0,1 M HCl and 1 N NaOH solutions.

The pH was selected to 5, to be approximately intermediate between the pK_a of CH (6.3) and ALG, pK_a of 3.38 and 3.65, as previously reported by other authors.[12,21] ALG and CH are two oppositely charged natural polyelectrolyte materials and very sensitive toward changes in external factor such as pH. At pH 5, the carboxylate group of ALG mainly exists in the form of COO^-, and the amino group of CH mainly exists in the form of NH_3^+. In this case, the presence of both COO^- and NH_3^+ along polymer backbone could enhance the electrostatic interaction of the network structure[22] (see Fig. 2).

Then, CH and ALG polyelectrolyte multilayer films were deposited over cotton by the LbL assembly, where CH was used as polycation and ALG as polyanion (see Fig. 2). For each layer deposition, the cotton substrate was immersed into the corresponding solution at room temperature for 5 min, followed by rinsing with deionized water to remove the excess of polyelectrolyte. Since the cotton samples were charged negatively, the CH was deposited as the first layer. Cotton samples treated during 30 min in TEMPO (designated by CT) and samples then functionalized by LbL were prepared with five layers (CH/ALG/CH/ALG/CH), six layers (CH/ALG/CH/ALG/CH/ALG), nine layers (CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH) and ten layers (CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH/ALG), designated, respectively, by

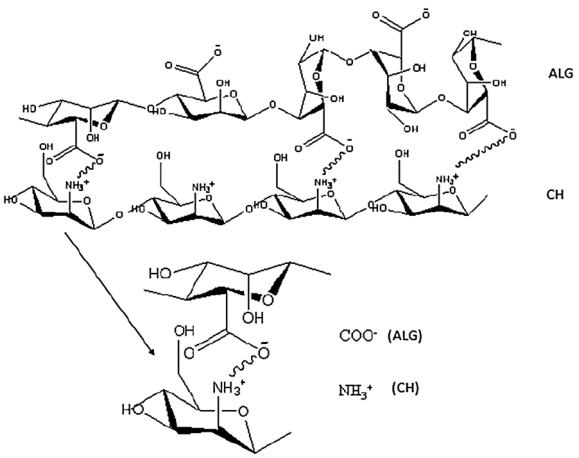

Figure 2. Schematic representation of the electrostatic interactions between the carboxylic groups of the ALG and the amine groups of the CH, adapted from Mi et al., 2002.[23]

polymers in advanced technologies

CT5, CT6, CT9 and CT10. After the last deposition, samples were dried in a desiccator at room temperature overnight. All chemicals were obtained from Sigma–Aldrich, are of analytical grade and were used as received.

Assessment of antibacterial activity – JIS L 1902:2002 - halo method

In order to evaluate the activity of antibacterial textiles, there are several standard methods available. The method used in this study and described in JIS L 1902 (Japanese Standard) appears to be the most commonly employed.

The culture medium Brain Heart Infusion used for the cultivation of the bacterial strains deployed in this work were prepared according with the instructions of the manufacturer. Culture media was dissolved directly after being weighed in deionized distilled water and then sterilized by autoclaving for 15 min at 121 °C. Agar was used to solidify the media before autoclaving. The strains of *S. aureus* and *Klebsiella pneumonia*e used were obtained from the American Type Culture Collection, ATCC 25923 and ATCC 13883, respectively. These strains were selected because they are indicated in several standards to evaluate antibacterial activity of textiles.[24]

The cell density of bacterial suspensions was determined by measuring the optical density (OD) of appropriately diluted samples using a spectrophotometer at a wavelength of 640 nm [OD_{640}]. 100 μl aliquots of the appropriate dilutions were plated onto the surface of agar plates with a Mueller–Hinton medium, and after approximately 10 min, the functionalized cotton samples were placed on the top of agar plates and incubated for 24 h at 37 °C.[25]

Assessment of antibacterial activity – JIS L 1902:2002 - absorption method

The JIS L 1902:2002 - absorption method is designed to quantitatively test the ability of textiles that have been treated with antibacterial agents to prevent bacterial growth and to kill bacteria, over an 18 h period of contact. This method is based on the quantitative determination of the potential effect and activity of functionalized samples, by the direct contact with a suspension of bacterial cells.

Textile samples with approximately 0.4 g, six control samples (without CH and ALG) and six samples functionalized with CH and ALG, previously sterilized in an autoclave at 121 °C for 15 min were tested for each bacterial strain. In order to calculate growth reduction rate, three samples were used to measure the number of live bacteria after inoculation (T_{0h}) and the other three to measure the number of live bacteria after incubation (T_{18h}).

Bacterial cell suspensions were collected from an overnight liquid culture in Nutrient Broth. After that, the bacterial concentration is adjusted to $1–2\times10^8$ cel/ml (equivalent to 0.5 McFarland), with the necessary dilutions to adjust the final bacterial concentration to $1 \pm 0.3\times10^5$ cel/ml.

Each textile sample was placed in a 50 ml Falcon tube, soaked with 200 μl of the inoculum previously prepared, and T_{18h} tubes were incubated for 18 h at 37 °C. For the release of bacterial cells from the textile samples, before and after the 18 h incubation period, 20 ml of 0.85% NaCl with surfactant Tween 80 (0.2%) was added to the samples in 50 ml Falcon tubes and vortexed. The resulting suspensions were used for the determination of viable counts using serial dilutions prepared in sterile 0.85% sodium chloride solution and plated. The plates were incubated at 37 °C for 18 h, and the number of colonies was counted visually using a microscope. This procedure was performed in triplicate.[25] The growth reduction rate of the bacteria was calculated using the equation:

$$\frac{T_{0h} - T_{18h}}{T_{0h}} \times 100\% = reduction\ \ rate\ \ (\%)$$

where, T_{0h} is the CFU/ml (CFU = colony forming units) of bacterial colonies at the initial stage (0 h), and T_{18h} is the CFU/ml of bacterial colonies after 18 h incubation.[24]

In order to carry out the judgement of test effectiveness, the growth value was calculated according to the following equation:

$$F = M_b – M_a$$

When the growth value is more than 1.5, the test is judged to be effective, and when the growth value is 1.5 or less, the test is judged to be not effective. When the test is not effective, a retest is necessary.

When the quantitative test has been effective, the bacteriostatic activity value should be calculated in accordance with the following equation:

$$S = M_b - M_c$$

and the bactericidal activity according to:

$$L = M_a - M_c$$

Where, F is the growth value, and S and L are the bacteriostatic and bactericidal activity values, respectively. M_a is the average of common logarithm of number of living bacteria of three test pieces immediately after inoculation of inoculum on standard cloth. M_b is the average of common logarithm of number of living bacteria of three test pieces after 18 h incubation on standard cloth. M_c is the average of common logarithm of number of living bacteria of three test pieces after 18 h incubation on antibacterial treated sample.[25] Traditionally, bacteriostatic means prevention of multiplication of bacteria without destroying them, whereas bactericidal effect implies forthright killing of the organisms[26]

RESULTS AND DISCUSSION

Assessment of antibacterial activity – JIS L 1902:2002 - halo method

The antibacterial efficacy of the CH/ALG multilayers was evaluated by assessing the reduction in bacterial growth on the functionalized cotton substrates. Antibacterial activity analysis revealed that on the CH/ALG functionalized cotton, the densities of attached bacteria decreased when compared with that of the control (cotton), as can be observed in Fig. 4 by the formation of a small halo on the functionalized samples. It appears that the effectiveness in preventing bacterial adhesion is high regardless of whether CH/ALG is deposited on cotton samples. This is in accordance with the expected. The use of CH as an anti-biofilm coating for medical applications has been suggested, as coating surfaces with CH is highly effective at restricting or preventing the formation *S. aureus* and *K. pneumoniae* biofilms.[27]

Cationic polyelectrolytes, as well as other molecules with a net positive charge, are capable of killing microorganisms. The mechanism of antibacterial action of cationic polyelectrolytes is not completely understood, but it has been suggested that these polymers can interact electrostatically with anionic groups at the bacterial cell walls causing an increase of membrane permeability and subsequent leakage of cellular proteins which ultimately leads to cell death.[28] In the context of this research, the observed antibacterial action of the polyelectrolytes is an interesting finding because in principle the antimicrobial activity of the functionalized samples can be adjusted using favorable layers number of polyelectrolytes on top of cotton.

On the other hand, according with several authors, the pH value affects the antibacterial effect, where an increase in pH leads to a decrease of the antibacterial action. In the case of CH, the high pH few amino groups in CH molecules will be free, but at pH below 6, the amino groups become ionized.[4] In this study, we use pH 5; this implies a higher number of side amino groups available.

Likewise, the results of the JIS L 1902:2002 - qualitative method were used to assess the antibacterial activity of textile specimens.

In Fig. 3, a positive control (sample known to have antimicrobial activity) presents a halo around the sample. Halo size provides some indication of the potency of the antimicrobial activity of textile samples and also that the antimicrobial agent is released from the textile. In contrast, the results of the functionalized cotton (Fig. 4) showed an ambiguous inhibitory effect against *S. aureus* and *K. pneumoniae*, although in terms of the surrounding clearing zone, the different samples show the same inhibitory effect against all tested microorganisms.

Figure 4 (a) sample CT5, (b) samples CT6, CT9 and CT10, showed a small but clear inhibitory zone for *S. aureus*. Figure 4 (c) sample CT5, (d) samples CT6, CT9 and CT10, showed a smaller halo for the *K. pneumonia*. However, Fig. 4 (a) samples CO (cotton) and CT, and Fig. 4 (c) samples CO and CT, showed a very thin inhibition line in the edges of the sample for both tested microorganisms. As a result, we conclude that for the *S. aureus* and *K. pneumonia*, the control samples (CO and CT) showed little microbial growth inhibition. In contrast, the inhibition was more significant in the presence of CH/ALG in samples CT5, CT6, CT9 and CT10.

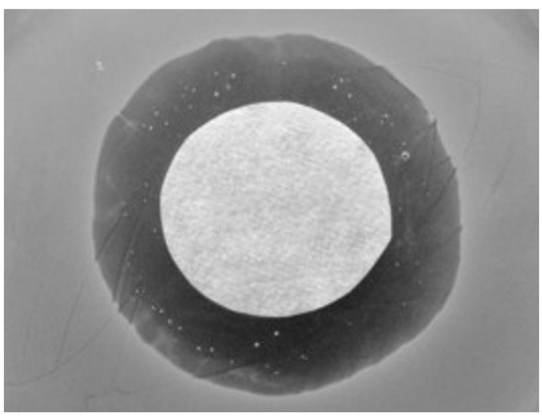

Figure 3. Image of a halo formation of a positive control (analysed using the standard method JIS L 1902:2002 - halo method, against *Staphylococcus aureus*).

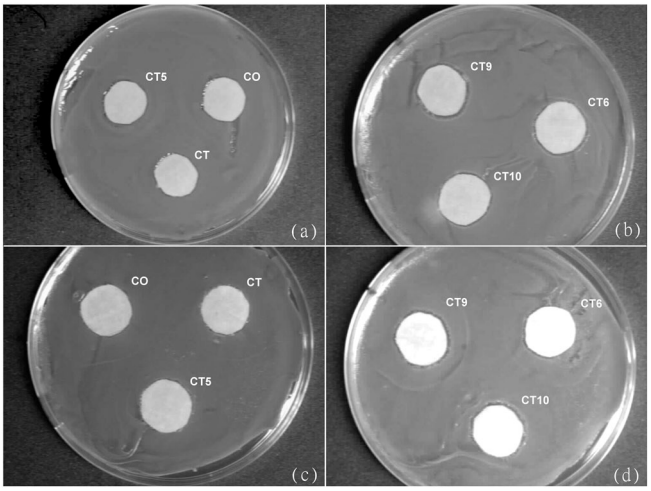

Figure 4. Images of samples tested according with the standard method JIS L 1902:2002 - halo method. a) and b), inhibition zone against *Staphylococcus aureus* for CO, CT, CT5, CT6, CT9 and CT10 samples. c) and d), inhibition zone against *Klebsiella pneumonia* for CO, CT, CT5, CT6, CT9 and CT10 samples.

From the little defined zone of inhibition obtained, it is apparent that the functionalized samples are bacteriostatic and not bactericidal. In addition, the results also showed that the antibacterial effect of CH/ALG occurred without migration of the active agents. As CH/ALG are in a solid form, only microorganisms in direct contact with the active sites of CH/ALG are inhibited because CH/ALG layers are incapable of diffusing through the adjacent agar medium, as discussed elsewhere.[29] As a result, and as described previously, the possible mechanisms for antibacterial activity are: (1) the CH/ALG on the surface of the cell can form a polymer membrane, which prevents nutrients from entering the cell. (2) CH/ALG entered the cell through pervasion, since CH could absorb the electronegative substance in the cell and flocculate them; it disturbs the physiological activities of the bacteria.[30]

Assessment of antibacterial activity – JIS L 1902:2002 - absorption method

The antibacterial effect of functionalized samples was tested according to the Japanese Industrial Standard JIS L Standard 1902:2002. This method is based on the quantitative determination of the potential effect and activity of functionalized samples, by the direct contact with a suspension of bacterial cells. The results of the bioactivity investigations are presented in Table 1.

All samples (CT, CT5, CT6, CT9 and CT10) showed a bacteriostatic activity and no bactericidal activity found against *S. aureus* and *K. pneumonia*, being in accordance with the results for antibacterial activity by the halo method. CT5 is the sample that has the highest value of bacteriostatic activity (1.9 for *S. aureus* and 1.5 for *K. pneumonia*), followed by the sample CT9 (1.8 for *S. aureus* and 1.3 for *K. pneumonia*). These samples have in common the last layer is composed for CH, suggesting more free amino groups from CT5 and CT9 and lower free amino groups from the CT6 and CT10, which are bonded with carboxylic groups of the ALG thus, reducing the activity. The experimental results provide tangible evidence in support of the hypothesis that the amino group on CH is a source of bacteriostatic activity.

polymers in advanced technologies

Table 1. Antibacterial activity (bacteriostatic and bactericidal activity)

Sample	*S. aureus*		*K. pneumoniae*	
	$M_b - M_c$	$M_a - M_c$	$M_b - M_c$	$M_a - M_c$
CO	0	−1.5	0	−1.8
CT	0.3	−1.1	0.2	−1.0
CT5	1.9	−0.5	1.5	−0.4
CT6	1.3	−1.0	1.1	−0.9
CT9	1.8	−0.6	1.3	−0.6
CT10	1.4	−0.7	1.1	−0.8

A-number of inoculated bacteria. B-number of bacteria on the standard sample contacted for 18 h. C-number of bacteria on the functionalized sample after incubation for 18 h. $M_a = \log$ A, $M_b = \log$ B, $M_c = \log$ C. Bacteriostatic activity level, $M_b - M_c$; bactericidal activity level, $M_a - M_c$.

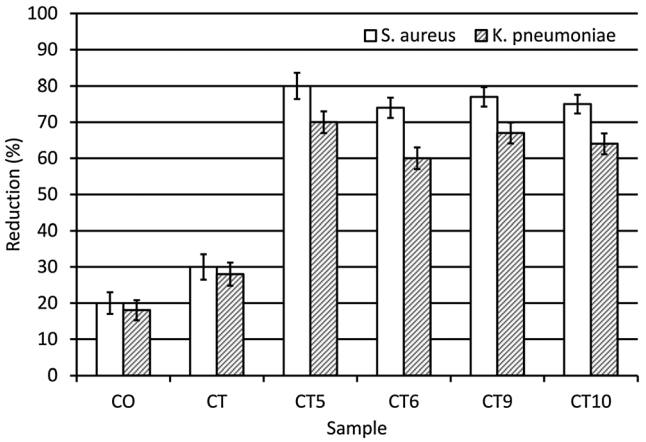

Figure 5. Reduction rate (%) of *Staphylococcus aureus* and *Klebsiella pneumoniae* in functionalized samples.

In literature, normally the CH exhibits a stronger bioactivity effect upon Gram-positive than Gram-negative bacteria. This observation may be explained by the higher deacetylation degree of CH used in this work (80%), which implies a higher number of side amine groups available for reaction.[4,27] On the other hand, the bioactivity effect also depends on the molecular weight; the inhibitory effect decrease slightly as the molecular weight increase. In this study, it was used CH of low molecular weight (420 kDa). CH is a cation that attracts the negative charges of the cell walls of bacteria, as claimed by several authors, being the cause for CH antibacterial action.[31–34] The surface of *S. aureus* includes the negatively charged teichoic acid within a thick peptidoglycan layer that lacks an outer membrane.[35] This should render the bacterial cell more attractive to and easier to be damaged through electrostatic-mediated contact-inhibition mechanisms when exposed to the positively charged CH layer (CT5 and CT9) than the negatively charged ALG layer (CT6 and CT10).

The lower bacteriostatic activity of the samples against *K. pneumonia* than that against *S. aureus* may be explained as a result of the different bacterial membrane structures. In contrast to that of *S. aureus*, *K. pneumonia* has a double protective layer, the outer lipopolysaccharide layer embedded with a number of small channels of porins and the inner peptidoglycan layer. The fact that it does not contain the negatively charged (teichoic acid) entities that can interact with positively charged molecules would potentially make it less sensitive to electrostatic binding with positively charged molecules like CH.[35]

The work on the effect of CH in strains of *K. pneumonia* and *S. aureus* found that CH promotes aggregation of bacterial cells and disorganization of bacterial cell wall and cytoplasmic membrane, which leads to the release of bacterial contents into the environment. These structural changes result in bacterial death.[36] These results suggest that the apparent difference action upon Gram-positive and Gram-negative microorganisms probably results from the intrinsic difference in their cell wall structure.

All functionalized samples (CT5, CT6, CT9 and CT10) showed antibacterial activity against *S. aureus* and *K. pneumonia* in solution. Figure. 5 shows the growth inhibition (cell reduction) of the *S. aureus* and *K. pneumonia* by the antibacterial activity of the functionalized samples with CH/ALG.

Analyzing the results, (Fig. 5) there is a reduction of 65–80% in bacterial growth on CT5 and CT9 samples, respectively, and a reduction of 60–75% in bacterial growth is achieved by CT6 and CT10 samples. An interesting observation is that all functionalized samples exhibit a high reduction of bacterial growth in solution although without a clear zone of inhibition assessed by the halo method. This may be because the concentration of CH and ALG on cotton samples is not sufficient enough for bactericidal activity as described elsewhere.[37] Singh and co-workers found that antimicrobial efficacy of a compound will vary when it is present in solution and when it is held intimately by a textile substrate.[37]

All of these results suggest that the functionalized samples with five numbers of layers (CH/ALG/CH/ALG/CH) are more active against *S. aureus* and *K. pneumonia* microorganisms.

Structurally, the cationic nature of CH is expected to interact strongly by ionic bonds with the anionic ALG, and the combination of ALG with CH has become quite commonplace for the development of potential wound healing materials as they showed no toxic effects to mammalian cells.[38] A recent study has shown that ALG/CH-based wound dressing films accelerated burn healing by modulating the epithelization, blood vessels formation and collagenization process.[39]

CONCLUSION

The results obtained confirm the possibility of using the LbL method for modification of the surfaces of cotton fabric in order to impart antibacterial properties to them. The most important potential application of these materials could be their use as external wound dressings. Their advantage toward the existing materials is that they are fully biocompatible and inexpensive.

Many applications are proposed based on this CH/ALG, which is the most investigated polyelectrolyte complex, especially for biomedical applications. Note that this technique (LbL) in textile is entirely new and is a simple and effective method with strong possibility of industrial application.

This new coating for cellulosic fibers is a new strategy and may open new avenues for the development of antimicrobial polymers with potential application in health-care field.

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Paper V

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New Biomaterial Based on Cotton with Incorporated Biomolecules

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New Biomaterial Based on Cotton with Incorporated Biomolecules

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ABSTRACT: The aim of this study was to investigate a method of embedding L-cysteine (L-cys), an antimicrobial agent, between layers of chitosan (CH) and sodium alginate (ALG) onto cotton samples obtained via a layer-by-layer electrostatic deposition technique via several embedding methods. The results show that the best way to incorporate L-cys into the layers was the one that used the property of gelling ALG. To monitor the L-cys embedding into the CH/ALG multilayer film, different methods were used: energy-dispersive X-ray spectrometry analysis to assess the presence of sulfur on the sample, Ellman's reagent method to analyze L-cys release from the sample, and attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (FTIR) to compare the ATR–FTIR spectra of the pure L-cys and L-cys embedded in the CH/ALG multilayer film to study the interaction between the L-cys and the CH/ALG multilayer films. Functionalized CH/ALG cotton samples were also investigated for their antibacterial properties toward *Staphylococcus aureus* and *Klebsiella pneumonia* with the Japanese Industrial Standard method JIS L 1902:2002, and the results show an enhancement of the antibacterial effect due to the presence of L-cys. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40519.

KEYWORDS: biomaterials; biomedical applications; functionalization of polymers; self-assembly; textiles

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INTRODUCTION

The continuous use of antibiotics has resulted in multiresistant bacterial strains all over the world. Consequently, there is an urgent need to search for alternatives for antibiotics. For this purpose, a new strategy is proposed here: to use natural bioactive agents as potential antibacterial agents for textiles for medical applications, such as wound dressings. In this way, L-cysteine (L-cys) was used as a model to find the best strategy to introduce active agents between the layers of chitosan (CH) and sodium alginate (ALG) because L-cys is an important biomolecule, which has been extensively used in pharmaceuticals, chemical synthesis, and so on.[1] L-Cys can be used for the conjugation of biomolecules, and this allows it to be used for biotechnological applications.[2]

To deposit several layers of CH and ALG on cotton samples, a layer-by-layer (LbL) technique was used. The LbL technique offers new opportunities for the preparation of functionalized biomaterial coatings and the incorporation of bioactive molecules between the layers.[3–5] This technique allows the preparation of nanoarchitectures exhibiting specific properties. Peptides, proteins, and active agents adsorbed or embedded in multilayer films have been shown to retain their biological activities,[6] whereas a covalent attachment to the biomaterial can reduce or even destroy their biochemical activity.[5] So, with the LbL technique, active agents can be directly integrated in the architecture without any covalent bonding with a biomaterial.[6,7]

The use of active agents coupled with polyelectrolytes constitutes a major advantage in comparison with direct chemical immobilization methods. On the other hand, the direct immobilization of active agents on a surface needs to be optimized for every individual agent/surface pair; thus, the resulting surfaces structures are much more difficult to characterize, and side reactions are detected.

An advantage of LbL is that the film can be assembled directly on the desired surface. The basic character of LbL, however, depends neither on the surface area of the support nor its shape but on the charge properties of the surface and assembling species. The layering process in LbL is repetitive and can be automated; this makes it suitable for commercial prospects in applications of technology.[8]

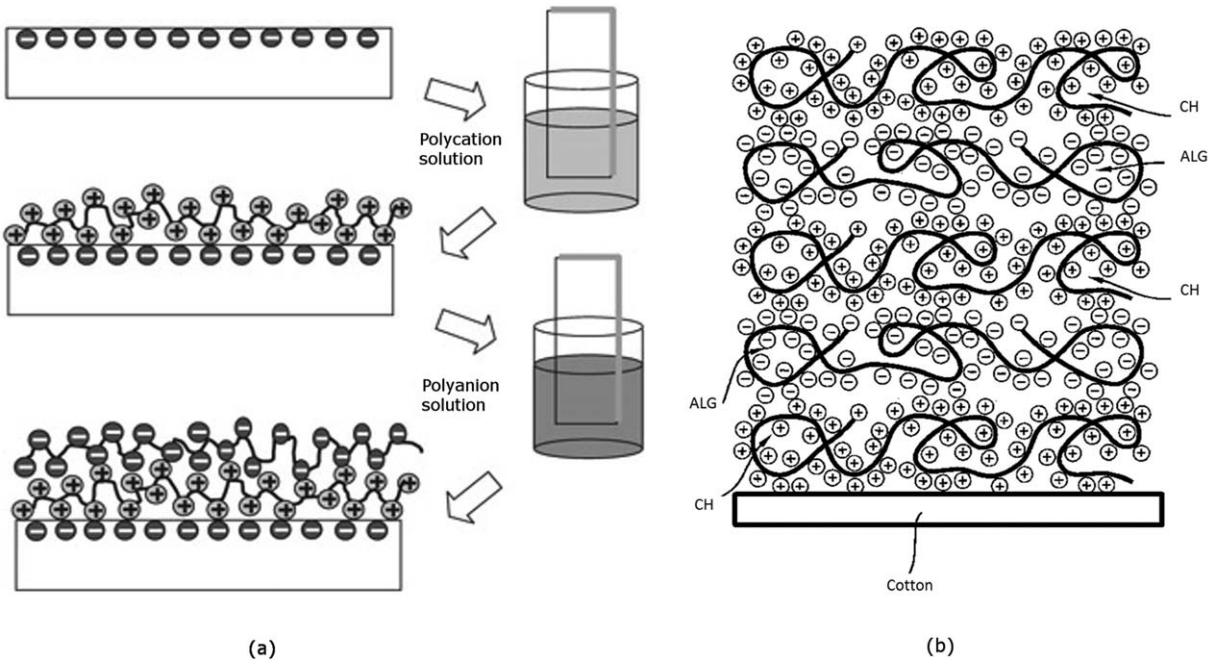

Figure 1. (a) Sequence of LbL electrostatic method in the negatively charged substrate with dipping into the polycationic solution (CH), deposited polycation layer, dipping into the polyanion solution (ALG), and deposited polyanion layer. (b) Structure of the functionalized sample.

The LbL method is based on the successive deposition of oppositely charged polymers onto solid surfaces,[9,10] as illustrated in Figure 1 (adapted from Nabok[11]).

The purpose of this study was to investigate the best method for the functionalization of cotton with polyelectrolyte multilayer films (CH and ALG) with incorporated L-cys. Our strategy was based on the use of multilayer films as reservoirs of active molecules. Nothing has been found in the literature concerning the aim of this study; therefore, all of the results were compared with works based on drug-delivery systems. These kinds of samples are promising examples for use in wound dressings. Natural cellulose (cotton) fiber is the basis of many wound dressings,[12] and wound dressings containing antibiotics have been developed for the inhibition of wound infection.[13–15] A variety of wound dressings that incorporate active agents is available on the market; they include iodine (Iodosorb by Smith & Nephew), chlorohexidime (Biopatch by J&J), and silver ions (Acticoat by Smith & Nephew, Actisorb by J&J, and Aquacel by ConvaTec).[16]

CH and ALG were selected to embed L-cys because these are natural biopolymers that are finding applications in food, cosmetics, biomedicals, and pharmaceuticals because they are biocompatible, biodegradable, and nontoxic. CH is widely used in wound dressings and has been shown to have mucoadhesive properties, a cationic nature, and antibacterial and hemostatic properties.[17,18] ALG is known to be nontoxic; it has hemostatic action and biocompatibility with a variety of cells. Because of these properties, ALG has been studied for application in biomaterials and wound dressings.[19] These natural polymers are now playing a significant role in the research field for skin, bone, vascular, nerve, and liver regeneration because of their demonstrated biocompatibility, relative abundance, and ease of processing.

ALG has the ability to form gels by reactions with divalent cations, such as Ca^{2+}. When in contact with calcium ions, ALG forms a reticulated structure that can be used to entrap drugs. ALG–RCOO⁻ groups can bind with Ca^{2+} to form an undissolved gel. The gelling and crosslinking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations and the stacking of these guluronic groups to form the characteristic egg-box structure, as shown in Figure 2 (adapted from Shilpa et al.[20]).

Biodegradable dressings made of natural polymers, such as CH[21,22] and ALG,[23] are already available on the market. Specifically, biological materials such as CH and ALG have been reported to perform better than conventional and synthetic dressings in accelerating granulation tissue formation and epithelialization.[22–24] In this context, a new biomaterial based on cotton with incorporated active agents would be advantageous for the progressive delivery of associated active agents.

EXPERIMENTAL

LbL Coating of Cotton

Cotton fabric obtained from James H. Heal & Co., Ltd., was used as a substrate. (2,2,6,6-Tetramethylpiperidin-1-yl)oxyl (TEMPO)-mediated oxidation, sodium bromide (NaBr), sodium hypochlorite (NaOCl) 5%, CH (low molecular weight, 420 kDa), acetic acid (CH_3COOH), ALG, sodium chloride (NaCl), sodium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Sigma-Aldrich. All of the chemicals were of analytical grade and were used as received. Polyelectrolyte CH (1 mg/mL) and ALG (1 mg/mL) solutions were prepared by the dissolution of CH and ALG in 0.1*M* CH_3COOH and 0.5*M* NaCl solutions, respectively. To apply the LbL technique, samples of the substrate (cotton) were charged by immersion in a TEMPO + NaBr + 5% NaClO (pH 10.5) solution under

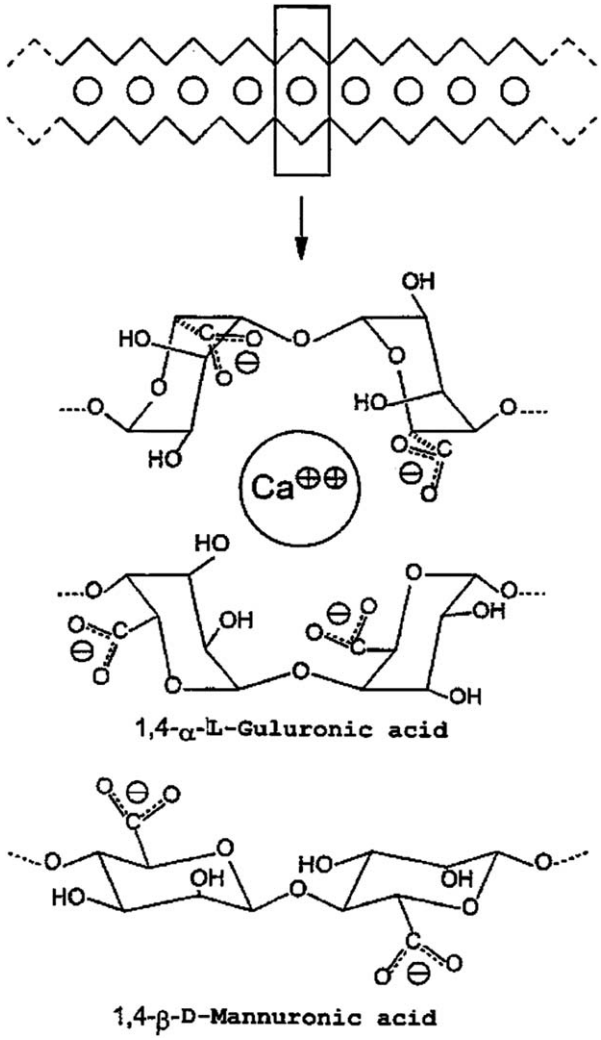

Figure 2. Schematic representation of an egg-box model showing the mechanism of the reaction between calcium ions and ALG that leads to gelation.

moderate stirring for 30 min; this was followed by a rinse with deionized water, as described elsewhere.[25–28]

The samples were manually prepared by the immersion of the cotton substrate alternately in polycation and polyanion solutions for 5 min. Between each polyelectrolyte exposure, the samples were rinsed with deionized water. For the CH and ALG polyelectrolyte layers, a pH of 5.0 was selected as an approximately intermediate value between the pK_a of CH (6.3) and the pK_a of ALG (3.38 and 3.65 for different residues).[29]

Embedding of L-Cys between Layers

The aim of this study was to investigate a method of embedding L-cys between layers of CH and ALG deposited on cotton obtained via an LbL technique.

L-Cys was incorporated into layers of CH and ALG by different methods:

Method 1. The use of L-cys as a polyelectrolyte.
Method 2. The use of L-cys by dissolution in the polyelectrolyte.
Method 3. The introduction of L-cys by direct microspray.
Method 4. Calcium–ALG gel entrapment of L-cys.
Method 4.1. Gelling of ALG followed by immersion in the L-cys solution.
Method 4.2. Immersion in the L-cys solution followed by the gelling of ALG.
Method 4.3. Dissolution of L-cys in the gelling solution.
Method 4.4. Method 4.2 with the gelling of ALG replaced by washing with deionized water containing calcium.
Method 4.5. Method 4.4 followed by coating with CH.
Method 5. Immersion in the L-cys solution without the gelling of ALG.

Method 1: The Use of L-Cys as a Polyelectrolyte. The pK_1 (—COOH), pK_2 (thiol or sulfhydryl), and pK_3 (NH_3^+) values of L-cys were 1.92, 8.37, and 10.70, respectively. Within a medium with a zwitterionic pH value of 5.02, there was no net charge on the molecule.[3] At pH values below and above 5.02, the molecule showed predominant cationic or anionic properties, respectively. With various solution pH values, the net charge could be changed from net positive at solution pH values more acidic than the isoelectric point to net negative at solution pH values more basic than the isoelectric point. At high pH values, the sulfhydryl group was also ionized and acquired a negative charge.

L-Cys was positively charged at pH 4, and this allowed it to be adsorbed onto a negative layer like a polycation. At pH 8 L-cys was negatively charged and could then be adsorbed onto a positive layer like a polyanion. Two embedding protocols were tested with the insertion of L-cys at the beginning and the end of the layer sequence considered.

Method 2: The Use of L-Cys by Dissolution in the Polyelectrolyte. In this method, a small amount of L-cys (1 mg/mL) was stirred together with the anionic polyelectrolyte (ALG) and together with the cationic polyelectrolyte (CH) to keep global negative and positive charges, respectively, after dissolution.

Method 3: Introduction of L-Cys by Direct Microspray. In this method, L-cys was introduced between the layers of the polyelectrolyte with a microspray during the process of the LbL method to ensure the use of a very small amount of L-cys to prevent any interference with the LbL process. Two solutions of L-cys (1% w/v) were used in the microspray, one with a pH of 1 and another with a pH of 12, to ensure positive and negative charges, respectively.

Method 4: Calcium–ALG Gel Entrapment of L-Cys. This method consists of the immobilization of L-cys in calcium–ALG gel by entrapment. ALG has a unique ability for gel formation in the presence of divalent cations, such as calcium ions. When sodium ALG is put into a solution of calcium ions, the calcium ions replace the sodium ions in the polymer. Hydrogels based on calcium-crosslinked ALG have been widely investigated for protein drug delivery. The crosslinking between sodium ALG and calcium ions leads to the gelling and entrapment of L-cys, which are dependent on the concentrations of both of these constituents. The ALG gel showed a positive degree of swelling at low calcium concentrations and a negative degree of swelling

at higher calcium concentrations.[30] Therefore, the concentration of ALG and the immersion time was optimized.

Calcium carbonate [$CaCO_3$ (5%)] was suspended in deionized water, and the $CaCO_3$ particles were dispersed ultrasonically for 10 min to form a homogeneous suspension.[31–33] The ALG hydrogel was prepared with a $CaCO_3$ solution, and glacial CH_3COOH was added to permit $CaCO_3$ solubilization. Acetic acid/$CaCO_3$ with a molar ratio of 2.5 was used.[33] The pH reduction (caused by proton diffusion into the aqueous phase) released Ca^{2+} ions from the insoluble calcium complex [eq. (1)] and caused gelling [eq. (2)]:[34]

$$2H^+ + CaCO_3 \rightarrow Ca^{2+} + H_2O + CO_2 \quad (1)$$

$$Ca^{2+} + 2Na^+ ALG^- \rightarrow Ca^{2+}(ALG^-)_2 + 2Na^+ \quad (2)$$

Taking into consideration the reaction between acetic acid and $CaCO_3$, each mole of $CaCO_3$ reacts with 2 mol of acetic acid. A $CH_3COOH/CaCO_3$ molar ratio slightly higher than the stoichiometric proportion (2.5/1) resulted in high encapsulation efficiencies.[33] Two different methods were applied.

Method 4.1: Gelling of ALG Followed by Immersion in the L-Cys Solution. After the gelling of ALG (last layer), the functionalized cotton (cotton with CH/ALG by the LbL technique) was immersed in a solution of L-cys (1% w/v) for 120 min.

Method 4.2: Immersion in the L-Cys Solution Followed by the Gelling of ALG. In this method, functionalized cotton was immersed in the L-cys (1% w/v) solution for 120 min, and then, the gelling of ALG was performed.

Method 4.3: Dissolution of L-Cys in the Gelling Solution. The functionalized cotton was immersed in a solution of ($CaCO_3$ + L-cys) to make the gelling and embedding of L-cys between the layers of CH/ALG occur simultaneously.

Method 4.4: Method 4.2 with the Gelling of ALG Replaced by Washing with Deionized Water Containing Calcium. The gelling process was replaced by washing with deionized water, where calcium was added. Longer exposure of ALG to the $CaCO_3$ solution induced a higher crosslinking degree, and the ALG-Ca^{2+} network limited the repulsion of the ALG chains and, hence, decreased the maximum L-cys uptake.

Crosslinking is an effective way to stabilize three-dimensional polymer networks for a variety of applications. Different types of crosslinking are used for different applications. Covalent crosslinking has been used in hydrogel formation with permanent three-dimensional structures, such as absorbents, lubricious coatings, and even some controlled release matrices, wound dressings, and cell culture substrates.[35] The covalent crosslinking reagents are usually toxic to cells. In this study, we used an ionic crosslinking system without any toxic chemicals to form homogeneous ALG gels. The calcium ions had only an instant crosslinking contact with ALG to form a gel and prevent the release of L-cys.

Method 4.5: Method 4.4 Followed by Coating with CH. According to studies from various authors concerning the encapsulation of active agents within ALG microspheres and crosslinked CH to reinforce the microspheres,[36,37] we prepared samples with method 4.4, where a final CH layer was added.

Method 5: Immersion in the L-Cys Solution without the Gelling of ALG. CH/ALG-functionalized cotton was immersed in a solution of L-cys (1% w/v) for 120 min.

Morphological and Structural Characterization of the Oxidized Cotton

Untreated cotton and TEMPO oxidized cotton samples were observed with scanning electron microscopy (SEM; Hitachi S-2700). To provide surface electrical conductivity, the samples were coated with a thin Au layer, which was applied by sputtering.

The same samples were also analyzed by X-ray diffraction. A Rigaku DMAX III/C instrument was used to make a 5–50° 2θ scan with the reflection method with an operation voltage of 30 kV and a current of 20 mA. The relative crystallinity was calculated according to eq. (3).

$$\text{Relative crystallinity} = (I_{\text{crystalline}} - I_{\text{amorphous}}) \times 100\% / I_{\text{crystalline}} \quad (3)$$

where $I_{\text{crystalline}}$ is the intensity at 22.5° and $I_{\text{amorphous}}$ is the intensity at 18.6°.[38,39]

Energy-Dispersive X-Ray Spectrometry (EDS) Analysis

To monitor the L-cys embedding on the CH/ALG multilayer film, EDS analysis was used to reveal the presence of sulfur (the chemical element only present in L-cys).

Ellman's Reagent

The amount of L-cys released from the functionalized cotton was measured by an Ellman's reagent assay. The degree of thiolation of functionalized cotton was determined by an Ellman's reagent [5,5-dithiobis(2-nitrobenzoic acid)] reaction, where 5,5-dithiobis(2-nitrobenzoic acid) reacted with thiol groups to release TNB^- ions. This further ionized to TNB^{-2}. This last ion showed a yellow color that could be detected by visible light at 405 nm.[30,40]

Attenuated Total Reflectance (ATR)–Fourier transform infrared (FTIR) Spectra

ATR–FTIR spectra of samples were acquired on a Thermo-Nicolet is10 FTIR spectrophotometer with OMNIC software with wavelengths of 500–4000 cm^{-1}. The spectra were collected at a resolution of 4 cm^{-1} with 64 scans per spectrum. A background spectrum was acquired and assigned for use on subsequent spectral acquisitions for each sample.

Assessment of Antibacterial Activity

The antibacterial effect of functionalized cotton was tested according to the Japanese Industrial Standard JIS L 1902:2002,[41] which is the most employed method. This method is designed to quantitatively test the ability of textiles that have been treated with antibacterial agents to prevent bacterial growth and to kill bacterial over an 18 h period of contact. This method is based on the quantitative determination of the potential effect and the activity of functionalized cotton by direct contact with a suspension of bacterial cells.

To judge the test effectiveness, the growth value (F) was calculated according to eq. (4):

$$F = M_b - M_a \quad (4)$$

When F is more than 1.5, the test is judged to be effective, and when F is 1.5 or lower, the test is judged to be ineffective. When the test is ineffective, a retest is necessary.

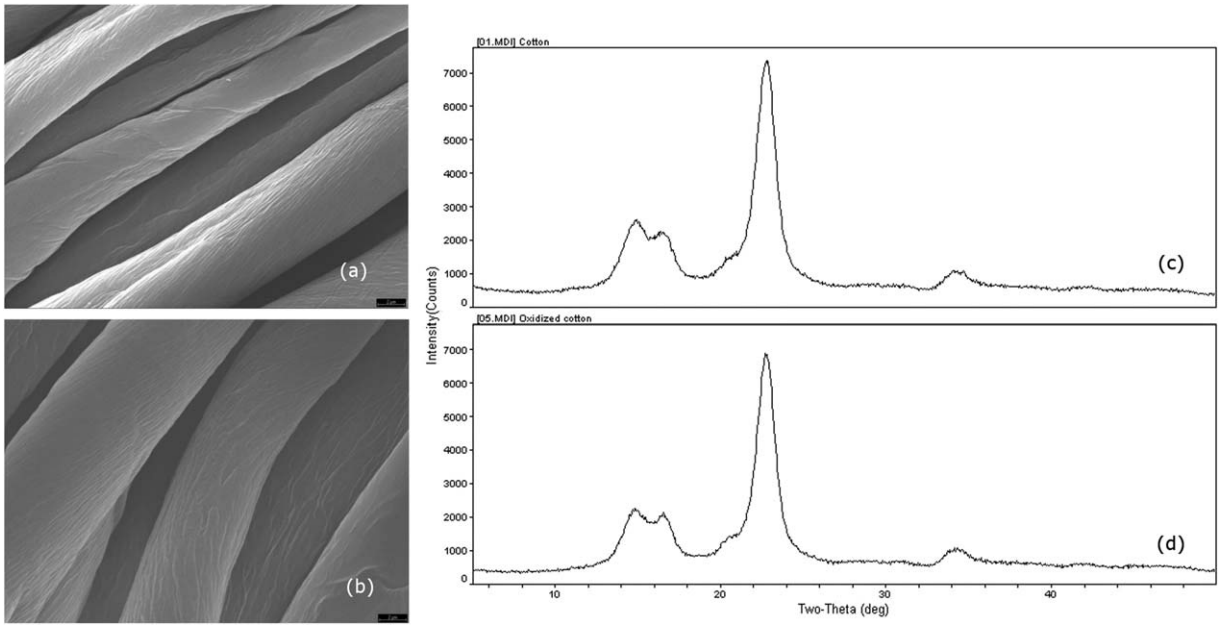

Figure 3. SEM images of the cotton sample (a) before and (b) after TEMPO-mediated oxidation and the corresponding X-ray diffraction patterns (c) before and (d) after TEMPO-mediated oxidation, Materials Data Inc (MDI).

When the quantitative test has been effective, the bacteriostatic activity (S) value can be calculated in accordance with eq. (5):

$$S = M_b - M_c \quad (5)$$

The bactericidal activity (L) was calculated according to eq. (6).

$$L = M_a - M_c \quad (6)$$

where M_a is the average common logarithm of the number of living bacteria of three test pieces immediately after the inoculation of the inoculum on standard cloth, M_b is the average common logarithm of the number of living bacteria of three test pieces after 18 h of incubation on standard cloth, and M_c is the average common logarithm of the number of living bacteria of three test pieces after 18 h of incubation on the antibacterial treated sample.[41] Traditionally, bacteriostatic means the prevention of multiplication of bacteria without their destruction, whereas a bactericidal effect implies the forthright killing of the organisms.[42]

The growth reduction rate of the bacteria was calculated with eq. (7):

$$\frac{T_{0h} - T_{18h}}{T_{0h}} \times 100\% = \text{Reduction rate}\,(\%) \quad (7)$$

where T_{0h} is the concentration (cfu/mL) of bacterial colonies at the initial stage (0 h) and T_{18h} is the concentration (cfu/mL) of bacterial colonies after 18 h incubation.[43]

RESULTS AND DISCUSSION

Morphological and Structural Characterization of the Oxidized Cotton

SEM images of the untreated cotton and oxidized cotton are shown in Figure 3. Figure 3(a) illustrates the original cotton sample, and the TEMPO-mediated oxidized cotton is shown in Figure 3(b).

A comparison between the SEM images of the original cotton and oxidized cotton showed that the used TEMPO-mediated oxidation conditions did not lead to any morphological change in the cotton samples.

Figure 3(c,d) illustrates the X-ray diffraction spectra of the initial cotton and oxidized cotton, respectively. Spectra are nearly identical, both in the sharpness and intensity of the diffraction. The comparison of the diffraction diagrams before and after surface oxidation indicated that the sample crystallinity was not affected by the oxidation treatment.

Comparing the original cotton samples [Figure 3(c)] with the oxidized cotton samples [Figure 3(d)], we observed that their polymorph type and crystalline degree did not show significant evolution upon the oxidation treatment. Such results agree with previously reported work.[44–46] The crystallinity degree of cotton was 88.06%, and the crystallinity degree of oxidized cotton was 88.50% and remained nearly constant during oxidation; this indicated that the fiber retained its crystal morphology. Generally, like other authors, we found that the process of oxidation with TEMPO did not reach the inside of the crystalline region.[38,39,44]

EDS Analysis

EDS analysis obtained from the different methods of embedding L-cys between layers of CH/ALG in functionalized cotton is shown in Table I.

The existence of L-cys on the functionalized cotton samples were determined by the amount of sulfur. Samples where sulfur was not detected had no L-cys embedded between the layers; in other words, the method used did not work. As shown in Table I, methods 1, 2, and 3 did not work because L-cys was not retained between the layers. That is, there were no

Table I. Results of the Embedding of L-Cys

Method[a]	Sample	Sequence	L-Cys pH	Sulfur (wt %)
1	1	Cotton-CH-ALG-(L-cys)-ALG-CH-ALG	4	—
1	2	Cotton-CH-ALG-CH-ALG-(L-cys)-ALG	4	—
1	3	Cotton-CH-(L-cys)-CH-ALG-CH-ALG	8	—
1	4	Cotton- CH-ALG-CH-(L-cys)-CH-ALG	8	—
2	5	Cotton-CH-(ALG+L-cys)-CH-ALG-CH-ALG	—	—
2	6	Cotton-CH-ALG-CH-(ALG+L-cys)-CH-ALG	—	—
2	7	Cotton-(CH+L-cys)-ALG-CH-ALG-CH-ALG	—	—
2	8	Cotton-CH-ALG-(CH+L-cys)-ALG-CH-ALG	—	—
3	9	Cotton-CH-ALG-(spray L-cys)-CH-ALG-CH-ALG	1	—
3	10	Cotton-CH-(spray L-cys)-ALG-CH-ALG-CH-ALG	12	—
3	11	Cotton-CH-ALG-CH-ALG-CH-(spray L-cys)-ALG	12	—
3	12	Cotton-CH-ALG-CH-ALG-(spray L-cys)-CH-ALG	1	—
4.1	13	Cotton-CH-ALG-gelation-L-cys (1%w/v, 120 min)	—	0.42
4.2	14	Cotton-CH-ALG-L-cys (1%w/v, 120 min)- gelation	—	0.70
4.3	15	Cotton-CH-ALG- gelation ($CaCO_3$+L-cys)	—	0.18
4.4	16	Cotton-CH-ALG- L-cys (1% w/v, 120 min)-wash (water with $CaCO_3$)	—	0.90
4.5	17	Cotton-CH-ALG-L-cys (1% w/v, 120 min)-wash (water with $CaCO_3$)-CH	—	0.85
5	18	Cotton-CH-ALG-L-cys (1% w/v, 120 min)	—	0.09

[a] 1, With L-cys as the polyelectrolyte; 2, with L-cys by dissolution in the polyelectrolyte; 3, introduction of L-cys by direct microspray; 4.1, gelation of ALG followed by immersion in an L-cys solution; 4.2, immersion in L-cys solution followed by the gelation of ALG; 4.3, dissolution of L-cys in the gelation solution; 4.4, method 4.2 with the gelation of ALG replaced by washing with deionized water containing calcium; 4.5, method 4.4 followed by coating with CH; 5, immersion in L-cys solution without the gelation of ALG.

electrostatic interactions between L-cys and CH and ALG. L-Cys is free and comes out easily during the LbL process. The result obtained by method 2 was in accordance with the results of the literature for drugs incorporated in biodegradable ALG.[47] The dissolution of the drug in polyelectrolyte led to good results when the drug was insoluble in water. Water-soluble drugs (in the case of L-cys) are not suitable for this technique because of the rapid loss of the external phase.[48]

In methods 4.1, 4.2, 4.3, 4.4, and 4.5, the presence of sulfur was detected. It was clear that the best result occurred when there was gelling. In method 4.3, there was less incorporation of L-cys compared with methods 4.1, 4.2, 4.4, and 4.5. The dissolution of L-cys (method 4.3) in the gelling solution interfered with the gelling process, and this resulted in lower values of incorporation of L-cys. The decrease in the L-cys content was a result of L-cys diffusion through the crosslinked ALG gel into the $CaCO_3$ solution. Comparing the results between methods 4.1 and 4.2 and methods 4.4 and 4.5, we observed that the process of gelling was not necessary because the addition of calcium in the wash water solution was enough to obtain desirable entrapment. Longer exposure of the ALG to the $CaCO_3$ solution induced a higher crosslinking degree, and the ALG-Ca^{2+} network limited the repulsion of the ALG chains and decreased the maximum L-cys uptake capacity. This evidence agreed with the results reported by Smrdel et al.,[49] where after 1 min of hardening, only the surface was crosslinked, whereas the interior of the beads was still liquid. On the other hand, a higher concentration of $CaCO_3$ resulted in a denser network; this prevented the ALG from eroding out of the film and delayed the kinetics of L-cys release.[50]

Method 4.4 led to better results with the greatest amount of detected sulfur; this meant that there was a larger amount of L-cys embedded between the CH/ALG layers.

In method 5, a very low amount of sulfur was detected. This indicated that a very small amount of L-cys was embedded between the layers of CH/ALG. In this method, gelling was not used, and a very low amount of L-cys between the layers was detected. This suggested that the gelling process was essential for the incorporation of L-cys between the layers of CH/ALG.

Ellman's Reagent

Samples 16 and 17 (samples with better results of L-cys incorporation, see Table I) were immersed in deionized water. Small amounts of liquid solution were collected and replenished by fresh deionized water and analyzed for different immersion times. The results for the absorbance obtained by the method of Ellman's reagent are show in Figure 4.

After immersion for 90 min in deionized water, the result of EDS analysis by weight percentage for sample 16 was 0.40% in sulfur and for sample 17 was 0.37% in sulfur. There was a reduction of 50% in the content of sulfur in each sample; this suggested that there was a release of 50% of L-cys for an immersion time of 90 min.

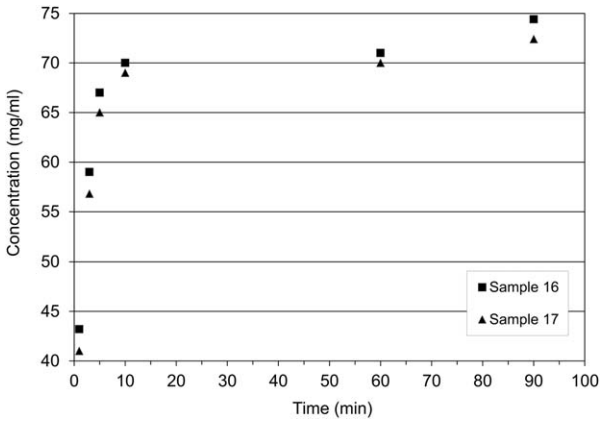

Figure 4. Ellman's reagent.

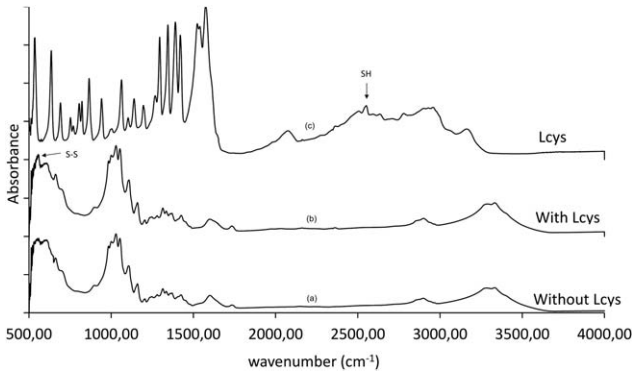

Figure 5. ATR–FTIR spectra of (a) a functionalized cotton sample with CH/ALG and without L-cys, (b) a functionalized cotton sample with CH/ALG and L-cys incorporated, and (c) L-cys.

ATR–FTIR Spectra

Figure 5(a,b) shows characteristic cellulose peaks around 1000–1200 cm^{-1}, which were those of the main components of cotton. Other characteristic bands related to the chemical structure of cellulose were hydrogen- bonded OH stretching around 3100–3550 cm^{-1}, C—H stretching around 2800 cm^{-1}, and asymmetrical COO^- stretching around 1600 cm^{-1}.[51–53] The characteristic peaks of CH were detected in the region around 1700–1500 cm^{-1} and corresponded to amino groups. The ALG spectrum showed the characteristic bands of carboxylate (COO^-) at 1600 and 1400 cm^{-1}.[54]

Pure L-cys showed bands at 1575 and 1390 cm^{-1} corresponding to the asymmetric and symmetric stretching of COO^-.[55] Characteristic peaks for L-cys resulting from amine bending vibrations modes were observed at 1523 and 1420 cm^{-1}.[55] The peak at 2551 cm^{-1} corresponded to the —SH group (thiol group of L-cys).[56] Figure 5(b) shows the spectrum of the functionalized cotton sample with L-cys, where the absence of the —SH band at 2551 cm^{-1}. This indicated sulfur–hydrogen bond breakage, and a new sulfur-sulfur bond appeared at 558 cm^{-1}.[2,57]

Comparing Figure 5(a) and 5(b), we observed that the curves were similar and had no displacement in the appearance of peaks. This indicated that there was no chemical bond between CH/ALG and L-cys. The absence of chemical bonds suggested that L-cys may have been coordinated with the nitrogen of the amino group of CH and the oxygen of the carboxylate group of ALG.

Best Configuration for the Samples

Considering all the results obtained in this study and the results reported in an already published article,[58] we found that five layers was the best setting for functionalized cotton with CH and ALG. So, the best settings in the preparation of the functionalized cotton were

Sample 19: Cotton–CH–ALG–CH–ALG–L-cys (1% w/v, 120 min)–wash (water with $CaCO_3$).
Sample 20: Cotton–CH–ALG–CH–ALG–L-cys (1% w/v, 120 min)–wash (water with $CaCO_3$)–CH.

Assessment of Antibacterial Activity

Table II presents the values of bacteriostatic and bactericidal activity levels for samples 19, 19 control, 20, and 20 control. The control samples had the same configuration of samples 19 and 20 but without immersion in the L-cys solution.

All samples (19, 19 control, 20, and 20 control) showed bacteriostatic activity and no bactericidal activity against *Staphylococcus aureus* and *Klebsiella pneumonia*.

For *S. aureus*, the bacteriostatic activity level in sample 19 (with L-cys) increased approximately 70% relative to the sample 19 control (without L-cys). Similarly, the bacteriostatic activity level in sample 20 (with L-cys) increased approximately 60% relative to the sample 20 control (without L-cys). For the *K. pneumonia*, the behavior was similar to the previous one, so the bacteriostatic activity level in sample 19 (with L-cys) increased approximately 90% relative to the sample 19 control (without L-cys). For sample 20 (with L-cys), the bacteriostatic activity level increased approximately 80% relative to the sample 20 control (without L-cys). By analyzing these results, we found that the presence of L-cys in the sample significantly increased the bacteriostatic activity level.

Sample 20 presented the highest values of bacteriostatic activity (2.9 for *S. aureus* and 2.2 for *K. pneumonia*). The last layer in this sample was composed of CH. Normally, CH exhibits a stronger bioactivity effect upon Gram-positive (*S. aureus*) and Gram-negative (*K. pneumonia*) bacteria.[59,60] This fact may be explained by the number of amine groups available for reaction. CH is a cation that will attract the negative charges of the cell

Table II. Bacteriostatic and Bactericidal Activity

	S. aureus		*K. pneumoniae*	
Sample	$M_b - M_c$	$M_a - M_c$	$M_b - M_c$	$M_a - M_c$
19	2.1	−0.2	1.9	−0.3
19 control	1.2	−0.6	1.0	−0.6
20	2.9	−0.1	2.2	−0.2
20 control	1.8	−0.4	1.2	−0.8

A, number of inoculated bacteria; *B*, number of bacteria on the standard sample contacted for 18 h; *C*, number of bacteria on the functionalized sample after incubation for 18 h. $M_a = \log A$, $M_b = \log B$, $M_c = \log C$. Bacteriostatic activity level = $M_b - M_c$. Bactericidal activity level = $M_a - M_c$.

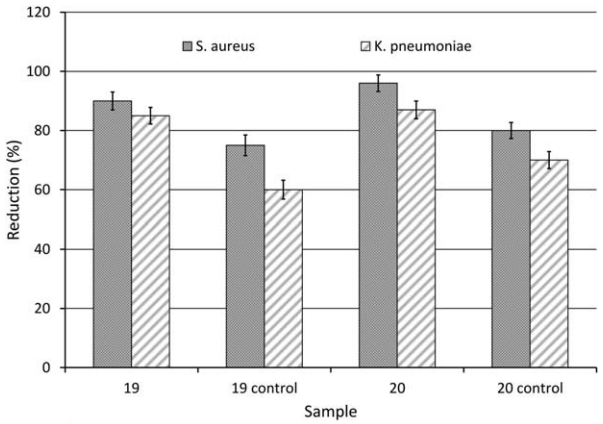

Figure 6. Reduction rate percentages of *S. aureus* and *K. pneumonia* in the functionalized samples with L-cys incorporated.

walls of bacteria and cause damage and sometimes even death.[61–64]

The sample 20 control presented lower values of bacteriostatic activity (1.8 for *S. aureus* and 1.2 for *K. pneumonia*) with respect to sample 20. These values were due to the layers of CH and ALG, which had antibacterial properties, but otherwise, there was no L-cys in this sample. This suggested that L-cys conferred a greater antibacterial effect to the samples.

Sample 19 presented values of bacteriostatic activity of 2.1 for *S. aureus* and 1.9 for *K. pneumonia*. As expected, the sample 19 control had lower values for bacteriostatic activity (1.2 for *S. aureus* and 1.0 for *K. pneumonia*) because it had no L-cys.

Figure 6 shows the growth inhibition (cell reduction) of *S. aureus* and *K. pneumonia* by antibacterial activity of the functionalized cotton with L-cys incorporated.

Analyzing the results (Figure 6) for *S. aureus*, we found a reduction of 90% in bacterial growth on sample 19 and a reduction of 95% on sample 20. For *K. pneumonia*, there was a reduction of 84% in bacterial growth on sample 19 and a reduction of 87% on sample 20.

For the 19 and 20 control samples for *S. aureus*, there were reductions of 72 and 80% in bacterial growth, respectively. For *K. pneumonia*, there were reductions of 60 and 70% in bacterial growth on the 19 and 20 control samples, respectively.

Sample 20 presented a greater reduction in bacterial growth than sample 19. This difference was due to the presence of CH in the last layer of sample 20. As discussed in an article already published[58] and based on the literature, CH exhibited a stronger bioactivity effect upon *S. aureus* and *K. pneumonia* bacteria.

From Figure 6, the difference in values was explained by the presence of L-cys in samples 19 and 20. The presence of L-cys gave a higher antibacterial activity to the functionalized cotton samples.

CONCLUSIONS

L-Cys, a bioactive agent, could be directly embedded between the layers of CH/ALG without any covalent bonding with a polyelectrolyte. With the results obtained taken into account, method 4.4 or 4.5 would be the most appropriate for that purpose. This method has many advantages; in particular, the bioactive agent was immobilized between layers (no chemical bond) without the necessary optimization for each bioactive agent because the agent could be embedded by methods 4.4 or 4.5.

In addition, LbL deposition allows the easy fabrication of multimaterial films, in which different layers carry different functionalities or repeat the same functionality several times to control the quality or the quantity of active agents.

Our results strongly suggest that biofunctionalized polyelectrolyte multilayered films containing L-cys represent a promising area for development in biomaterials and biotechnology. Thus, these unique structures are potentially very useful as wound dressings.

ACKNOWLEDGMENTS

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Paper VI

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Incorporation of antimicrobial peptides on functionalized cotton gauzes for medical applications

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Incorporation of antimicrobial peptides on functionalized cotton gauzes for medical applications

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Abstract:

A large group of low molecular weight natural compounds that exhibit antimicrobial activity has been isolated from animals and plants during the past two decades. Among them, peptides are the most widespread resulting in a new generation of antimicrobial agents with higher specific activity. In the present study we have developed a new strategy to obtain antimicrobial wound-dressings based on the incorporation of antimicrobial peptides into polyelectrolyte multilayer films built by the alternate deposition of polycation (chitosan) and polyanion (alginic acid sodium salt) over cotton gauzes. Energy dispersive X ray microanalysis technique was used

to determine the depth at which the antimicrobial peptides penetrated within the films. FTIR analysis was performed to assess the chemical linkages, and antimicrobial assays were performed with two strains: *Staphylococcus aureus* (Gram-positive bacterium) and *Klebsiella pneumonia* (Gram-negative bacterium). Results showed that all antimicrobial peptides used in this work have provided a higher antimicrobial effect (in the range of 4 log-6 log reduction) for both microorganisms, in comparison with the controls, and are non-cytotoxic to normal human dermal fibroblasts at the concentrations tested.

Keywords: Antimicrobial peptides; hBD-1, ß-Defensin-1, Human; Dermaseptin; Cys-LC-LL-37; Magainin 1; biocompatibility; wound-dressing.

1. Introduction

Several authors found that there was a significant absorption of antibiotic, when it is placed directly on the wound as a cream, which may increase the risk of cytotoxicity of the treated tissues, because in this case easily excessive amounts that can be used and it is difficult to control the optimal amount of cream. (Boosalis, McCall, Ahrenholz, Solem & McClain, 1987; Mi et al., 2002; Wang, Wang, Zhang, Zapatasirvent & Davies, 1985). Likewise, it is important to develop a method to control the release of antimicrobial agents.

It also has been reported that higher concentrations of some compounds are toxic to tissue and may be a burden to organs or lead to the development of antibiotic resistance (Boateng, Matthews, Stevens & Eccleston, 2008; Dave, Joshi & Venugopalan, 2012; Hidalgo & Dominguez, 1998). Compounds most commonly incorporated into dressings to control or prevent infection are silver (Boateng, Matthews, Stevens & Eccleston, 2008), povidone-iodine (Misra & Nanchahal, 2003) and polyhexamethylene biguanide (Motta, Milne & Corbett, 2004). On the other hand, semi-solid preparations such as silver sulphadiazine cream (Hudspith & Rayatt, 2004) and silver nitrate ointment (Moir & Serra, 2012) are used to treat bacterial infection on the surface of the wound but direct application onto open wounds can be very painful (Thakoersing, Gooris, Mulder, Rietveld, El Ghalbzouri & Bouwstra, 2012) and the scientific evidence for the efficacy of these agents in wounds is scarce. Common topical antibiotics also include mupirocin (Rode, Hanslo, Dewet, Millar & Cywes, 1989), neosporin (Sinha, Agarwal & Agarwal, 1997) and tetracycline (Kumar, Bai & Krishnan, 2004). However, these antibiotics are ineffective when resistant bacteria colonize the wound (Cookson, 1998; Hetem & Bonten, 2013). Moreover, it is important that slow release of antimicrobial agent from wound dressing have the advantage of treating infected wounds in a mild way (Elsner, Berdicevsky & Zilberman, 2011; Kostenko, Lyczak, Turner & Martinuzzi, 2010).

Since the beginning of the antibiotic era in the 1940s, the use of antibiotics has resulted in the continual emergence of resistant strains of bacteria, further complicating the clearance of infection in cutaneous wounds (Gibson et al., 2012). Therefore, a new and innovative strategy is needed to combat infected cutaneous wounds. For this purpose a new strategy foresees the

use of antimicrobial peptides (AMPs) as potential antibacterial for wound dressing application (Boateng, Matthews, Stevens & Eccleston, 2008). AMPs are a potential therapeutic compounds, they are essential components of the human innate immune system and as such contribute to the first line of defence against infections (Nizet et al., 2001; Zasloff, 2002).

AMPs produced in bacteria, insects, plants, invertebrates and vertebrates, are an important component of the natural defences of most living organisms. AMPs exhibit potent killing of a broad range of microorganisms, including Gram-negative and Gram-positive bacteria, fungi and viruses (Dai, Huang, Sharma, Hashmi, Kurup & Hamblin, 2010; Leguen, Chassepot, Decher, Schaaf, Voegel & Jessel, 2007; Marshall & Arenas, 2003). AMPs are diverse in their sequence and structures. They are generally small (10-50 aminoacids) and have at least two positive charges (da Silva & Machado, 2012). Besides antibacterial and antifungal activities, some of AMPs also possess antiviral or anticancer properties. AMPs exert their antifungal or antibacterial effects by interacting and destabilizing the microbial membrane, leading to cell death (Sato & Feix, 2006; Wimley & Hristova, 2011). The exact mechanism by which AMPs exert their antimicrobial properties is yet unknown, but it is generally accepted that cationic AMPs interact by electrostatic forces with the negatively charged phospholipid head groups on the bacterial membrane and cause disruption, resulting in bacterial killing (da Silva & Machado, 2012; Zasloff, 2002).

There are different methods based on physical or chemical immobilization of AMPs to develop antibacterial surfaces. In covalent immobilization the AMPs chemically react with a given surface to form stable antimicrobial coatings (Onaizi & Leong, 2011). Surfaces that are not reactive towards AMPs can undergo some surface treatment to introduce the desired functional groups that will allow the grafting of AMPs in a further step (Banerjee, Pangule & Kane, 2011). Among the physical immobilization methods Layer by Layer (LbL) can be a promising technique to immobilize AMPs on materials surfaces. This method is based on the alternate adsorption of polycations and polyanions on a solid substratum (Ariga, Hill & Ji, 2007). In this work AMPs can be simply embedded in the multilayer structure to prepare functional cotton gauzes.

From reports in the scientific literature, a group of 4 AMPs was selected for the present study: hBD-1, ß-Defensin-1, Human; Dermaseptin; Cys-LC-LL-37 and Magainin 1. All of these AMPs have been described to have an antimicrobial activity against different microorganisms (Guani-Guerra, Santos-Mendoza, Lugo-Reyes & Teran, 2010; Jiang et al., 2012; Nascimento, Franco, Oliveira & Andrade, 2012; Nicolas & El Amri, 2009). Another important factor of these AMPs there are cysteine residues, which promote the formation of disulfide bonds in the molecular structure, making them resistant to proteases, temperature and pH (Bulet, Stocklin & Menin, 2004).

Defensins are cysteine-rich cationic antimicrobial peptides that play an important role in innate immunity they are known to contribute to the regulation of host adaptive immunity and capacity of re-epithelialisation of healing skin (Sakamoto et al., 2005).

Dermaseptin is a linear polyatomic peptide, composed of 34-residue anionic, which are structured in amphipathic α-helices in apolar solvents. Several Dermaseptins have been reported to inhibit the activity of microbial cells, rapidly, efficiently and irreversibly without toxic effects on mammalian cells (Marshall & Arenas, 2003).

LL-37 induces keratinocyte migration required for re-epithelialization of the wound. LL-37 is also an important factor in the proliferation and formation of vessel-like structures, and induces functional angiogenesis important for cutaneous wound neovascularization. LL-37 has antimicrobial activity against both Gram-positive and Gram-negative bacteria, stimulates wound vascularization and re-epithelialization of healing skin and has antitumor activity. The human cathelicidin LL-37 also has been associated with host stimulatory events important to the wound repair process (Izadpanah & Gallo, 2005).

In this work we used a new line of LL-37 from AnaSpec, Inc., Cys-LC-LL-37. This is a new AMP like the LL-37, but has a broad range antimicrobial activity. This new AMP was obtained with one cysteine, where LC is a 6-carbon linker.

Magainin 1 is a 23-amino acid cationic AMP, which has a α-helical structure and is characterized by being a cationic and amphipathic molecule. Magainin 1 reveals multiple functions related to

membrane interactions, being active toward multiple pathogens. This peptide also carries a positive net charge at a neutral pH level and has hydrophobic residues that are essential for antimicrobial activity (Nascimento, Franco, Oliveira & Andrade, 2012; Speranza, Taddei & Ovidi, 2007). This AMP has broad-spectrum, nonspecific activity against a wide range of microorganisms, including viruses, Gram-positive and Gram-negative bacteria, protozoa and fungi, may also be haemolytic and cytotoxic to cancer cells and is a bactericide (Zairi, Tangy, Bouassida & Hani, 2009). These observations suggest AMPs serve a dual role in wound healing: killing bacteria and stimulating complex host repair phenomena.

The biomaterials chosen for the functionalization of cotton gauze were chitosan (CH) and alginic acid sodium salt (ALG), both known as biodegradable, nontoxic and biocompatible polymers. CH is widely used as wound dressings and has been shown to have mucoadhesive properties, cationic nature, anti-bacterial and haemostatic properties (Alves, Picart & Mano, 2009; Jayakumar, Chennazhi, Muzzarelli, Tamura, Nair & Selvamurugan, 2010).

ALG is known to be nontoxic, having hemostatic action and biocompatible with a variety of cells, ALG has been studied for application as biomaterials and as wound dressings (de Moraes & Beppu, 2013). Due to its properties CH and ALG are already widely used in biomedical applications (Caridade, Monge, Gilde, Boudou, Mano & Picart, 2013; Lee & Mooney, 2012; Martins, Merino, Mano & Alves, 2010)

An ideal wound dressing can restore the milieu required for the healing process, while simultaneously protecting the wound bed against bacteria. This has encouraged the development of improved wound dressings that provide an antimicrobial effect by eluting germicidal compounds such as iodine or most frequently silver ions. Such dressings are designed to provide controlled release of the active agent through a slow but sustained release mechanism which helps to avoid toxicity and yet ensures delivery of a therapeutic dose to the wound (Peles, Binderman, Berdicevsky & Zilberman, 2013).

Based on the previous concept, in this study, we have incorporated AMPs onto a substrate of cotton gauze functionalized with layers of CH and ALG. Functionalized cotton gauzes with CH and ALG were obtained via LbL electrostatic deposition, as described in a work already

published (Gomes, Mano, Queiroz & Gouveia, 2012). The aim of this work is to incorporate AMPs between the layers of CH and ALG. These layers are based on the alternate deposition of oppositely charged polyelectrolyte layers (CH is a polycation and ALG is a polyanion), this deposition was made on cotton gauze.

The embedding of active agents by LbL is a very recent area of research receiving great interest due to the advantage of obtaining control over drug release. Not so recent and with a large number of published papers, LbL was developed for drug delivery systems through microcapsules (Johnston, Cortez, Angelatos & Caruso, 2006; Quinn, Johnston, Such, Zelikin & Caruso, 2007; Sukhishvili, 2005; Tang, Wang, Podsiadlo & Kotov, 2006; Wang, Angelatos & Caruso, 2008). LbL deposited thin films were first developed by Decher and co-workers (Decher, 1997). They proposed a protocol for the preparation of thin films based on alternate and repeated adsorption of polycations and polyanions on the surface of a solid substrate from solution. A diversity of materials have been employed as building blocks for LbL films, including synthetic polymers, biopolymers, inorganic nanoparticles, etc (Ariga, Hill & Ji, 2007). Consequently, a variety of components and functionality can be incorporated into LbL films, which forms the basis for the development of stimuli-sensitive LbL films for drug delivery.

2. Materials and methods

2.1 AMPs

Sequence of the AMPs used is shown in table 1. The lots containing lyophilized powder of hBD-1, ß-Defensin-1, Human (ANASPEC); Dermaseptin (ANASPEC); Cys-LC-LL-37 (ANASPEC) and Magainin 1 (ANASPEC) were stored at -20ºC. When the AMPs solution was prepared the content present in the lots (1 mg) was dissolved in 1 mL of sterile water and a stock solution of AMPs of 10 µg/mL was prepared.

Table 1 - Structure for AMPs

Peptide	Size (kDa)	Amino acid sequence
hBD-1, ß-Defensin-1, Human	3.9	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK (Disulfide bridge: 5-34, 12-27, 17-35)
Dermaseptin	3.4	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
Cys-LC-LL-37	4.7	C-LC-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
Magainin 1	2.4	GIGKFLHSAGKFGKAFVGEIMKS

2.2 Determination of minimal inhibitory concentration (MIC)

By definition MIC is the lowest concentration of antimicrobial agent that prevents the visible growth of a microorganism on susceptibility testing. MICs were determined using the broth microdilution method, as described by CLSI M7-A7 standard method for bacterial strains *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumonia* (ATCC 13883) (Matthew A. Wikler, 2006). According to the guidelines, the MIC was determined by serial dilution (1:2) in Mueller-Hinton Broth (MHB). The inoculum was prepared from fresh overnight liquid cultures and the turbidity was adjusted to obtain turbidity to 0.5 McFarland (approximately $1x10^8$ CFU/ml, CFU=colony forming units) with 0.85 % NaCl and microorganisms, and then diluted to give a final concentration of $1x10^5$ CFU/ml. For this 10µL of bacterial suspension, 990 µL of MHB were added. After the preparation of the bacterial suspension, a solution of AMPs of the initial concentration 10 µg/mL was used as starting point to perform the successive volumetric dilutions in a ratio of 1:2. To each well of the microdilution, 50 µL of the work suspension was added with 50 µL of AMPs in different dilutions. Inoculated microdilution was incubated at 35° C for 16-20 hours.

2.3 Polyelectrolyte multilayer film preparation and AMPs incorporation

Sterile cotton gauzes obtained from Albino Dias de Andrade, Lda (www.ADA.pt) were used as substrate. (2,2,6,6- tetramethylpiperidin-1-yl)oxyl designated by TEMPO, Sodium Bromide (NaBr), Sodium Hypochlorite (NaOCl) 5%, CH (low molecular weight, 50-190 kDa and 80% degree

of deacetylation), Acetic Acid (CH_3COOH), ALG (molecular weight 120-190 kDa, ratio of mannuronic acid to guluronic acid is 1.56 and viscosity 15-20 cP, 1% in H_2O), Sodium Chloride (NaCl), Sodium Hydroxide (NaOH) and Hydrochloric acid (HCl) were purchased from Sigma-Aldrich. All chemicals were of analytical grade and used as received. Polyelectrolyte CH (1mg/mL) and ALG (1mg/mL) solutions were prepared by dissolving CH and ALG in 0.1M CH_3COOH and 0.5M NaCl solutions, respectively. To apply LbL technique, cotton gauzes were charged by immersing in (TEMPO + NaBr + NaClO 5%, pH=10.5) solution under moderate stirring, for 30 minutes, followed by a rinse with deionized water, as described elsewhere (Dang, Zhang & Ragauskas, 2007; Diez, Eronen, Osterberg, Linder, Ikkala & Ras, 2011; Gomes, Mano, Queiroz & Gouveia, 2012; Saito, Okita, Nge, Sugiyama & Isogai, 2006).

Samples were manually prepared by immersing the cotton gauze substrate alternately in polycation and polyanion solutions, for 5 minutes. Between each polyelectrolyte exposure, the sample was rinsed with deionized water. For the CH and ALG polyelectrolyte layers, a pH of 5.0 was selected, to be approximately an intermediate value between the pK_a of CH (6.3) and ALG (3.38 and 3.65 for different residues) (Maurstad, Morch, Bausch & Stokke, 2008).

Functionalization of cotton gauzes was prepared with four layers (cotton gauze/CH/ALG/CH/ALG), according with the optimization made in our previous work (Gomes, 2010; Gomes, Mano, Queiroz & Gouveia, 2012, 2013; Gomes, Mano, Queiroz & Gouveia, 2014). In these published papers, we have shown that there was an exponential growth of the layers, resulting in a coating thickness in range of 0.6 -1μm (four layers), as we can see in the TEM image of figure 1. The samples were examined using a HITACHI HT 7700 TEM.

Note that previous work reported by Deng et al, 2010, where the LbL method was assembled on cellulose (main constituent of cotton) nanofiber highly compact and crystalline surface obtained by electrospinning, the estimated thickness of CH/ALG bilayer formed on fibers was in the range of 8-15 nm. For the same polyelectrolytes (CH and ALG), a study on polypropylene substrate showed the thickness of 160 nm (Caridade, Monge, Gilde, Boudou, Mano & Picart, 2013) and in quartz substrate the thickness of CH/ALG was found to be 15 nm (Martins, Mano & Alves, 2010).

Actually, in linearly growth of layer thickness each bilayer interacts only with bilayers that directly neighbor it (above or below) with very little inter-penetration. However, there are systems that showed exponential increases in film thickness with each deposition cycle, this type of growth was attributed to a diffusion of polyelectrolyte “in” and “out” of the film during each bilayer step (Foster & DeRosa, 2014), which is the case of cotton porous fibers whereas polyelectrolyte diffusion is expected. The typical thickness of a linearly growing film constituted of 20 bilayers is of the order of 100 nm but the thickness of exponentially growing films can reach 10 μm or more after the deposition of a similar number of layers (Richert et al., 2004) which is the case of the coating obtained in this study.

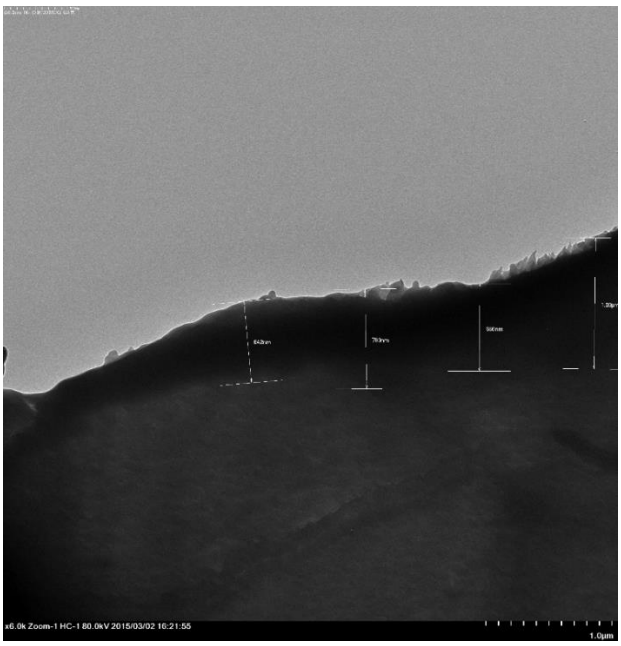

Figure 1 – TEM image of sample cotton/CH/ALG/CH/ALG

Then, the functionalized cotton gauze were immersed in a solution of AMPs in concentration higher than the MIC values, 10μg/mL for 24 hours. The selected concentration aimed to ensure that a significant amount would be absorb, giving the expected antimicrobial activity. Absorption rates of the AMPs were monitored after 6, 12, 18 and 24 hours through the Bradford method described in 2.8 sub-section. The functionalized samples of cotton gauzes were washed with deionized water containing calcium in order to occur the ALG gel formation, finally a final

CH layer was added. With this procedure we were able to obtain the expected coating with embed AMPs.

2.4 Energy dispersive X ray microanalysis technique

Energy dispersive X ray microanalysis technique (EDS) has been a common elemental analysis method used to determine the composition of particles and thin films in sample analysis. EDS technique is based on volume analysis, and as such, the electron penetration of the specimen is a direct function of acceleration beam voltage and sample density. The accelerating voltage determines the force of the electron beam. In general, one can see that as the electron beam voltage increases, the penetration depth increases. Higher accelerating voltages, which apply more force to the electrons in the beam, can allow them to penetrate deeper into the sample. For example, using 5 kV, 10 kV, 15 kV, 20 kV, 25 kV and 30 kV acceleration voltages for carbon sample, penetration depth is about 0.34 μm, 1.20μm, 2.2 μm, 4.10 μm, 6.1 μm and 8.5 μm respectively (Hua, 2004; Lee, Hua, Zhao & Mo, 2006).

In this study, Scanning Electron Microscopy (SEM) and EDS analyses were carried out on a Hitachi S 2700 using different electron beam accelerating voltages and 12 mm working distance.

2.5 ATR-FTIR spectra

All samples were analyzed in absorption mode using a Nicolet iS 10 FT-IR spectrometer (Thermo Scientific) accommodated with a smart ITR accessory for ATR sampling. The smart ITR accessory is equipped with a single bounce diamond crystal. Prior to spectrum recording the sample was pressed directly on the diamond crystal by usage of the smart, ITR high pressure clamp. Each spectrum was measured at a spectral resolution of 4 cm^{-1} with 64 scans per spectrum. Spectrum recording was performed in the range of 4000 to 600 cm^{-1}. A spectrum was obtained in three different locations for each sample to ensure even chemical composition. Before each sample a blank was measured to check the crystal for contamination. Every hour a background spectrum was measured against air using identical instrumental conditions as the samples.

Spectral data were obtained using the OMNIC Software (Thermo Scientific). After each measurement, the crystal was cleaned using a soft tissue soaked with ethanol and left to dry in ambient air.

ATR-FTIR reveals information about the molecular structure of chemical compounds and is useful for the characterization of biopolymers. These analyses were made in order to investigate the way the AMPs were incorporated between the layers of CH/ALG of functionalized cotton gauzes.

2.6 Assessment of antibacterial activity

The AMPs antibacterial effect of functionalized cotton gauzes with CH/ALG was tested according to the Japanese Industrial Standard JIS L 1902:2002 (Standard, 2002), which is the most employed method. This method is designed to quantitatively test the ability of textiles that have been treated with antibacterial agents to prevent bacterial growth and to kill bacteria, over an 18 hour period of contact. This method is based on the quantitative determination of the potential effect and activity of functionalized samples, by the direct contact with a suspension of bacterial cells.

The cotton gauze sample sizes are approximately 18x18 mm. We prepared 6 control samples with CH/ALG and without AMPs and 6 functionalized samples with CH/ALG and AMPs, these samples were tested for each bacterial strain. In order to calculate growth reduction rate, three samples were used to measure the number of live bacteria after inoculation (T_{0h}) and the other three to measure the number of live bacteria after incubation (T_{18h}).

Bacterial cell suspensions were collected from an overnight liquid culture in Nutrient Broth. After that, the bacterial concentration is adjusted to 1-2x10^8 cel/mL (equivalent to 0.5 McFarland), with the necessary dilutions to adjust the final bacterial concentration to 1±0.3 x10^5 cel/mL.

Each sample was placed in a 50 mL Falcon tube, soaked with 200 µL of the inoculum previously prepared, T_{18h} tubes were incubated for 18 h at 37 ºC. For the release of bacterial cells from

the cotton gauze samples, before and after the 18 h incubation period, 20 mL of 0.85 % NaCl with surfactant Tween 80 (0.2 %) was added to the samples in 50 mL Falcon tubes and vortexed. The resulting suspensions were used for the determination of viable counts using serial dilutions prepared in sterile 0.85 % sodium chloride solution and plated. The plates were incubated at 37 ºC for 18 h, and the number of colonies was counted visually using a microscope. This procedure was performed in triplicate (Standard, 2002). The growth reduction rate of the bacteria was calculated using the equation:

$$\frac{T_{0h} - T_{18h}}{T_{0h}} \times 100\% = reduction\ rate\ (\%)$$

where, T_{0h} is the CFU/mL of bacterial colonies at the initial stage (0h) and T_{18h} is the CFU/mL of bacterial colonies after 18 h incubation (Park & Park, 2010).

2.7 Cytotoxicity assay

The possibility of application of these functionalized samples as wound dressings is also evaluated by cytotoxicity test. Cytotoxicity of the AMPs was evaluated by an MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay (Freshney, 2005) 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 37ºC 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 concentrations of hBD-1, ß-Defensin1; Dermaseptin and Cys-LC-LL-37 of 5.00 µg/mL, and Magainin 1 with two concentrations of 0.20 and 4.17 µg/mL and incubated at 37ºC, in a 5% CO_2 atmosphere, for 48 hours. The concentrations chosen were the MIC values against *S. aureus* and *K. pneumoniae*.

Untreated cells were used as control. Afterwards the liquid content of the wells was removed and it was replaced with 200 µL of MTT solution of 1mg/mL in PBS. The multi-well plates were incubated for 4 hours, at 37ºC, 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 cristals and to stabilize the colour, respectively. The absorbance of each well was measured at 570 nm using a Biochrom Anthos 2020 microplate reader. The extent of cell viability was expressed as the percentage of viable treated cells in comparison with control cells. All experiments were done in triplicate.

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.

2.8 Absorption/desorption rates of the AMPs (Bradford Reagent)

The absorption and release of the AMPs into and from samples was determined using the colorimetric assay of Bradford reagent. The procedure is based on the formation of a complex between the dye, Coomassie Brilliant Blue G, and proteins (AMPs) in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption/desorption is proportional to the protein present (Bradford, 1976).

The Bradford method is the most commonly used in quantitative protein determination. This method is popular because it uses a single addition of the dye reagent to the sample, it is rapid and it is done at room temperature. However, it is still an open question how reliable this method is if the formulation also involves polymer excipients. Carlsson et al. 2011, demonstrated the potential perturbations in the Bradford assay by chitosan which can interact directly with the anionic Coomassie Blue dye and perturbs its absorption spectrum. They also found that above 5 µg/mL chitosan is not as critical as at chitosan concentrations below 5 µg/mL (Carlsson, Borde, Wolfel, Akerman & Larsson, 2011).

To overcome this problem in this work two standards curves are prepared. Firstly a calibration curve was made using bovine serum albumin (BSA) with selected concentrations (0, 2.5, 5, 7.5, 10 µg/mL). Secondly a calibration curve was made with the same substrate (cotton/CH/ALG) as the final samples. So, cotton/CH/ALG samples were used with various concentrations of BSA as standards. The Bradford reagent was then added and to each tube containing 1 mL of the sample it was added 1 mL of dye and mixed thoroughly. After 15 min incubation, the absorbance of each sample was read at 595 nm wavelength, and for each concentration of BSA three independent measurements were made and the best estimate taken as their mean. The concentration of AMPs in the solution was determined by a calibration curve using standard protein BSA. The absorption rate concentrations were estimated through the difference of the amount of AMPs in the solution before (10 µg/mL) and after the sample immersion. For the release assay, absorbance was monitored for 1, 2, 3, 4, 6, 12 and 24 hours, directly on a solution containing the functionalized cotton gauzes immersed in a 20 mL of 0.85 % NaCl, in order to establish the release profile for each AMPs incorporated.

3. Results and Discussion

3.1 Determination of minimal inhibitory concentration (MIC)

MIC is important to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. Table 2 shows the calculated values of the MICs of AMPs for *Staphylococcus aureus* and *Klebsiella pneumonia*. The data indicate that MIC values for hBD-1, ß-Defensin-1, Human; Dermaseptin and Cys-LC-LL-37 are identical for the two microorganisms, being 5.00 µg/ml to *Staphylococcus aureus* and 5.00 µg/ml to *Klebsiella pneumonia*. For Magainin 1 the MIC value was 0.20 µg/ml for *Staphylococcus aureus* while for *Klebsiella pneumonia* was 4.17 µg/ml. Thus, is visible that all AMPs exhibit MIC values very low, which is a major benefit in comparison with other antimicrobial agents for wound dressings.

Table 2 - MICs of AMPs for *Staphylococcus aureus* and *Klebsiella pneumoniae*

MIC (µg/ml)	***Staphylococcus aureus***	***Klebsiella pneumoniae***
hBD-1, ß-Defensin-1, Human	5.00	5.00
Dermaseptin	5.00	5.00
Cys-LC-LL-37	5.00	5.00
Magainin 1	0.20	4.17

The MIC value found in the literature for ß-Defensin and Dermaseptin was a value of 10 µg/mL for Gram-positive and Gram-negative bacteria (Li, Zhao, Song, Huang & Zhao, 2013; Zairi, Tangy, Ducos-Galand, Alonso & Hani, 2007), twice high to that found by us. In previous works, LL-37 showed a MIC value of 3.6 µg/mL for *Staphylococcus aureus* and MIC values between 0.4 and 5.7 µg/mL for Gram-negative bacteria (De Smet & Contreras, 2005).

Among the different AMPs tested, we found that Magainin 1 was the more potent to inhibit *Staphylococcus aureus* growth with the lowest MIC value of 0.20 µg/mL.

3.2 Energy dispersive X ray microanalysis technique

The functionalized samples of cotton gauze were analyzed by different values of beam acceleration voltages (5, 10, 15, 20, 25 and 30 kV) to determine how deep each AMPs diffuses between the layers of CH and ALG. This analysis was performed by determining the elemental percentage of sulfur, because sulfur is the only chemical element that is present in each AMPs and is not present in cotton gauze, CH and ALG. These values are summarized into table 3, from this table it can understand the diffusion depths of the different AMPs in the functionalized cotton gauzes through the different values of mass percentage of sulfur according to the different electron beam acceleration voltage.

Table 3 - EDS analysis (beam accelerating voltages, Kv *vs*. percentage elemental of sulfur, wt%) for the functionalized cotton gauzes in AMPs solutions , overtime (18 h and 24 h).

18 h of absorption of the AMPs	**%Sulfur (5 kV)**	**%Sulfur (10 kV)**	**%Sulfur (15 kV)**	**%Sulfur (20 kV)**	**%Sulfur (25 kV)**	**%Sulfur (30 kV)**
hBD-1, ß-Defensin-1, Human	0	0	0.51 ±0.10	0.19 ±0.10	0	0
Dermaseptin	0	0.20 ±0.07	0.09 ±0.06	0	0	0
Cys-LC-LL-37	0	0	0.22 ±0.05	0.09 ±0.06	0	0
Magainin 1	0	0	0.10 ±0.05	0.27 ±0.10	0.08 ±0.08	0
Control	0	0	0	0	0	0
24 h of absorption of the AMPs	**%Sulfur (5 kV)**	**%Sulfur (10 kV)**	**%Sulfur (15 kV)**	**%Sulfur (20 kV)**	**%Sulfur (25 kV)**	**%Sulfur (30 kV)**
hBD-1, ß-Defensin-1, Human	0	0	0.54 ±0.10	0.25 ±0.09	0	0
Dermaseptin	0	0.25 ±0.08	0.10 ±0.05	0	0	0
Cys-LC-LL-37	0	0	0.28 ±0.07	0.10 ±0.04	0	0
Magainin 1	0	0	0.12 ±0.04	0.30 ±0.10	0.10 ±0.05	0
Control	0	0	0	0	0	0

Note: At each point with the highest value of beam accelerating voltage, was subtracted the value obtained in the previous point of less beam accelerating voltage.

Figure 5 a) show the absorption kinetic of AMPs into functionalized cotton gauze, where it can be seen that the equilibrium phase is between 18-24 hours, being therefore the selected time for the EDS analysis. Analyzing the values in table 3 it can be seen that for immersion times of 18 h and 24 h there are no differences on the AMPs diffusion depth, only a slight increase in the % of sulfur from 18 to 24 hours, due to higher amount absorbed overtime.

Results show that Magainin 1 had a higher diffusion between the layers of the functionalized cotton gauzes, because sulfur was detected to a higher value of acceleration beam voltage. This is due to the small size of the Magainin 1 (2.4 kDa) and the electrostatic attractive forces between Magainin 1 (cationic peptide) and ALG (anionic polyelectrolyte).

Dermaseptin have a lower diffusion, in other words, it is incorporated in the surface of the functionalized cotton gauzes. Dermaseptin is an anionic peptide then there will be electrostatic

repulsive forces between ALG and the Dermaseptin, so Dermaseptin will not penetrate deep inside the layers of CH/ALG.

Cys-LC-LL-37 and hBD-1, β-Defensin-1, Human had an intermediate diffusion through the layers of CH/ALG. In this way, it can be concluded that the depth diffusion of AMPs into the CH/ALG layers of the cotton gauzes is dependent of their structural features, ionic charges and size. With this analysis we can know if the AMPs are on the surface or inside the layers of the functionalized cotton gauzes.

This method constitutes a starting point for determining the conditions for optimizing the process of incorporation of the AMP for a particular application and to monitor the diffusion and attachment of the AMPs into the CH/ALG layers with success. This analysis is in agreement with other published works by Hua, Y. (2004) that used the technique of analysis by EDS in various materials, to know the depth of penetration of the electron beam, for example the study of semiconductor and analysis of thin film layers (Si_3N_4 , SiO_2 and TiW) in wafer fabrication (Hua, 2004; Lee, Hua, Zhao & Mo, 2006).

3.3 ATR-FTIR spectra

Figure 2, shows characteristic cellulose peaks around 1000 – 1200 cm^{-1}, which are the main components of cotton (Chung, Lee & Choe, 2004; Wang, Fan, Gao & Chen, 2006; Yan, Hua, Qian, Wang, Du & Chen, 2009). Other characteristic bands related to the chemical structure of cellulose were hydrogen – bonded OH stretching around 3100 – 3550 cm^{-1}, the C-H stretching around 2800 cm^{-1} and the asymmetrical COO^- stretching around 1600 cm^{-1}, if the carboxylate existed in ionized form (COO^-), it would show two peaks at 1600 and 1400 cm^{-1} for the asymmetric and the symmetric stretching of COO^- ion, respectively (Chung, Lee & Choe, 2004;

Wang, Fan, Gao & Chen, 2006; Yan, Hua, Qian, Wang, Du & Chen, 2009; Zhao, Tang, Wang & Lin, 2010).

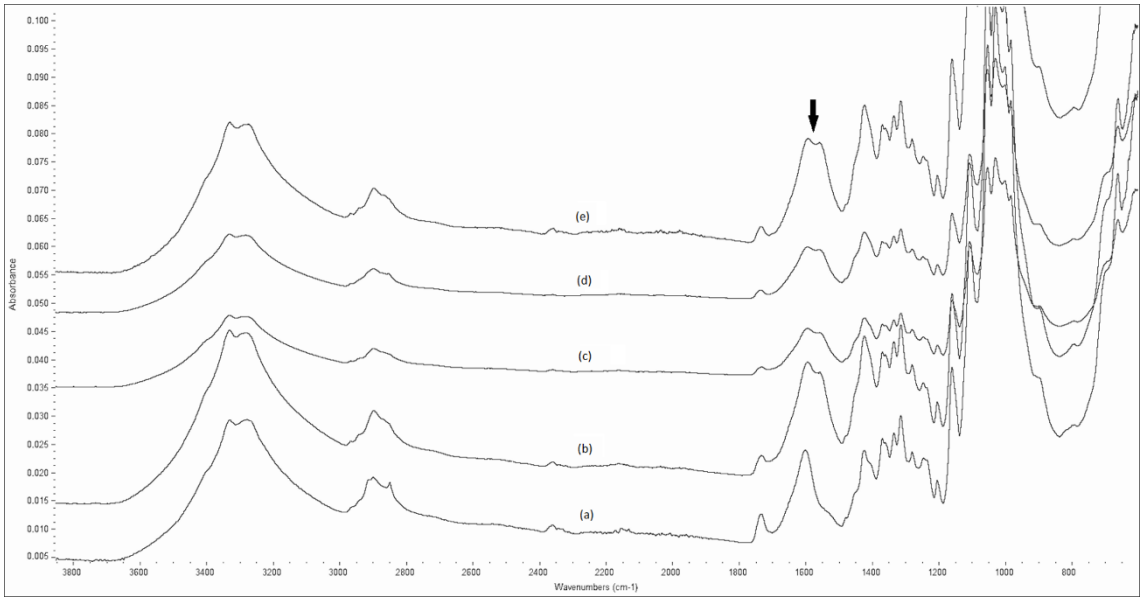

Figure 2 – ATR – FTIR (a) functionalized cotton gauze with CH/ALG, (b) hBD-1, ß-Defensin-1, Human incorporated in functionalized cotton gauze, (c) Dermaseptin incorporated in functionalized cotton gauze, (d) Cys-LC-LL-37 incorporated in functionalized cotton gauze, (e) Magainin 1 incorporated in functionalized cotton gauze.

Absorptions in the region 3200-2700 cm^{-1} , if the main absorption is below 3000 cm^{-1} , the compound is probably aliphatic and probably contains a long linear aliphatic chain (Coates, 2000). In figure 2 (b), (c), (d) and (e) it is found that this band decreases in intensity relatively the same band in figure 2 (a), maybe due to the breaking of long aliphatic chain during the AMPs embedding.

Absorptions in the region 1850-1650 cm^{-1} more specifically in the range of 1750-1700 cm^{-1} , means that the compound is probably a simple carbonyl compound or a carboxylic acid (Coates, 2000). It was observed in figure 2 (b), (c), (d) and (e) that this peak is slightly shifted to lower values, meaning that there is a conjugation with another carbonyl group or aromatic ring, indicating the presence of AMPs.

In figure 2 (b), (c), (d) and (e) it can be noted the presence of peaks at 1558 and 1600 cm^{-1} (marked with an arrow in figure 2). In figure 2 (a) the peak around 1600 cm^{-1} is typical of the antisymmetric stretching of COO^- group of ALG. In figure 2 (b), (c), (d) and (e) in addition of 1600 cm^{-1} there is a peak around 1558 cm^{-1} corresponding the NH amide group of AMPs because the peak around 1420 cm^{-1} is characteristic for CH amide (Mocanu, Nichifor, Mihai & Oproiu, 2013). Note that the peak around 1558 cm^{-1} is also due to aromatic ring (AMPs).

Finally, it can also be observed that the AMPs are not bound to CH and ALG, so the AMPs are able to be released, which can be an important issue as a requirement of antimicrobial activity for a wound-dressing.

3.4 Assessment of antibacterial activity

Figure 3 show the growth inhibition (cell reduction) of the *Staphylococcus aureus* and *Klebsiella pneumonia* by the antibacterial activity of the cotton gauzes, designated CO (without CH/ALG and AMPs); functionalized sample of cotton gauzes with CH/ALG, designated control; and functionalized cotton gauzes with CH/ALG and AMPs. The incorporation of each AMPs was performed by incubation for 24 hours on solution by functionalized cotton gauzes with CH/ALG.

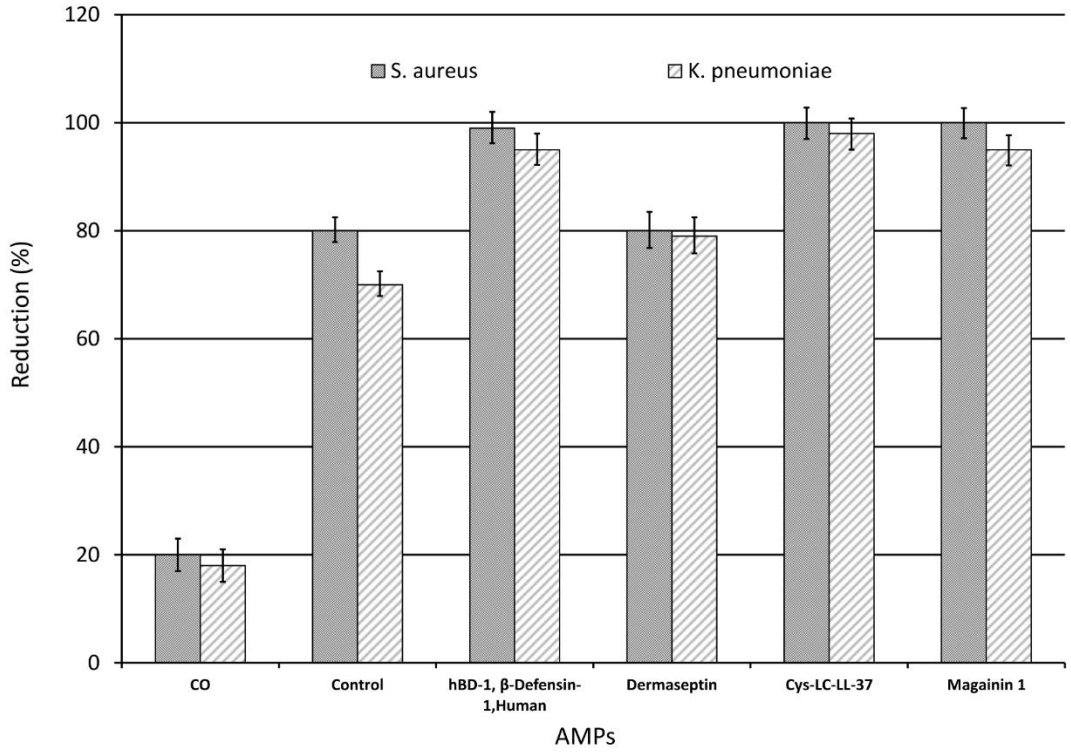

Figure 3 – Reduction rate (%) of *S. aureus* and *K. pneumoniae* in functionalized cotton gauzes with AMPs.

CO: cotton gauze; Control: cotton gauze/CH/ALG/CH/ALG/CH; Hbd-1, ß-Defensin-1, Human incorporated in Control; Dermaseptin incorporated in Control: Cys-LC-LL-37 incorporated in Control; Magainin 1 incorporated in Control

Analyzing the results (fig. 3), it appears that cotton gauze (CO) have some reduction of growth inhibition (20%) of *Staphylococcus aureus* and *Klebsiella pneumonia*, this is because cotton in solution has a negative charge. We can see in figure 3 that functionalized cotton gauzes with hBD-1, ß-Defensin 1; Cys-LC-LL-37 and Magainin 1 have growth inhibition similar for the two microorganisms (95-100%), these values show the bactericidal power of AMPs. These samples have an increase about 20-30% in growth inhibition for two bacteria relatively samples without AMPs. Despite the fact that AMPs cause an increase of 20-30% in reduction of bacterial growth, it is important to achieve a 100% reduction (6 log reduction = 99.9999% reduction) to be consider a very good antimicrobial textile material. In addition, there are several benefits in use AMPs in wound healing. The continuous use of antibiotics has resulted in multi-resistant bacterial

strains all over the world. Consequently, there is an urgent need to search for alternatives to synthetic antibiotics. AMPs are an effective alternative because until today it was not been proven that AMPs induce resistance. Other advantages of AMPs in wound dressings include pain relief, reduction of inflammation, angiogenesis and acceleration of the healing process

Functionalized cotton gauzes with Dermaseptin has lower growth inhibition rate for the two microorganisms (79-80%). In this case there is a minor increase in growth inhibition for *Klebsiella pneumonia* and no increase in growth inhibition for *Staphylococcus aureus,* possibly is due to Dermaseptin is an anionic peptide, giving him low antibacterial activity, and due to repulsion between negative charge of Dermaseptin and negative charge of ALG (layer directly involved in the process of incorporating AMPs), which led to the entrance of a small amount of Dermaseptin between multilayers of CH and ALG.

The indications of US FDA and their European counterparts consider that exist antibacterial properties in the case of bacterial reduction to be at least ≥ 99.99% (4 log reduction). Analyzing the results of figure 3, cotton gauzes with Cys-LC-LL-37 and cotton gauzes with Magainin 1 there is 100% reduction (6 log reduction) against Staphylococcus aureus, but the other samples have values below 99.99 %. (< 4 log reduction), while cotton gauzes with CH/ALG and without AMPs have growth inhibition rates of 70-80%). Incorporation of AMPs is also important due to several properties, such as:

- AMPs show broad spectrum antimicrobial activities against various microorganisms, including Gram-positive and Gram-negative bacteria, fungi and viruses (Zasloff, 2002), and have rapid onset of activity.
- Low amounts of AMPs are needed, as can be seen by the values of MIC.
- Many AMPs are effective against multi drug resistant bacteria and possess low propensity for developing resistance (Marr, Gooderham & Hancock, 2006; Mygind et al., 2005), probably due to their distinguished mode of action.

The problems caused by drug resistant bacteria have created an urgent need for the development of alternative therapeutics. In this respect, AMPs are considered as promising antimicrobial agents for producing new generation antibiotics. However, with all published

work in the last two decades, there is no AMPs agent currently approved by FDA (Fjell, Hiss, Hancock & Schneider, 2012)

3.5 Cytotoxicity assay

To complement our bacterial growth inhibition studies, we performed a simple set of experiments to evaluate AMPs-loaded functionalized cotton gauzes toxicity against mammalian cell. The purpose of these experiments was to evaluate whether AMPs-loaded functionalized cotton gauzes could have utility in wound healing where they would be in contact with human or animal tissue. MTT viability assay was used to determine the AMPs cytotoxicity on NHDF. Figure 4 shows the levels of cytotoxicity exhibited by the four AMPs used in this work toward NHDF. hBD-1, ß-Defensin-1 and Cys-LC-LL-37 did not exhibit cytotoxicity to NHDF at concentrations of MIC values (5.00 µg/mL). Such as indicated in the introduction hBD-1, ß-Defensin-1 and Cys-LC-LL-37 stimulates wound vascularization and reepithelialization of healing skin, because of this the cytotoxicity have a little value above 100%. Dermaseptin at concentration of 5.00 µg/mL and Magainin 1 at concentration of 0.20 and 4.17 µg/mL, there was a decrease of 5% in NHDF viability.

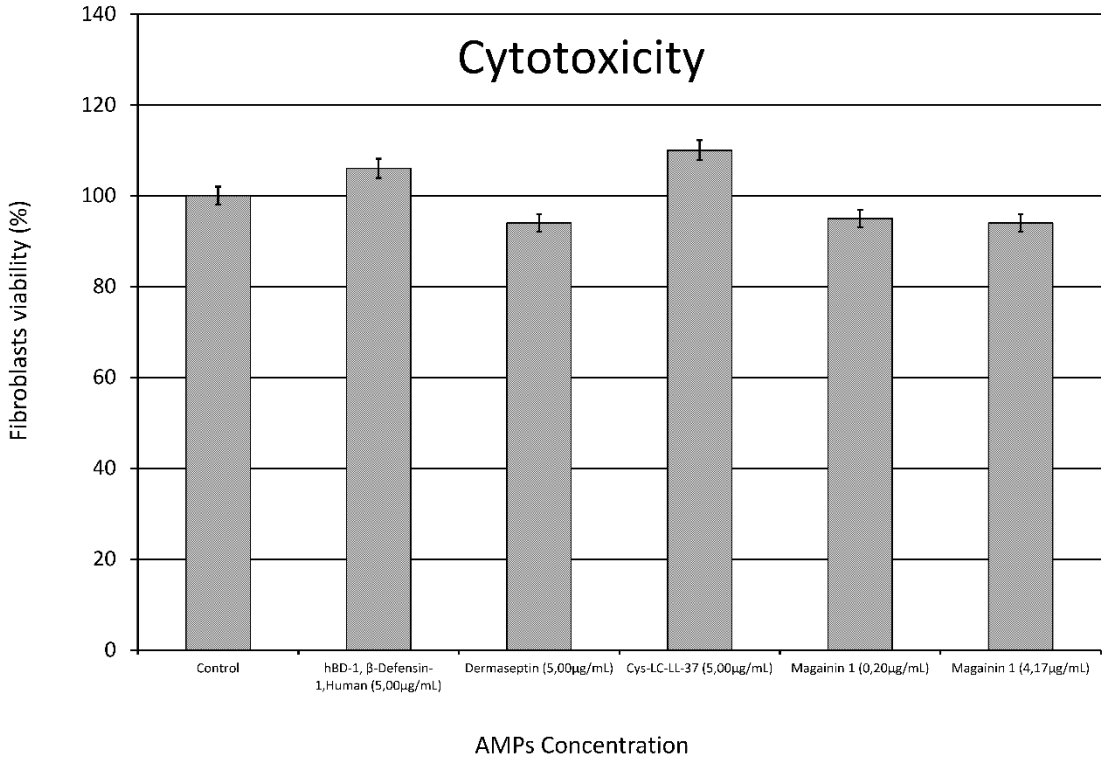

Figure 4 – Fibroblasts viability when in contact with to functionalized cotton gauzes with AMPs and with the control (functionalized cotton gauzes without AMPs)

These results mean that none of the Dermaseptin and Magainin 1 concentrations caused cytotoxic effect in NHDF, since according to Gouveia et al., 2012 (Gouveia, Sa & Henriques, 2012), only an alteration above 30% in comparison with control is considered cell-toxic (Gouveia, Sa & Henriques, 2012). Consequently, these AMPs were considered safe to be applied as antimicrobial agents to contact with the human skin without 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. The analysis of the results (figure 3 and 4) showed that only cotton gauze with Cys-LC-LL-37 and with Magainin 1 have a 100 % growth inhibition for *Staphylococcus aureus* and lower cytotoxicity. These results suggest that cotton gauze with Cys-LC-LL-37 and with Magainin 1 can be successfully incorporated into layers of CH/ALG and could be used for wound healing applications with minimal cytotoxicity to the surrounding tissue.

3.6 Absorption/desorption rates of AMPs (Bradford Reagent)

The AMPs concentration in solution was calculated by comparison to a standard curve (calibration curve), formed by known concentrations of BSA. Analysis of AMPs concentrations was carried out in the solution of the incubation bath, before and after the AMPs incorporation on functionalized cotton gauzes by measuring the absorbance of the solutions.

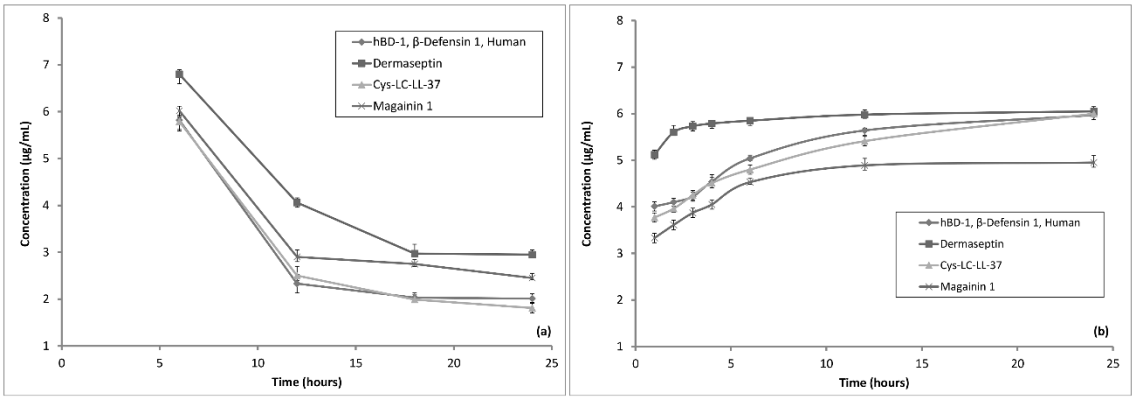

Figure 5 – Results of the assay of Bradford reagent in solution. (a) absorption of the AMPs into functionalized cotton gauzes, (b) release of the AMPs from functionalized cotton gauzes.

Results showed after 6 hours of immersion, there are higher levels of diffusion of the AMPs within functionalized cotton gauzes (figure 5a). Figure 5a also shows that the diffusion of the Dermaseptin in the sample is slower, most probably due to electrostatic repulsion between the Dermaseptin and ALG, both negatively charged. After 24 hour of incubation (immersion of functionalized cotton gauzes in the AMPs solution) the absorption rate is completed for all the peptides and the higher value is approximately 8µg/mL. Figure 5b shows the variation in the release of AMPs over 24 hours. It is noted that the Dermaseptin has a level of faster release. In contrary, Magainin 1 has a slower release. hBD-1, ß-Defensin 1, and Cys-LC-LL-37 both have a gradual level of release over time. We also observed that there was a release of AMPs in solution from 5-6 µg/mL. These values are higher than the values of MIC, which is an advantage, because AMPs quantities high than the MIC values should be released in order to eradicate all bacteria and prevent infection.

For the AMPs release profile, the samples were immersed in phosphate buffered saline (PBS, pH=7.0) at 37 ºC in order to determine the AMPs release from cotton gauze functionalized with CH and ALG. The medium was completely removed periodically, at each sampling time (1, 2, 3, 4, 6, 12 and 24 h) and fresh medium was introduced. The results are given in cumulative release data.

The AMPs release profiles obtained from the functionalized cotton gauze showed a moderate burst effect (45-67%) during the first 6 h, accompanied by a stage of continuous decrease in release rate during the next 18h. The obtained release profile can be beneficial for our application of AMPs-eluting wound dressings.

According to the work of Harrison-Balestra et al. 2003, the onset of an infection, it is crucial to immediately respond to the presence of large numbers of bacteria (Harrison-Balestra, Cazzaniga, Davis & Mertz, 2003). The goal of prophylactic topical antimicrobial therapy is to control microbial colonization and prevent wound infection. Unprotected burn wounds are colonized by bacteria within 12-24 h with microbial levels reported to reach 100 million microbes per gram of tissue within 48h (Loke, Lau, Yong, Khor & Sum, 2000). So, during the first hours of the wound, it is essential to release a relatively high amount of antibacterial compound in order to eliminate various infections that were not eliminated during wound cleansing and might create a resistant biofilm. This work overcomes one of the major limitation regarding the delivery of antimicrobial in a biomaterial model: the effect of burst-release. Burst-release is consistent with an initial high and rapid release of the antimicrobial. It is one of the major challenges of modern drug delivery but after the first hours should continue a low release of the antimicrobial agent to healing wound.

In this work we found that during the first 6 hours there is a high release of AMPs. From figure 5 (a), the absorption of AMPs is approximately 8 μg/mL and from figure 5 (b) the releasing of AMPs is in the range of 5-6 μg/Ml. This implied that about 25% of the absorbed AMPs still remained in the functionalized cotton gauze after 24 hours.

Another important issue is the effect of ionic strength on the releasing of AMPs from the functionalized cotton gauze. LbL film structures are low densely packed so it allows an easy

diffusion of materials through the films. In LbL multilayers constituted of weak polyelectrolytes (like chitosan and alginate) charge ratio changes drastically around their pKa. Furthermore, electrostatically assembled layers are usually destabilized to a certain pH and ionic strength (Sato, Yoshida, Takahashi & Anzai, 2011). Several works in the area of delivery drugs systems using chitosan/alginate as substrate, showed that if ionic strength increased, the difference in concentration of mobile active agent between multilayers of chitosan/alginate and the surrounding media was reduced (Chen, Wu, Mi, Lin, Yu & Sung, 2004; Yang, Chen, Pan, Wan & Wang, 2013; Zhang, Wei, Lv, Wang & Ma, 2011). However, at pH 7.4 (near to pH of wound) the carboxylic acid groups of alginate hydrogel became ionized and the hydrogel can swell more significantly. The amount of active agent released at low pH was relatively low, while that released at high pH increased significantly. As the cotton-based bioactive gauzes are expected to be applied as wound-dressings, the phenomena above described will help in peptide release when infections are higher (higher pH).

In the present work LbL cotton AMPs dressings proved to be able to decrease bacterial presence and are expected to have a similar behavior in the wound bed, thus preventing and treating infection. Consequently, in one application in which is important to have a rapid release Dermaseptin should be chosen. In order to have a gradual release, it should be used the hBD-1, ß-Defensin 1 or Cys-LC-LL-37.

Considering the emerging need for new classes of antimicrobial agents, the AMPs represent a new alternative and may present great advantages: are usually small, have a broad spectrum of action and typically high affinity for membranes of microorganisms, they are generally protease-resistant, they have fast action and limiting the development of resistance by microorganisms.

4. Conclusions

We found that Magainin 1 is the AMP more potent to inhibit *Staphylococcus aureus* growth, because have a lowest MIC value. This AMP have a higher diffusion between the layers of

CH/ALG, probably is due to the small size and the electrostatic attractive forces. On the other hand Dermaseptin has a lower diffusion, so, is incorporated in the surface of sample.

By ATR-FTIR analysis, we found that the AMPs are not bound to CH and ALG, so the AMPs are able to be released, which can be an important issue as a requirement of antimicrobial activity for a wound-dressing.

This study demonstrates that cotton gauze with Cys-LC-LL-37 and with Magainin 1 have a 100 % growth inhibition for *Staphylococcus aureus* and lower cytotoxicity. These results suggest that cotton gauze with Cys-LC-LL-37 and Magainin 1 can be successfully incorporated into layers of CH/ALG and could be used for wound healing applications with minimal cytotoxicity to the surrounding tissue. The AMPs release profile exhibited a fast effect, followed by a decreasing release rate. The release mechanism is based mainly on diffusion through the layers of CH/ALG.

Samples prepared in this study are expected to be useful in biomedicine specially in wound healing. These dressings proved to be able to decrease bacterial presence without cytotoxicity and at very low concentrations and are expected to have a similar behavior in the wound bed, thus preventing and treating infection.

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Chapter 4

General discussion of the results

Coating is an important way to functionalize textiles in order to achieve an antibacterial activity with potential application on medical products. There are several coating processes to obtain bioactive textiles. LbL technique is a simple and versatile method for coating various surfaces by alternate deposition of polyelectrolytes with opposite electrical charges, but LbL is a technique with few applications in textile materials, especially by using natural polyelectrolytes and biomolecules to give bioactive function to natural fibres as cotton.

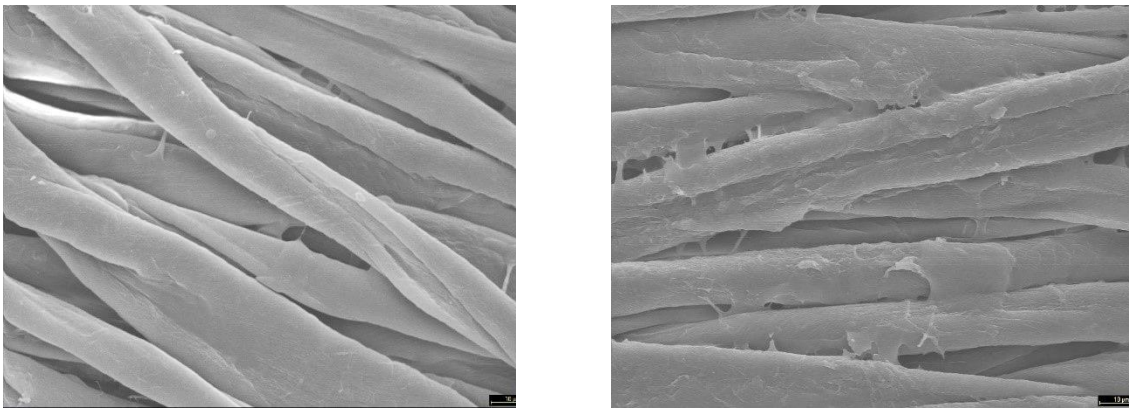
In this work cotton was selected as substrate, CH and ALG as cationic and anionic polyelectrolytes respectively. In addition, L-cys and four different AMPs were incorporated between layers of CH and ALG obtained by LbL over the cotton, to increase antimicrobial activity.

To ensure the success of LbL technique application onto cotton materials, some procedures have to be carried out, in particular:

- Activation of the substrate with TEMPO-oxidation is required to impart negative charges onto cotton surface for LbL assembly application.
- When polyelectrolytes are weak (which is the case of CH and ALG), there are two important parameters to control, in the deposition process: the pH and concentration of polyelectrolyte.

CH and ALG are two oppositely charged natural polyelectrolyte materials and are very sensitive toward changes in pH. The pH was selected to 5, because is approximately intermediate between the pK_a of CH (6.3) and ALG, pK_a of 3.38 and 3.65. At pH 5 the carboxylate group of ALG mainly exists in the form of COO^- and the amino group of CH mainly exists in the form of NH_3^+ . In this case the presence of both COO^- and NH_3^+ along polymer backbone can enhance the electrostatic interaction of the network structure. The CH polyelectrolyte concentration was (1mg/ml) in 0.1 M CH_3COOH solution and for ALG was (1mg/ml) in 0.5 M NaCl. These values of pH and concentration were selected after some preliminary experiments and also because they have been used in several other works, being extensively tested.

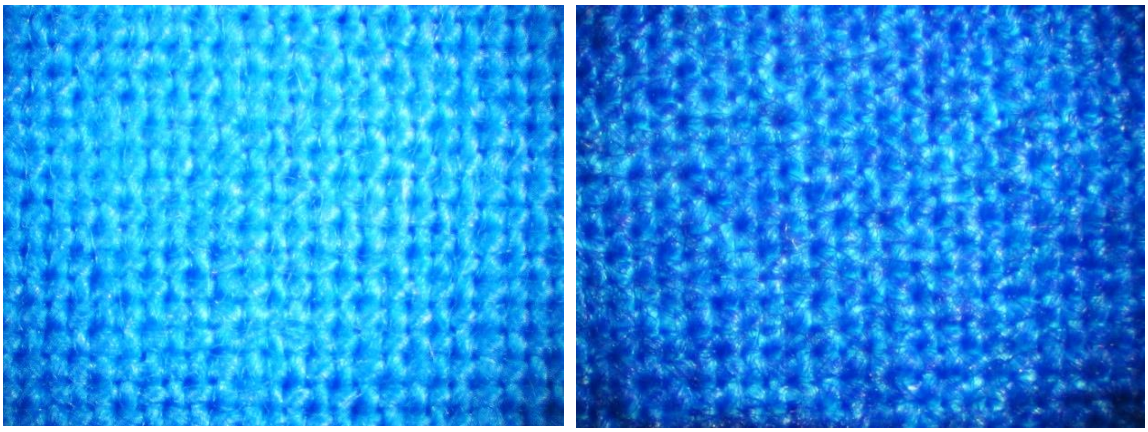
Figure 1a shows a SEM image of cotton and figure 1b shows a SEM image of functionalized cotton with six layers of deposition (Cotton/CH/ALG/CH/ALG/CH/ALG). Looking at the two images it can be seen that the functionalized cotton sample shows a large deposition of polyelectrolytes.



(a) (b)

Figure 1 – SEM images. (a) Cotton sample, (b) Cotton/CH/ALG/CH/ALG/CH/ALG (6 layers of deposition over cotton)

In addition, a cationic dye, methylene blue, was used to evaluate the negative charges on the cotton surface sample and cotton pre-treated with TEMPO. Figure 2a shows an image of cotton sample dyed with methylene blue and figure 2b show an image of cotton sample pre-treated with TEMPO and subsequently dyed with methylene blue. It was found that TEMPO activated cotton has a deeper more intense blue color, as it can be seen in figure 2b. This is due to the cotton sample pre-treated with TEMPO get additional negative charges, so there is a greater absorption of the cationic dye, methylene blue. This would also explain the larger deposition of polyelectrolyte, as show in fig.1b.



(a) (b)

Figure 2 – Methylene blue test. (a) Cotton, (b) Cotton pre-treated with TEMPO.

In the LbL technique the adsorption process is typically dominated by electrostatic attraction. When polyelectrolyte concentration is low, adsorption of polyelectrolytes from a solution onto a solid surface can be considered as a polyelectrolyte interaction with the charged surface. The polyelectrolyte is adsorbed on the charged surface in order to have a compensation of surface charge. In these conditions, the adsorbed amount is large and the layer is dense. Cotton pre-treated with TEMPO is negatively charged, so, when in contact with CH (cationic polyelectrolyte), it adsorbs sufficient quantity, so the cotton surface becomes positively charged. This charge is then reversed when in contact with ALG (anionic polyelectrolyte), the charge is compensated and the cotton surface becomes negatively charged.

Three characterization methods were tested to assess electrostatic LbL deposition. The first was contact angle between a liquid (water) and the sample surface in order to determine the wettability of the sample with different layers of CH and ALG. We found out that the surface wettability is very sensitive to the surface composition of the outermost layer.
Table 1 presents the contact angle of cotton with 5, 6, 9 and 10 layers. Samples where the last layer is CH, a higher contact angle was achieved, showing the higher hydrophobic character of CH, in comparation with ALG, being these results in agreement with the literature.

Table 1 Contact angle

Sample	Description of sample	Contact angle (º)
CT5	Cotton/CH/ALG/CH/ALG/CH	114.6 ± 4.46
CT6	Cotton/CH/ALG/CH/ALG/CH/ALG	67.67 ± 13.10
CT9	Cotton/CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH	114.63 ± 10.13
CT10	Cotton/CH/ALG/CH/ALG/CH/ALG/CH/ALG/CH/ALG	82.65 ± 12.07

Contact angle changes according to the polyelectrolyte of the outermost layer (CH or ALG) (measured at 100 ms).

CT means that cotton was pre-activated with TEMPO

For all samples (see table 1), the contact angle exhibits zigzag feature relatively with last layer indicating the alternate assembly of CH and ALG on the cotton surface. This result indicates the successful application of LbL technique onto cotton. It is also interesting to note that the contact angle value of CT10 is near the contact angle value of CT9, revealing the sequential continuity of LbL process thus showing a small interpenetration of layers.

The successful of LbL application onto cotton was also confirmed by the methylene blue test, as described before, and by ATR-FTIR. LbL technique theoretically covers any surface when it is charged, as widely described in literature, but is still emergent in textile materials. This work

proves the applicability of LbL on cotton samples, besides inherent characteristics related with porosity and fiber heterogeneity.

It was also found that the TEMPO-oxidation can be used on cotton to give negative charge to ensure the success of LbL technique, without introducing changes in cotton fibers.

The functionalized cotton samples with CH/ALG were tested for their antibacterial activity towards *Staphylococcus aureus* and *Klebsiella pneumoniae* using the international standard method JIS L 1902:2002. Figure 3 shows the images of samples tested according with the standard method JIS L 1902:2002 – halo method.

The samples showed a small but clear inhibitory zone for *Staphylococcus aureus* and a smaller halo for the *Klebsiella pneumonia*. However, halo size is not possible to quantify through the images of figure 3. It is interesting to note, that for both bacteria, the control sample (cotton without CH/ALG, designated by CO) showed little bacterial growth inhibition, but significant bacterial growth inhibition is show in the presence of CH/ALG, as expected, since cotton is not an antimicrobial material. The results showed that the antibacterial effect of CH/ALG occurred without migration of CH and ALG, because only bacteria in direct contact with the CH/ALG are inhibited, (discussed in paper III and IV).

In paper IV it was found that the functionalized samples with five layers (CH/ALG/CH/ALG/CH) are more active against *S. aureus* and *K. pneumoniae* microorganisms through the assessment of the bacteriostatic activity.

CH is known in the wound management field for its haemostatic properties and other biological activities that helps in faster wound healing. Wound dressings based on ALG are well described in literature and have an important property in retaining exudate away from the wound bed, a problem in the wound healing since there is a tendency for dressing adherence to the wound surface, and when the dressing is removed, considerable damage is inflicted on the newly formed epithelium. To resolve this problem the ALG gel helps in removing the dressing without much trauma and reduces the pain experienced by the patient during the change of dressing. It also provides a moist environment that leads to rapid granulation and re-epithelization. Systems simultaneously composed of CH and ALG offer the advantages of both materials to wound dressing.

In this context a new method is proposed to improve the performance of the functionalized cotton. L-cys, an antimicrobial agent, was embedded between layers of CH and ALG onto cotton obtained by LbL technique. In this technique, functionalized cotton (cotton/CH/ALG/CH/ALG) was immersed in the L-cys solution and then washed with deionized water, where calcium was added for ALG gelling and finally CH layer was added:

Cotton/CH/ALG/CH/ALG-L-cys-wash (water with $CaCO_3$)/CH,

designated by **LbLCotton-L-cys** (sample 20 in paper V)

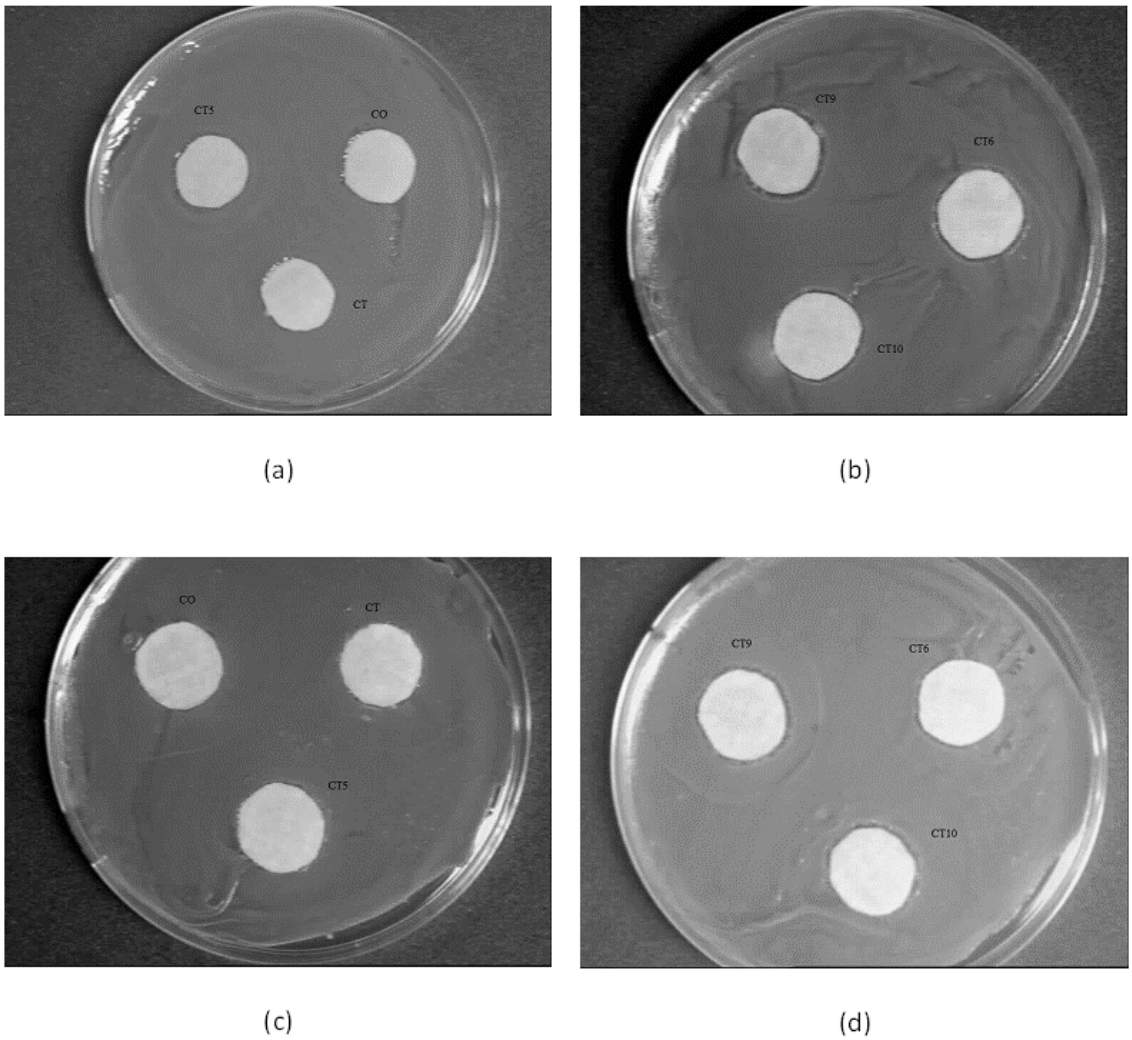


Figure 3 – Images of samples tested according with the standard method JIS L 1902:2002 – halo method. a) and b), inhibition zone against *Staphylococcus aureus* for CO (Cotton), CT5, CT6, CT9 and CT10 samples. c) and d), inhibition zone against *Klebsiella pneumoniae* for CO (Cotton), CT5, CT6, CT9 and CT1O samples. CT is cotton treated with TEMPO and NaBr.

In order to quantify the antibacterial activity JIS L 1902:2002 – absorption method, was used because it allows to assess the bacteriostatic and bactericidal activity level.

Table 2 presents the values of bacteriostatic and bactericidal activity levels for samples without/with L-cys.

Table 2 – Bacteriostatic and bactericidal activity

Sample	S. aureus		K. pneumoniae	
	$M_b - M_c$	$M_a - M_c$	$M_b - M_c$	$M_a - M_c$
Cotton	0	-1.5	0	-1.8
LbLCotton	1.9	-0.5	1.5	-0.4
LbLCotton-L-cys	2.9	-0.1	2.2	-0.2

A-number of inoculated bacteria. B-number of bacteria on the standard sample contacted for 18h. C-number of bacteria on the functionalized sample after incubation for 18h. M_a = log A, M_b = log B, M_c = log C. Bacteriostatic activity level, $M_b - M_c$; bactericidal activity level, $M_a - M_c$.

All samples (except cotton) showed a bacteriostatic activity and no bactericidal activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*. This is in accordance with the results for antibacterial activity by the halo method. In addition, and as expected, LbLCotton-L-cys is the sample that has the highest value of bacteriostatic activity, 2.9 for *S. aureus* and 2.2 for *K. pneumoniae*.

The LbLCotton sample has small bacteriostatic activity level, because this sample has no incorporated biomolecules, as L-cys. Analyzing the results of table 2, it can also be found that the presence of L-cys in the samples increases significantly the bacteriostatic activity level for both strains.

The L-cys molecular structure exhibits biofunctional terminal groups on each side: the thiol (-SH) group on one side and the amino (-NH2) and carboxyl (-COOH) groups on the other side. L-cys is a highly reactive compound, which in the presence of oxygen oxidize to Cystine. Cystine is the amino acid dimmer formed when a pair of L-cys molecules are joined by a disulfide bond through oxidation. Both L-cys and Cistine are important for cell survival and growth.

ATR-FTIR of functionalized cotton with L-cys (paper V) shows the sulfur-hydrogen bond breakage of L-cys, and a new sulfur-sulfur bond appeared (cystine). Depending on the conditions of the medium, dry sample or in solution there is always L-cys and/or Cistine between the layers of CH/ALG.

Wound dressings containing antibiotics have been developed for the inhibition of wound infection. But, during the last decades, the availability of a wide variety of antibiotics and its extensive use to combat infections resulted in a progressive increase in the resistance of strains of pathogenic microorganisms to these compounds, limiting its use. Consequently there is an urgent need to search for alternatives for antibiotics. The AMPs are molecules of the immune system present in most living organisms, which may have potent antimicrobial activity against a broad spectrum of microorganisms and at the same time low or no toxicity to animal cells.

Moreover, in general, the acquisition of resistance of a microorganism to a specific AMPs seems to be very small.

Major infection in skin wounds arise from introduction of bacteria into deep tissue sections. These infections are particularly dangerous, which makes AMPs a great selection due to their diffusivity ability to move into wound deep tissue. Another important property of these AMPs is related to cysteine residues, which promote the formation of disulfide bonds in the molecular structure, making AMPs resistant to proteases, temperature and pH.

Considering AMPs the new antimicrobial agents, and the results from incorporation L-cys (paper V), we have develop antimicrobial textiles based on the incorporation of AMPs into multilayers of CH/ALG, using cotton gauzes as substrate. hBD-1, ß-Defensin-1, Human; Dermaseptin; Cys-LC-LL-37 and Magainin 1 were selected based on the broad spectrum of antimicrobial activity and all properties mentioned above. Each AMPs was incorporated between multilayers of CH/ALG by embedding the functionalized cotton gauze in AMPs solution by the same process of embedding L-cys.

Figure 4 show the growth inhibition (cell reduction) of the *Staphylococcus aureus* and *Klebsiella pneumoniae* by the antibacterial activity of the functionalized cotton gauze (cotton/CH/ALG/CH/ALG/CH) designated LbLCotton; functionalized cotton gauze with L-cys designated LbLCotton-L-cys; and functionalized cotton gauze with AMPs designated LbLCotton-AMPs.

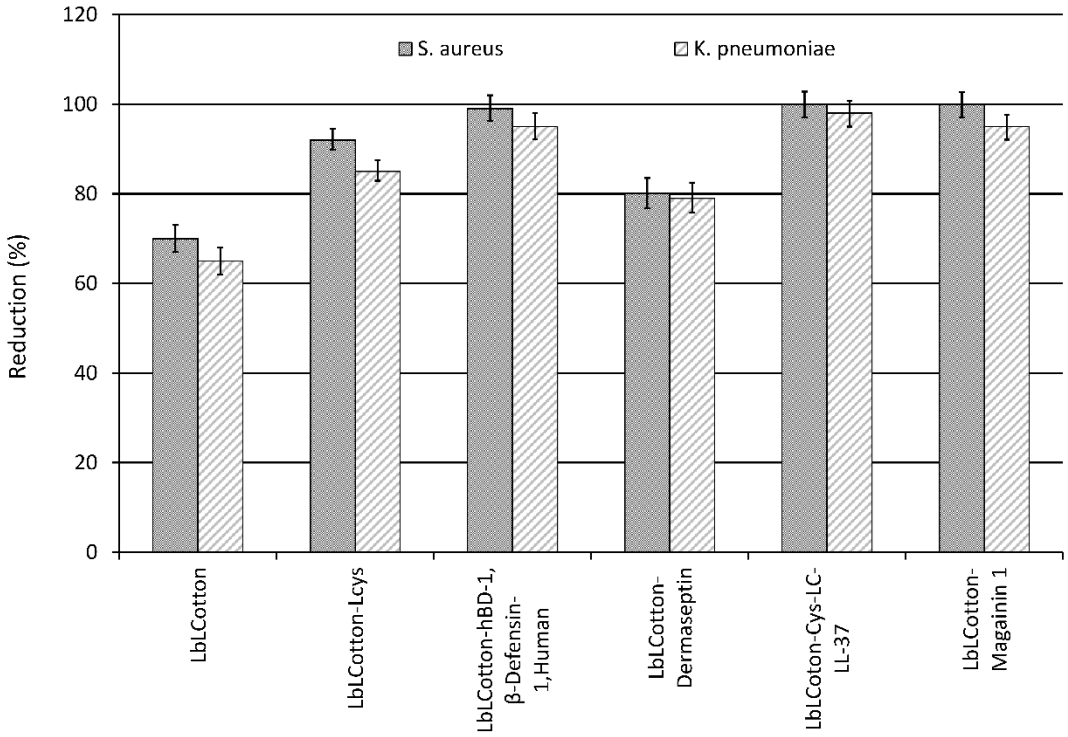


Figure 4 – Reduction rate (%) of *S. aureus* and *K. pneumoniae*

Analyzing the results (fig. 4), LbLCotton sample has the lowest reduction of growth inhibition against the two bacteria. The antibacterial activity is due the CH and ALG, as these are well-known biopolymer with good antibacterial activity. The increase in growth inhibition for sample LbLCotton-L-cys is due to incorporation of L-cys between multilayers. Samples with embedded AMPs: hBD-1, ß-Defensin-1, Human; Cys-LC-LL-37 and Magainin 1, have the highest values of growth inhibition rates, reaching 100%, showing the excellent bactericidal character of the AMPs. An interesting result is seen in sample with Dermaseptin, in which the growth inhibition rate is between the values for control sample (LbLCotton) and samples with others AMPs. This is probably due to repulsion between negative charge of Dermaseptin and negative charge of ALG, which led to the entrance of a less amount of Dermaseptin between multilayers of CH and ALG.

To complete bacterial growth inhibition we have evaluated samples cytotoxicity against mammalian cell. The results (paper VI – Cytotoxicity assay) demonstrated that the selected AMPs can be considered safe to be applied as antimicrobial agents to be in contact with human skin without causing any cutaneous adverse reaction in the concentrations tested (MIC values).

To complete the study and taking into account the potential application of this research work as novel wound-dressings, the release profile of the AMPs was studied over 24 hours. Dermaseptin has a faster level of release, most probably because this AMPs is located on the surface of cotton. Magainin 1 has a slower level of release because it is deeper incorporated into multilayers, as shown by the results (paper VI). In contrast, Cys-LC-LL-37 and hBD-1, ß-Defensin-1 Human have a gradual level of release over time. The AMPs release profiles obtained in solution showed a moderate burst effect (45-65%) during the first 6h, accompanied by a stage of continuous decrease in release rate during the further 18h. This is important, because in the onset of an infection it is crucial an immediately respond to the presence of large numbers of bacteria and subsequent continuous release for several hours. With these functionalized samples, it is ensured that during the first 6 hours a high release is obtained and a continuous release is ensured through the subsequent 24 hours.

Table 3 shows a summary of AMPs characteristics and results.

Hbd-1, ß-Defensin-1, Human; Cys-LC-LL-37 and Magainin 1 have higher % of growth inhibition against the two bacteria, most probably because they are cationic AMPs. It is know that the positive charge of AMPs interact electrostatically with anionic groups at the bacterial cell walls, causing an increase of membrane permeability and subsequent leakage of cellular proteins which ultimately leads to cell death.

Table 3 – Characteristics of AMPs

	Hbd-1, β-Defensin-1, Human	Dermaseptin	Cys-LC-LL-37	Magainin 1
Level of realese	gradual	faster	gradual	slower
Growth inhibition of bacteria (%)	99% *S. aureus* 95% *K. pneumoniae*	80% *S. aureus* 79%*K. pneumoniae*	100% *S. aureus* 98% *K. pneumoniae*	100% *S. aureus* *95% K. pneumoniae*
cytotoxicity (%)	106	94	110	95
Observation	cationic	anionic	cationic	Cationic, more potent to inhibit *S. aureus* growth

It is also very interesting to observe that the values 106% and 110% for cytotoxicity results of Hbd-1, ß-Defensin-1, Human and Cys-LC-LL-37, respectively, clearly indicates that the incorporation of these AMPs will able to stimulate wound vascularization and re-epithelialization of healing skin.

Analyzing the results and characteristics presented in table 3 it can be concluded that all the AMPs have high growth inhibition rates for the two tested bacteria and low cytotoxicity. Thus, AMPs serve a dual role in wound healing: killing bacteria and stimulating complex host repair phenomena with the inherent advantage of the rapidly ability to destroy the target cells. AMPs are characterized by amphipathic molecules, having both a hydrophobic region that interacts with lipids, as a hydrophilic positively charged region, capable of interacting with anionic residues.

The general purpose of this work was achieved, and a new process for the bio-functionalization of cotton with an antimicrobial effect was identified. In addition, the results obtained are very promising for potential application as wound dressings.

Wound infections are caused by the deposition and multiplication of microorganisms in the surgical site of a susceptible host. There are a number of ways microorganisms can get into wounds.

The microorganisms that typically infect wounds and the skin depend on what is present in the environment, the state of the person immune system, and the depth of the wound. The most

common causative organisms associated with wound infections include: *Staphylococcus aureus, Streptococcus pyogenes, Enterococci, E. coli, Klesiella pneumoniae and Pseudomonas aeruginosa*.

Generally wounds have alkaline pH and ALG gel structure opens for alkaline pH, therefore this type of functionalized cotton when in contact with the wound causes the ALG gel structure opening and release of the AMPs to the outside. This is an important property because there is an interaction between the wound and the wound-dressing.

It is imperative that modern wound dressings undergo another revolution with the view of becoming more interactive and smart in nature, whereby they not only absorb exudates, but also interact with the wound to encourage healing and thus a reduction in exudate will ensue.

Conclusion

The first part of this work was dedicated to validate the feasibility of using LbL assembly deposition of natural polyelectrolytes onto cotton, as a novel technique for textile bio-functionalization. It was found that the pre-treatment of the cotton samples with TEMPO is required to create a substrate that is able to support the multilayer films. The successful of application of LbL onto cotton was confirmed by several analysis, in particular by the contact angle between a liquid (water) and the sample surface, dyeing test with methylene blue and ATR-FTIR.

These results abled to confirm the deposition of alternate layers of CH and ALG, indicating the success of LbL technique onto cotton as a novel process to obtain bioactive textiles with potential medical application.

The second part was devoted to find a strategy to have a "reservoir" where it can be loaded bioactive agents for subsequent release. Therefore, a new method was optimized for embedding a bioactive agent between layers of CH and ALG, with success. Moreover, the new method allows to embed the active agents by exposing the functionalized cotton with CH/ALG to an active agent solution to incorporate the bioactive agent into layers of CH and ALG,using the ability of ALG for gelling. In this way, Lcys and AMPs were embedded between multilayers without any covalent bonding with CH and/or ALG with success.

The last part of this thesis was dedicated to evaluate the antimicrobial activity and to assess the biological characterization of this new bio-functional textile. Antibacterial assays were performed against two strains *Staphylococcus aureus* (Gram-positive bacterium) and *Klebsiella pneumoniae* (Gram-negative bacterium) using the international standard method JIS L 1902:2002. Results showed that all AMPs used in this work have shown an antibacterial effect toward both microorganisms and no cytotoxicity to normal human dermal fibroblasts (NHDF) at Minimal Inhibitory Concentration was found. The AMPs release profiles obtained in solution showed a moderate burst effect (45-65%) during the first 6 hours, accompanied by a stage of continuous decrease in release rate during the next 18 hours.

The results showed a promising eco-friendly and simple technique to give bioactive function to textiles with antimicrobial properties using natural polyelectrolytes and bioactive agents. This method can open new avenues towards the development of non-toxic and safe biomedical textiles being highly promising for potential application as wound dressings.

Future perspectives

This thesis describes a new process for the biofunctionalization of cotton-based materials. Some of the suggestions that should be taken into consideration for further investigations are given below.

- The *in vitro* antimicrobial and cytotoxicity of biofunctionalized cotton were evaluate, the next step is to perform *in vivo* tests of biofunctionalized cotton.
- In order to implement this process in the industry and to put this kind of samples in the market it is necessary to request Infarmed authorization.
- As an alternative approach, the LbL technique, should be tested in other natural fibres, such as, wool, silk and bamboo.