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Bioactive microsphere-based coating for biomedical-textiles with encapsulated antimicrobial peptides (AMPs)

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Abstract

In this study a sonochemical method was used to prepare chitosan-based-micro/nanoparticle with incorporated antimicrobial-peptides as a novel antimicrobial coating for cotton-gauzes. Characterization in terms of size, morphology and stability was evaluated and microspheres were approximately 2 µm in-size and were further coated by the layer-by-layer deposition of alginate (Alg) and chitosan (CS). In addition, an antimicrobial-peptide Dermicidin-1-L was incorporated into the microspheres (CS/AMP microspheres) to give more effective antimicrobial activity against a wider range of microorganisms. Results showed a much higher antimicrobial activity of the CS/AMP-microsphere-coated-cotton in comparison with LbL-Alg/CS-microsphere-coating, without cytotoxicity.
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1. Introduction

Recently, several methods depending on the active agent and type of fiber have been developed or are being developed to confer antimicrobial activity to textiles. The antimicrobial agents can be applied to the textile substrates by exhaustion techniques, impregnation, coating, spraying, microencapsulation and foams. However, desired antimicrobial properties related to the effectiveness of these application methods are not always achieved with a high success rate. Thus, new developments and processes are expected around the world [1,2].

The area of functional fibers and technical textiles has encouraged industry to use microencapsulation processes as a mean of transmitting properties and finishes for textiles, which would not be profitable using other techniques. Microcapsules have been used in several areas such as adhesives, cosmetics, pesticides, pharmaceuticals, medicine, food, etc. but they have been more recently introduced in textile area [3-5]. Therefore, controlled release of bioactive substances encapsulated in polymeric microspheres and microcapsules deposited on textiles may be a new strategy that opens new perspectives for applications of textiles.

The sonochemical method developed by Suslick and co-workers [6] can be used to produce micrometer-sized gas-or liquid-filled proteinaceous microspheres from various kinds of proteins. This one-step procedure yields microspheres with a long shelf life and high stability. Ultrasound offers the prospect of an escalation of reaction rates, improved yields, or a better quality product due to the better homogenization of the constituent chemicals [7,8].

In addition, the ability to readily tailor the properties (e.g., size, composition, porosity, stability, surface functionality, colloidal stability) capsules prepared by

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the layer-by-layer (LbL) technique have also attracted particular interest. These capsules allow the introduction of multiple functionalities, thus providing opportunities to engineer a new class of materials with unprecedented structure and function and can be assembled from suite materials, including synthetic and natural polyelectrolytes, nanoparticles and bio-macromolecules. Moreover, various bioactive compounds can be sequestered into the capsule for drug delivery, sensing, or catalysis applications, and capsule surface can be modified to alter the functionality and/or improve the colloidal stability of the capsules [9,10]. The polyelectrolyte multilayer coatings may serve multiple purposes such as stabilizing alginate hydrogels against dissolution in biological environments and providing barrier membranes for alginate hydrogels to slow release of encapsulated drug or biomolecules [11]. On the other hand, many of the antimicrobials used as finishing agents for textile materials are toxic which means that there is a growing public concern about the possible effects of antimicrobial finishes in environmental and biological systems. Thus, the antimicrobial finishing of textiles should be able to destroy undesirable microorganisms, as well as be safe and environmentally friendly [1,2,12]. A wide range of extracts and natural products with antimicrobial properties has been reported during the last years due to the increased multidrug resistance of many human pathogenic microorganisms as well as the appearance of undesirable side-effects of certain antibiotics [12]. In accordance to this, antimicrobial peptides (AMPs) [13-15] and natural bioactive compounds [2,12] have been recently reported as new candidates to overcome the disadvantages of the currently in use antibiotics and synthetic biocides. Consequently, considerable efforts have been expended to exploit the therapeutic potential of AMPs, especially regarding the pharmaceutical industry [14]. Moreover, because of the membrane-disturbing mode of action of most AMPs, there is a reduced likelihood of the acquisition of resistance by bacteria [13-15].

In this way, the association between chitosan and AMPs may be suitable for further enhancing the antimicrobial properties of chitosan and/or other antimicrobial polymers and increase the action against a broader spectrum of microorganisms. Consequently, a new approach that is developed in this study aim to investigate the more suitable conditions for surface functionalization of textile materials with potential use as wound-dressings, with embedded microspheres, capable of providing a controlled antimicrobial action

increased by the AMPs in order to prevent microbial infections.

Therefore, this study aims to investigate the development of chitosan-based microspheres using the sonochemical and LbL techniques in order to produce more stable and resistant microspheres to be used in the functionalization of textile materials. More important, the embedment of an antimicrobial peptide (AMP) was analyzed to assess the increase and effectiveness of the microsphere-based coating as an antimicrobial method for textiles. To authors´ knowledge, this is the first report associating the latest innovation in terms of new biocides, i.e. AMPs and textiles, in particular a new finishing method based on chitosan-AMPs microsphere-coating for textiles as a potential for the design of new and non-toxic bioactive textiles with a high effectiveness of antibacterial activity, showing no cytotoxicity.

2. Experimental

2.1. Materials and reagents

Terephthalic Acid (98%), Sodium Hydroxide (> 98%), Phosphate Buffer pH 7.4, Chitosan (low molecular weight), Alginic Acid sodium salt, Acetic Acid glacial (95%), Dodecane ($\geq$ 90%), Sodium Chloride ($\geq$ 99.5%) was purchased from Sigma-Aldrich.

AMP Dermicidin was chosen due to the anionic charge and the present application in this study, with potential as a wound-dressing. Therefore, Dermcidin DCD – 1L (from AnaSpec) was purchase to Genentec (Brussels).

The textile materials used were 100% cotton gauzes and were selected due to their common use in medical textiles.

2.2. Microorganisms

The microorganisms used in all assays were clinical multiresistant species of *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 4352) as described in the international standard JIS L 1902-2002.

2.3. Methods

2.3.1. Minimal Inhibition Concentration of DMD-1L

The minimal inhibitory concentration (MIC) against multiresistant (clinical) *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 4352) was determined using the broth macrodilution method, as

described by CLSI M7-A6 standard method (International standard CLSI M7-A6). The choice of using clinical species aimed to assess the effectiveness against multiresistant bacteria. According to the guidelines, the minimal inhibitory concentration was determined by serial dilution in Mueller-Hinton Broth (MHB) (Sigma-Aldrich, St. Louis, MO) with concentrations of DCD-1L ranging from 0.2 µg/mL to 8 µg/mL. The inoculums were prepared from fresh overnight liquid cultures and the turbidity was adjusted to 0.5 McFarland (approximately 1x108 CFU/mL) with 0.85% (w/v) NaCl, and then diluted to give a final concentration of 1x10^5 CFU/mL. 1mL of inoculum was added to each tube containing 1 mL of antimicrobial agent in the dilution range prepared from a stock solution of 10 µg/mL. The inoculated macrodilution tubes were incubated for 24 hours (h) at 37 ºC to assess antibacterial activity. Control tubes of the medium, broth and broth with DCD-1L were also incubated.

2.3.2. Preparation of the Chitosan Microspheres

Chitosan microspheres were synthesized sonochemically from aqueous solutions of chitosan. Concentration ranged from 0.20 to 1.0 mg/mL. The n-dodecane was used as the co-solvent to make the emulsion which is necessary for microsphere formation. Temperature ranged from 20, 25, 30, 35 and 40 ºC, and sonication time from 5, 10, 15, 20, 25 and 30 min. sonicated with 32 kHz.

It was also tested an AMP (DCD-1L, 5.0 µg/mL. highest MIC value) dissolved in the CS solution at the optimum conditions described below, in order to evaluate the possibility of incorporating bioactive molecules into the microsphere which, in this case, may provide a higher antimicrobial effect against a larger spectrum of microorganisms.

After the synthesis, the mixture solution was stored at 4ºC during 12 hours to able the separation of all phases (dodecane, microspheres and chitosan or chitosan/AMP solution).

2.3.3. LBL Self-Assembly of Alg/CS layers on Chitosan Microspheres

LbL deposition of polyelectrolytes over the microsphere shell was assessed to evaluate the influence on microsphere stability overtime and in storage conditions. The polyelectrolyte solutions used for alternating deposition of micro layers of Alg/CS on the CS microspheres were prepared at concentrations of 1 mg/mL For the LbL deposition alginate was dissolved

in an aqueous solution of 0.5M sodium chloride as described elsewhere 16 and Chitosan was dissolved in aqueous solution of 0.1 M acetic acid. The pH values were adjusted to 3 using HCl 0.1 M and NaOH 0.1 M solutions.

For the deposition of each layer, 900 µL of polyelectrolyte solution was added to a test-tube containing 100 µL of microsphere suspension and stirred for 10 min on a magnetic stirrer. Afterwards they were washed with 400 µL of deionized water, and stirred on a magnetic stirrer for 5 min. This process was repeated (2-6 layers) using the oppositely charged polyelectrolyte until expected multilayer pattern was obtained, where chitosan was used as polycation and alginate as polyanion. The layer sequence was (Alg/CS)n. as described by Liu and co-workers and illustrated in figure 1 [11].
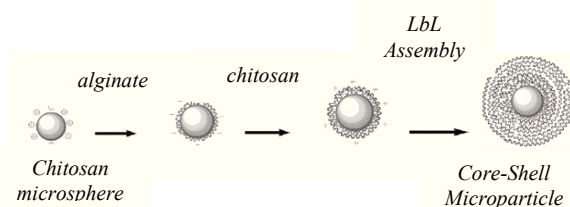

Fig. 1. Chitosan-template polyelectrolyte multilayer microcapsules loaded with Alginate (Adapted from Liu and co-works [11]).

2.3.4. Functionalization of the cotton gauzes

Optimum conditions, namely: 3.9 mL of CS solution (0.8 mg/L) and 2.6 mL of n-dodecane or 5 µg/mL AMP dissolved in the CS solution, in the case of CS-AMP microspheres, were used to produce and bind simultaneously the microspheres onto textile materials (50 mg), which were immersed in the solution, before sonication, and sonicated for 10 min at 37 ºC.

For the case of the LbL CS/Alg coated CS microspheres, the cotton gauzes (50 mg) were immersed into a test-tube with microsphere suspension prepared in the above described optimum conditions, in a proportion to obtain a solution volume of 6.5 mL (5.85 mL polyelectrolyte and 0.65 mL microspheres) and was sonicated with 32 kHz for 10 min at 37 ºC.

2.3.5. Characterization of microspheres

The size and the morphology of the microspheres were observed under a HITACHI S2700 scanning electron microscopy (SEM) and an inverted microscopy OLYMPUS CKX 41. SEM samples were prepared by the application of a drop of microspheres suspension onto the glass and then dried at room temperature (20 ±

2 ºC); which were further coated with a thin layer of sputtered gold in a gold EMITECH-K550 evaporator. The samples for the inverted microscopy were prepared by the application of a drop of microspheres suspension in a plate and observed under a microscope.

2.3.6. Measurement of the Stability of Chitosan Microspheres and LBL Chitosan Microspheres

The stability of the chitosan microspheres, CS/AMP microspheres and LbL Alg/CS coated chitosan microspheres, was assessed by following the changes in the microspheres which were monitored under different temperatures (-5, 5, 20, 35, 37 and 40 ºC), taking into account the possible conditions of storage (-5, 5 and 20 ºC) and the possible contact with human body (37 and 40 ºC), in case of wound-dressings. Stability to pH (4.5, 7 and 9.5) was also evaluated due to the different pH of the skin. Stability in all conditions was monitored for 30 days.

2.3.7. Assessment of antibacterial activity by the JIS L 1902-2002 Halo-method

Antibacterial activity of LbL2-Alg/CS and CS/AMP microspheres, the more stable microspheres produced, as well as controls (unfunctionalized cotton gauzes), was tested against two bacterial multiresistant clinical strains - a Gram positive strain *Staphylococcus aureus* (ATCC 6538) and a Gram negative strain *Klebsiella pneumoniae* (ATCC 4352), adapted from the Japanese Industrial Standard JIS L Standard 1902:2002 (Testing for antibacterial activity and efficacy on textile products).

Bacterial inoculums were prepared from an overnight liquid culture in Nutrient Broth and incubated at 110 rpm, 37 ºC for 24 h. Bacterial concentrations were then adjusted to 1-2 x 10^8 cel/mL (equivalent to 0.5 McFarland) and working standards were prepared to a final concentration of 1 ± 0.3 x 10^5 CFU/mL. The cotton samples coated with the different microspheres were placed in a 50 mL Falcon tube and 200 µL of the working standards previously prepared was added. The T_{24h} tubes were incubated for 24 h at 37 ºC and the T_{0h} samples were analyzed. To release the bacterial cells from the cotton samples, before and after the 24 hours incubation period, 20 mL of 0.85% NaCl with surfactant Tween80 (0.2 % (v/v)) was added to the samples in 50 mL The resulting suspensions were diluted in sterile 0.85 % sodium chloride solution (1:10; 1:100; 1:1000) and plated to determine the viable counts. The plates were incubated at 37 ºC for 24 h, and

the number of colonies was determined. This procedure was performed in triplicate. The growth reduction rate of the bacteria was calculated using the equation (Eq. 1)

$$\frac{T_{0h} - T_{24h}}{T_{0h}} \times 100\,\% = reduction\ rate\ (\%) \quad (1)$$

where, T_{0h} is the CFU/mL of bacterial colonies at the initial stage (0h) and T_{24h} is the CFU/mL of bacterial colonies after 24 h incubation.

2.3.8. Evaluation of cytotoxicity

This procedure was performed according to E DIN EN ISO 10993-5. Initially, it was prepared a perspiration extract of the CS/AMPs bioactive microsphere-coated cotton gauzes, which presented the major inhibition of bacterial growth. The bioactive material was incubated with an acid perspiration solution, for 24 h at 37 ºC under slight shaking. The resulting perspiration extract was set up to a pH value of 7.3–7.4 with sodium hydroxide and filtrated in sterile conditions. A cell culture of Connective tissue cells L 929 [ATCC No. CCL1, NCTC clone 929 L (DSMZ), was treated for 68–72 h with the perspiration extract diluted about 33.3% – 4.4%. To confirm the validity of the test system, controls were carried out along the experiment. Control tests made included: solvent control (phosphate buffered solution diluted in culture medium corresponding to the test material), positive control (5% DMSO in culture medium) and negative control (culture medium). Then, the test group of the material presented concentrations of the test material in culture medium of 4.4%, 6.6%, 9.9%, 14.8%, 22.2%, and 33.3%. After the incubation period, the protein content of the test groups cultures (Culture medium: DMEM with 10% FBS) was compared with the protein content of the controls and from that the cell growth was determined in the presence of the test material. In the presence of cell-toxic substances there is a modified proliferation and partition rates of the cells (growth inhibition test).

3. Results and discussion

3.1. Determination of the minimal inhibitory concentration of DCD-1L

The results showed a MIC for *Klebsiella pneumoniae* of 2.92 µg/mL and for *Staphylococcus aureus* of 5.00 µg/mL. Therefore, the 5.00 µg/mL (5x10-5% (w/v) concentration was used in the preparation of the CS/AMPs microspheres.

The different MIC for the two strains can be due to the fact that most anionic and amphipathic antimicrobials act easier against Gram negative bacteria (*K. pneumoniae*) because of the composition of Gram negative outer membrane, most possible through hydrophobic interaction with the phospholipids of the membrane.

The results are as expected very important since these MIC values (5×10^{-5} % (w/v) in the case *K. pneumoniae*) are significantly lower in comparison with others used as antimicrobial agents for textiles, as for example for chitosan (0.05% (w/v) against *S. aureus* and *K. pneumoniae*) and for triclosan (6 % (w/v) against *S. aureus*) [16-17].

3.1.1. Production and characterization of the Chitosan Microspheres

Chitosan microspheres were prepared by sonication (Ultrasons-H, Selecta) in optimal conditions at frequency of the 32 kHz, for 10 min. The optimum conditions were found to be, 3.9 mL of chitosan 0.8 mg/mL dissolved in aqueous solution of 1 M acetic acid placed in a test-tube with 2.6 mL dodecane, to give a total volume of 6.5 mL, which was also found to be the best amount in the equipment used (data not shown). The results showed that at temperature of 37 ºC sonicated for 10 min, occurred the higher formation of CS microspheres, i.e., ± 1 mL. With regards with the sonication time, results showed that the best time was 10 min, where microspheres resulted stable for many hours.

In the above described conditions, the CS microspheres have spherical shape and a size about 2 µm, as it can be seen in the inverted microscopy images and SEM images presented in figure 2. Thus they have the shape and size that are in accordance with the previous results obtained by Shao and co-workers [10] with the advantage of being obtained in a much faster and simple method than the reported by Agnihotri and co-workers[18] who studied the preparation of chitosan nanoparticles by emulsion cross-linking or by Shao and co-workers [10] whereas ultrasonication required 30 min to generate monodisperse CS microspheres with 1 µm size and spherical shape, still a little more time in comparison of the 3 minutes reposted by Shimanovih and co-workers [4], probably due to the difference in using BSA instead of CS, that can provide protein crosslinking through SS bonds.

Therefore, in comparison with other methods which used CS as the primer polymer for the microsphere shell formation, the results obtained are promising since they show microspheres with size ± 2 µm through a simple and fast process (only 10 minutes sonication).
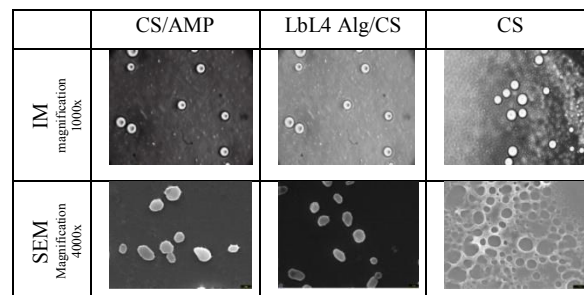

Fig. 2. Micrographs of the different produced microspheres (IM-inverted microscopy and SEM).

3.1.2. LbL deposition of Alg/CS nanolayers on Chitosan Microspheres

The morphology of microspheres with deposition of Alg/CS layers was characterized by inverted and SEM. Inverted microscope images show that it appears that the microspheres of chitosan with LBL4 (Alg/CS) are, as well, spherical and the layers are deposited on the shell of the CS microspheres as shown in the figure 3. Observing the SEM images (figure 3) it appears that the microspheres with LbL layers have spherical shape and monodispersitivity of about 1 µm in size, although it was also visible some microspheres with non-spherical shape most probably because the microsphere cores were totally removed when completely dried before testing in SEM analysis, resulting in microcapsules with shrunken surfaces as reported by Xie and co-workers [19].
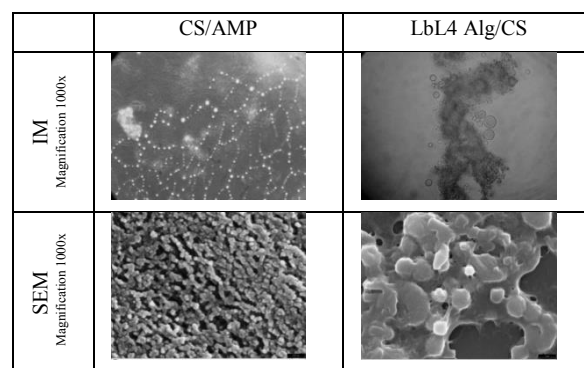

Fig. 3. Images of the cross-linking and agglomeration effect of the LbL6 Alg/CS microspheres (IM- inverted microscopy and SEM).

Chitosan microspheres with LbL (Alg/CS)6 presented some problems such as cross-linking, as visible in figure 3 due to the agglomeration promoted by the solutions of CS and Alg for the LbL deposition. However, cross-linking of chitosan microspheres was expected, in this case, because it's in agreement with the results obtained by Mei and co-workers [20]. CS microspheres took the shape of irregular long strips interconnecting with each other, which is typical for macromolecular structure because of the straight chain. The agglomeration observed is in agreement with results of Genç and co-workers [21].

The results show the success of the LbL assembly of the polyelectrolytes on the CS microsphere shell as expected, increasing the thickness of the membrane and the stability of the microspheres, as shown in the stability assays discussed below.

3.1.3. Chitosan Microspheres with AMPs

Chitosan microspheres with AMP DCD-1L were sonicated using the same optimal conditions studied for CS microspheres.

The images acquired of CS/AMP microspheres with AMPs in the inverted microscope and SEM are shown in figure 2. The microspheres present a spherical shape and monodispersivity, being in agreement with the previous results where CS solutions were used instead of CS/AMP solution, because they present the same size (2 µm). Pedro and co-workers [22] studied the entrapping of citronella oil in chitosan microspheres produced by the emulsions technique, with different particles sizes, ranging from 11 ± 3 µm to 225 ± 24 µm. In addition, it was also reported that the smallest microparticles showed the biggest release rate, most probably because they have a larger specific surface area, causing the oil release rate to be faster. Therefore, for certain applications in which a smaller size might be required, this sonication process reveals to be very effective.

3.2. Stability of the microspheres

3.2.1. CS microspheres

In all the preliminary assays for the production of CS microspheres, they have shown very low stability. For the different concentrations, temperatures and sonication time, microspheres were stable for only several hours, in the solution (data not shown). In contrast, the microspheres produced under the optimum conditions (at frequency of the 32 kHz, for 10 min, the

optimum conditions were 3.9 mL of chitosan 0.8 mg/mL dissolved in aqueous solution of 1 M acetic acid placed in a test-tube with 2.6 mL dodecane, to give a total volume of 6.5 mL) are very stable in solution, at all pH and all temperature studied for at least 1 month. However, at pH 7 and temperature of 40 ºC they present lower stability (only some hours). This is in agreement with previous studies performed by Avivi and Gedanken [23]. The microspheres have to stay in solution in order to last several weeks, otherwise, as for example in dry air, they break out very easily (in few hours).

3.2.2. Microspheres with multilayers of Alg/CS and AMP/CS microspheres

The microspheres with 4 and 6 layers of Alg/CS showed high stability at all pH and temperatures for at least 1 month, in solution being in accordance with the stability studies reported by Shao and co-workers [10]. In the case of 6 layers of Alg/CS deposition, it was however observed some interconnection and agglomeration of the microspheres, as is visible in figure 3. Therefore, LbL Alg/CS4, with 4 layers of LbL Alg/CS were further investigated concerning the antibacterial activity.

Moreover, the microspheres with LbL Alg/CS layers and the CS/AMP microspheres do not break out for one week, when exposed to dry air, which is in agreement with the study of Shao and co-workers [10] being much more stable than the microspheres of chitosan that only last in moisture conditions. In the case of the CS/AMPs this might be due to the ionic binding through the amino and carboxylic groups of the CS and of the peptide, since no LbL deposition was made in this case. In addition, disulfide bonds may occur between the AMP molecules, as described for L-cysteine-BSA microspheres Gouveia [5] giving a higher stability to the structure, comparable to the LbL coated CS microspheres.

All these results are in agreement with the expected and the LbL deposition has shown to be fundamental to provide microsphere´s stability.

3.2.3. Microspheres-based Coating for Textile Materials

The adhesion of the microspheres onto the fibers was evaluated by optical microscopy. Figure 4 shows the AMP/CS microspheres adhered to the fibers tested. Adhesion is visible most probably due to the surface

structure of the fiber and the negative charge exhibit by these cellulosic fibers, especially because the outer layer is composed by CS which has an opposite charge to the fiber and may enhance electrostatic interaction.
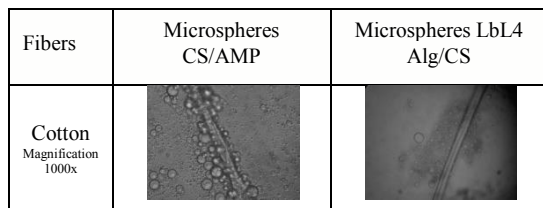
Fibers	Microspheres CS/AMP	Microspheres LbL4 Alg/CS
Cotton Magnification 1000x		

Fig. 4. Images of microsphere-based coating on the cotton fibers.

3.2.4. Antibacterial activity

In this work, the antibacterial efficacy of the LbL Alg/CS multilayer's and CS/AMPs coated cotton samples was assessed against multiresistant bacteria by the evaluation of bacterial activity according with qualitative method JIS L 1902-2002.

The Control samples (cotton gauze without microspheres) did not present any inhibition, as expected (see Table 1). Quite different were the results presented by the samples coated with Alg/CS multilayers in chitosan microspheres, since inhibition rates of 28.33% and 29.00% were obtained against *K. pneumoniae* and *S. aureus*, respectively, an antibacterial effect comparable with the qualitative results given by Shimanovih and co-workers [4] since no quantitative average was provided.

On the other hand, samples treated with chitosan microspheres produced with AMPs showed significant higher inhibition rates against the test specimens revealing that the sonication process and the embedding into CS microspheres doesn't alter the peptide activity and that a major antibacterial activity is achieved in comparison with LbL-Alg/CS microspheres coating of the cotton gauzes. Rates were 75.33% and 99.86% against *K. pneumoniae* and *S. aureus* multiresistant species, respectively, being in agreement with the fact that there is a greater inhibition growth in the case of *K. pneumoniae*, as shown by the MIC values. This is in accordance with the expected due to the release of the AMP, in this case DCD-1L, that is entrapped in the CS layer of the microspheres being released over time, increasing the antibacterial effect of the bioactive textile, in comparison with LbL Alg/CS microsphere-based coating. These inhibition rates are very high and target multiresistant bacteria with success avoiding their natural growth when in close contact with this

novel CS/AMP microsphere coated gauzes, as it is expected in the case of new biocides as AMPs [14]. Results of these samples tested against *K. pneumoniae* and *S. aureus* species can be observed in table 1.

Table 1. Antimicrobial activity of the LbL Alg/CS and CS/AMP coated cotton samples.

	control	LbL4 Alg/CS	CS/AMP
S. aureus reduction rate (%)	0	28.33	75.33
K. pneumoniae reduction rate (%)	0	29.00	99.86

3.2.5. Cytotoxicity analysis

The perspiration extract of the test material (Cotton gauze coated with CS/AMPs microspheres) showed a growth inhibition of 20 % in the cytotoxicity test under the mentioned conditions (see Table 2). A growth inhibition of more than 30% in comparison with the solvent control is assessed as a clear cell-toxic effect. Therefore, the cytotoxicity test for these novel bioactive cotton gauzes revealed less than 30% of cellular viability reduction, making CS/AMP-microspheres a safe antimicrobial agent.

Table 2. Cytotoxic effect of the CS/AMP coated cotton samples.

	Average of perspiration extracts	Standard deviation	Growth Inhibition (%)
Blanc	0.1902	± 0.0082	
Positive control	0.1935	± 0.0310	99
Negative control	1.3504	± 0.0253	0
Solvent control	1.3604	± 0.0405	0
Test material AMP/CS cotton	1.2707	± 0.0502	20

In accordance to this, it can be concluded that no cytotoxic substances are released from the functionalized novel CS/AMP microsphere-based coating process avoiding the risk of irritations with skin contact. Moreover, despite some emerging reports in this area [2-3], none of them clearly indicated a safe and non-toxic effect.

Conclusions

This work describes a novel method to give a CS/AMPs antimicrobial microsphere-based coating for cotton gauzes using non-toxic and biodegradable agents. To the best of authors´ knowledge, this is the first report on the simultaneous formation and coating of textiles through a single-step sonochemical method, incorporating AMPs and giving antibacterial effect against clinical multiresistant bacteria without cytotoxicity.

The major advantages of this method in comparison with other techniques that are commonly used to incorporate microspheres/microcapsules onto textile materials, are the non-toxicity both to the potential users and to the environment, and the possibility of being carried out in a simple step process with short reaction time and without using cross-linking agents such glutaraldehyde or epoxy resins that are normally required to produce microspheres or to bind them onto the textile materials. The other advantage is the incorporation of antimicrobial agents such as AMPs, effective against a wide range of microorganisms, inclusive multiresistant bacteria where other agents failed, and which is a major issue for medical textiles.

Consequently, this can be a very promising strategy that may open new avenues for the design of *in situ* textile-based antimicrobial delivery systems for skin-contact interaction.

Acknowledgements

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