

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



Frederico Nogueira^{a,b}, Luíza Granadeiro^a, Claudia Mouro^b, Isabel C. Gouveia^{b,*}

^a CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Portugal

^b FibEnTech – Fiber Materials and Environmental Technologies, University of Beira Interior, Portugal

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ABSTRACT

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

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1. Introduction

Silk, produced by silkworm *Bombyx mori*, consists of fibroin and sericin. Fibroin, which is the structural protein of silk fibers, comes out from the silkworm gland in the form of filaments enclosed in a water-soluble sericin glue-like coating [1]. Silk fibroin (SF) is composed of heavy (350 kDa) and light chain (25 kDa) polypeptides linked by a disulfide bond [2,3]. Its structure is mainly composed by the amino acids glycine (46%), alanine (29%), and serine (12%), which are amino acids having small side chains. Also, SF presents tyrosine (4.8%), which has an aromatic side chain [4]. They form flexible and stretching resistant secondary structure beta-sheets, which render silk its characteristic mechanical strength [5–7]. Silk is non-toxic, non-immunogenic, biocompatible, and hydrophilic. Although it is stated that the itching of AD is exacerbated by spiky

fibers of wool and by the wild texture of cotton [8,9], silk, on the other hand, with its extremely smooth and its comfortable properties reduces these effects and therefore has been increasingly researched for applications related to atopic dermatitis (AD). Furthermore, Sugihara et al. [10] postulated that wounds treated with a silk dressing healed 7 days faster, by promoting the enhanced synthesis of collagen and epithelialization [10].

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

* Corresponding author at: FibEnTech R&D – Fiber Materials and Environmental Technologies, University of Beira Interior, Rua Marquês d'Ávila e Bolama, 6201-001 Covilhã, Portugal.

E-mail address: igouveia@ubi.pt (I.C. Gouveia).

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

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

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

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

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

2. Materials and methods

2.1. Materials

Silk was obtained from the cocoons of larvae of the silkworm *B. morii* (Brazil). Silk was knitted at a rectilinear needle loom with 7 Picks per inch, with a Jersey knit structure and a linear density of 21.68 number metric (Nm). Its sericin was removed with dichloromethane in a soxhlet apparatus. Silk fibroin (SF) was then functionalized in a L-Cysteine (L-Cys) (Sigma–Aldrich) solution (ddH₂O) at pH's 5.5 and 9.5. Covalent bonds at tyrosine (Tyr) were performed with NaCNBH₃ dissolved in NaOH:HCl buffers (Panreac). *S. aureus* (ATCC 6538) strain was grown on Nutrient Agar (NA) and Nutrient Broth (NB) (Panreac).

2.2. Methods

2.2.1. Minimal inhibitory concentration (MIC) evaluation

Minimal inhibitory concentration (MIC) determination was performed with the microdilution method in accordance with M07-A6

guidelines applied to *S. aureus* (ATCC 6538) – Standard JIS L 1902:2002. Briefly, L-Cys was serially diluted in Mueller-Hinton Broth (MHB) (Sigma–Aldrich) to yield concentrations from 0.3 to 20 mg/mL. Overnight liquid *S. aureus* cultures were turbidity-adjusted to 0.5 McFarland (1×10^7 CFU/mL for *S. aureus*) with sterile H₂O, from which 500 μ L were blended with 4500 μ L MHB, forming the bacterial work suspension. 50 μ L of work suspensions – L-Cys dilutions and stock solution – were added into 96-multi well plate. Samples were incubated for 24 h at 37 °C. Deposited microorganisms (dot-shaped) in the bottom of each well were examined. The last well in the dilution series that showed deposit (microbial killing) corresponded to MIC of the antimicrobial agent. Triplicates were used.

2.2.2. Antioxidant activity: determination of DPPH

(2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

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

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

Efficient concentration (EC₅₀ μ g/mL) was defined as substrate concentration to produce 50% reduction of the DPPH°.

2.2.3. Tumoral activity assay

The cytotoxicity of L-Cys was performed, adapting the procedures from Standard E DIN EN ISO 10993-5. It was selected the concentration of L-Cys chosen for this work, 1%, and its half 0.5%, on breast cancer cell line (NCI-PBCF-HTB22 (ATCC® HTB-22™)). Incubation was done at 37 °C for 48 h under a humidified atmosphere containing 5% CO₂. MTT viability assay was performed. Solvent, positive and negative controls were included.

2.2.4. Silk substrate preparation

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

2.2.4.2. Grafting/functionalization processes. SF knitted fabric samples were functionalized by means of an exhaustion process at 90 °C, 15 rpm, in a Mathis-BFA12 finishing machine. Single covalent strategy (SF-g-L-Cys): samples were immersed in a 1% over the weight of the fiber (owf) L-Cys solution (ddH₂O) adjusted to pH's 5.5 and 9.5, for 3 h. Fig. 1 A. Double covalent strategy (SF-g-L-Cys/Tyr-g-L-Cys): samples were immersed in a 1% over the weight of the fiber (owf) L-Cys solution (ddH₂O) adjusted to pH's 5.5 and 9.5, for 3 h, and were added 0.3 (w/V) NaCNBH₃ solution from the beginning of second hour. Samples were rinsed three times with ddH₂O and dried at 30 °C (Fig. 1B).

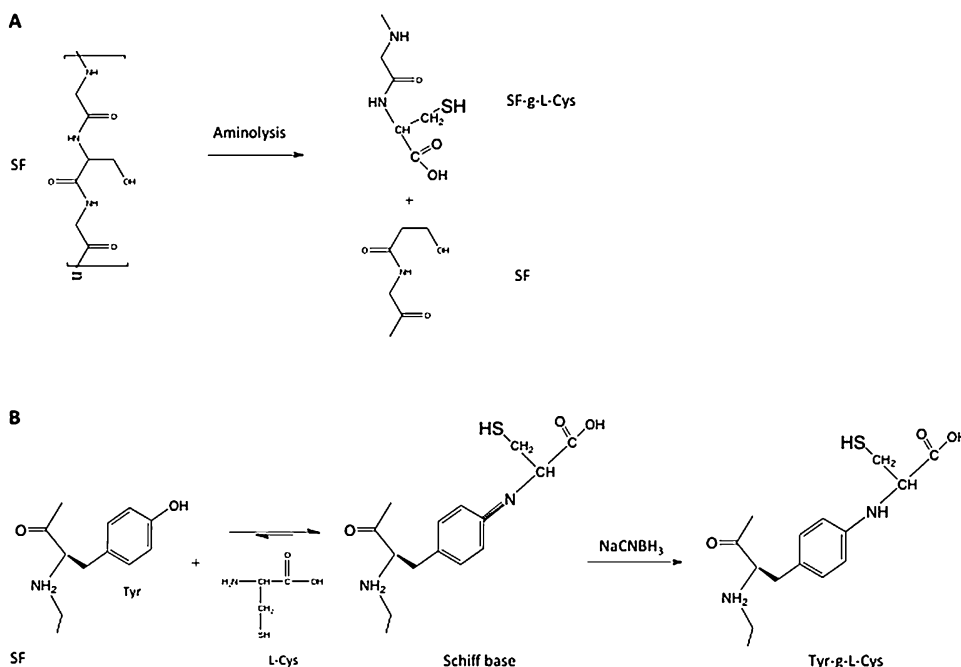


Fig. 1. Strategies for the activation of silk fibroin. (A) SF-g-L-Cys; (B) SF-g-L-Cys/Tyr-g-L-Cys.

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

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

2.2.5. Grafted-silk characterization

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

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

2.2.5.2. Fourier transform infrared spectroscopy (FT-IR). The chemical composition of SF, SF-g-L-Cys, and SF-g-L-Cys/Tyr-g-L-Cys was analyzed by means of FT-IR. After three rinses (no washing cycles), 5 washing cycles, and 25 washing cycles FT-IR spectra were analyzed. Measurements were done with a Thermo-Nicolet iS10 FTIR spectrophotometer. Each sample was scanned 64 times, with a spatial frequency resolution of 4 cm⁻¹.

2.2.5.3. Exhaustion rates: degree of functionalization. In order to assess the efficiency of the functionalization, i.e. the quantity of L-Cys that the substrate SF was able to crosslink, exhaustion rate of functionalization assays were performed for each condition. Ellman's reagent, (5,5'-dithio-bis-(2-nitrobenzoic acid/DTNB) (Sigma)

produces a yellow color when bonded to free thiol groups in solution. After reacting with free thiol groups (of L-Cys) there is formation of a disulphydic compound and a colored species – 2-nitro-5-thiobenzoic acid (TNB). This colorimetric assay was performed by measuring and comparing the functionalization solution before and after functionalization, after doing a calibration with known concentrations of L-Cys. For each sample 250 µL was added to a test tube containing 2.5 mL of sodium phosphate (pH 8) and 50 µL of Ellman's reagent. Test tubes were vortexed and were incubated for 15 minutes before their absorbance being read at 412 nm.

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

2.2.6. Antimicrobial activity assessment

The antimicrobial behavior of SF samples functionalized with L-Cys (-g-L-Cys and -g-L-Cys/Tyr-g-L-Cys) was tested according to Japanese Industrial Standard JIS L 1902:2002, specific for textile samples. Gram-positive *S. aureus* (ATCC 6538) strain was chosen once it plays an important role in atopic dermatitis (AD). Briefly, inoculum of *S. aureus* was prepared with a final concentration of 1–5 × 10⁶, bacteria/mL. 200 µL of *S. aureus* were inoculated on 0.4 g square samples. Samples were incubated for 24 h. Antimicrobial activity was assessed by a quantitative method to determine the percentage of bacterial growth inhibition (% of Inhibition) at 24 hours:

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

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

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

Concentration $\mu\text{g/mL}$	% of remaining [DPPH]
0	100
0.05	8.459185758
0.04	47.30720708
0.03	48.37492768
0.02	76.5334419
0.1	4.770788268
EC50	0.029957282

2.2.7. Scanning electron microscopy (SEM)

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

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

2.2.8. Transmission electron microscopy (TEM)

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

2.2.9. Statistical analysis

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

3. Results

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

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

3.2. Antioxidant activity

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

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

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

3.3. Tumoral activity assay

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

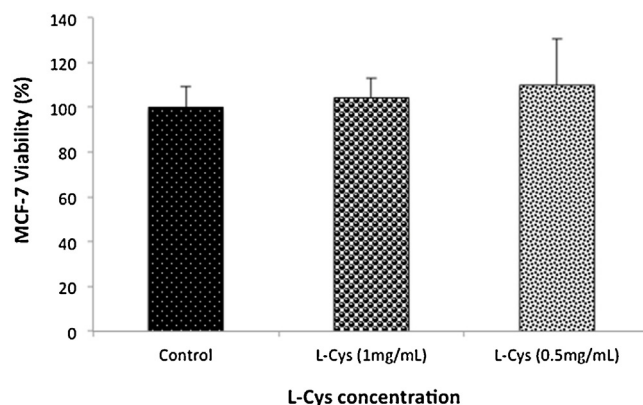


Fig. 2. MCF-7 breast cancer cell line viability percentage when in contact with two concentrations of L-Cys and the untreated controls.

3.4. Silk characterization

3.4.1. Contact angle

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

3.4.2. Fourier transform infrared spectroscopy (FT-IR)

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

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

3.4.3. Efficiency of functionalization/grafting

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

3.4.4. Tensile strength assays

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

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

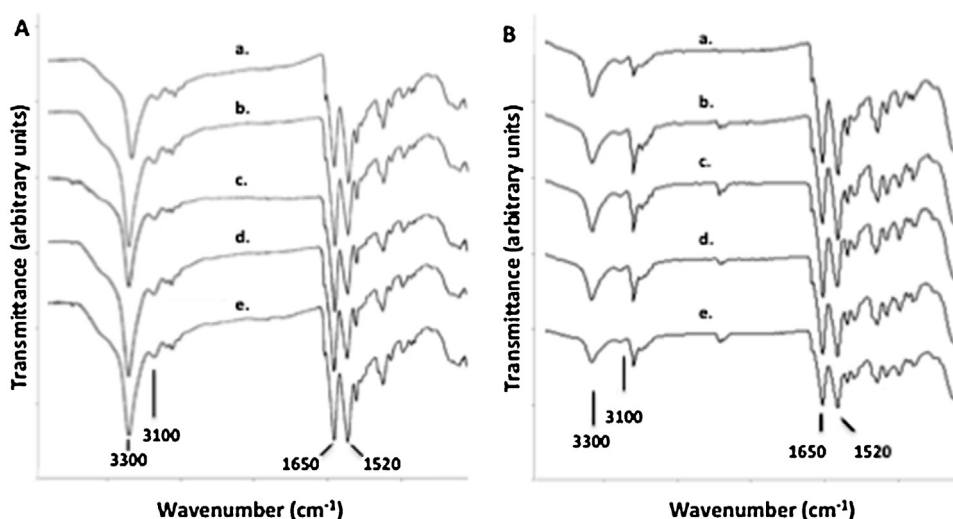


Fig. 3. IR spectra of unmodified (a) and modified silk fibroin with different parameters of surface-activation after (A) 0 washing-cycles and (B) 25 washing-cycles, with 1% owf L-Cys functionalization. (b) SF-g-L-Cys at pH 5.5 90 °C; (c) SF-g-L-Cys at pH 9.5 90 °C; (d) SF-g-L-Cys/Tyr-g-L-Cys at pH 5.5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

Table 2

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

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

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

3.5. Antimicrobial assays

3.5.1. Quantitative method (suspension)

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

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

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

3.6. Scanning electron microscopy (SEM)

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

3.7. Transmission electron microscopy (TEM)

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

4. Discussion

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

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

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

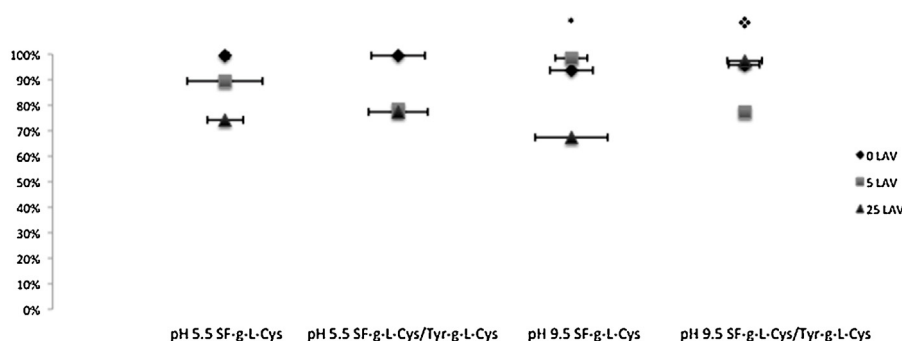


Fig. 4. Percentage of microbial inhibition against *S. aureus* at 24 h – all of these values were obtained by comparison with results of non-functionalized silk fibroin. * statistically significant for 5 washing cycles. ◆ statistically significant for 25 washing cycles ($p < 0.05$).

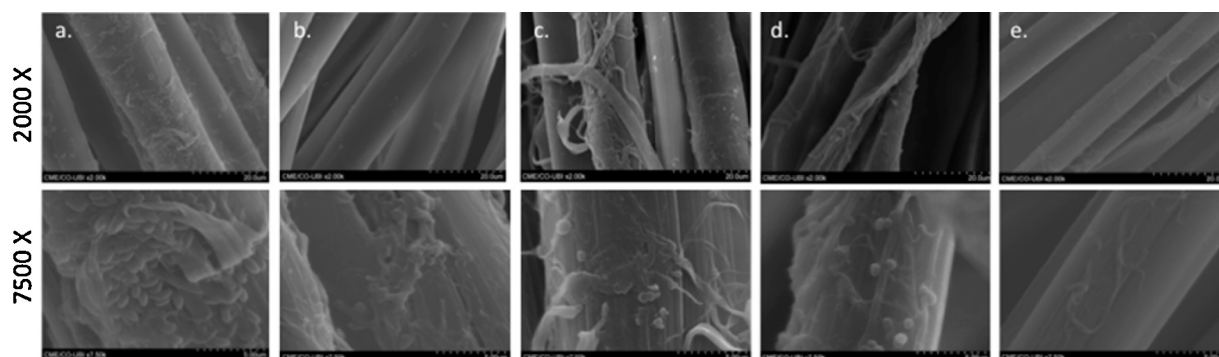


Fig. 5. SEM images of adsorbed *S. aureus* on silk fibroin surface at different conditions. (a) Control; (b) SF-g-L-Cys at pH 5.5 90 °C; (c) SF-g-L-Cys/Tyr-g-L-Cys at pH 5.5 90 °C; (d) SF-g-L-Cys at pH 9.5 90 °C; (e) SF-g-L-Cys/Tyr-g-L-Cys at pH 9.5 90 °C.

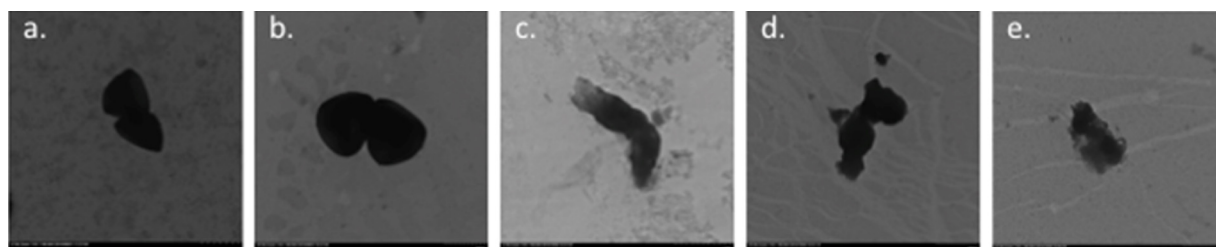


Fig. 6. TEM images of detached *S. aureus* on silk fibroin surface at different conditions. Magnification of 10 000 \times . (a) Control; (b) SF-g-L-Cys at pH 5.5 90 °C; (c) SF-g-L-Cys/Tyr-g-L-Cys at pH 5.5 90 °C; (d) SF-g-L-Cys at pH 9.5 90 °C; (e) SF-g-L-Cys/Tyr-g-L-Cys at pH 9.5 90 °C.

esterification and “saponification”, respectively. Also, the presence of further carboxylates in SF is also described in literature [26]. Amino groups of L-Cys were then covalently linked by reacting with these carboxylic acids by aminolysis (SF-g-L-Cys). The second process relied on covalent modification of further 4.8% of SF structural tyrosine aromatic side chain. Aldehydes of tyrosine side chains were blended with the amines of L-Cys, forming unstable Schiff base intermediates (or imines). These intermediates were chemically stabilized by reductive amination with NaCNBH₃ [27], forming ultimately a covalent bond. In the first process, Schiff base intermediates also formed at these sites but reverted, once they were not stabilized by reduction. Furthermore, in this process of grafting L-Cys on Tyr, important issues as Tyr localization, and its influence on backbone conformation and packing were regarded [4]. L-Cys carries a thiolate group in its molecular structure which kills microorganisms through disulfide bridge establishment with some bacterial wall structural components [28] or even intracellular components if internalized.

The ability of L-Cys to inhibit the growth of microorganisms in solution was given by Minimal Inhibitory Concentration (MIC) studies of soluble L-Cys against *S. aureus* which was 5 mg/mL. This

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

It has already been demonstrated that topical application of antioxidants decreased wound healing time [31]. The total antioxidant capacity of L-Cys was assessed by using spectrophotometric methods with DPPH $^{\circ}$ (2,2-diphenyl-1-picrylhydrazyl) [22]. The L-Cys concentration that caused a decrease in the initial DPPH $^{\circ}$ by 50% (EC₅₀) was found to be 0,029957 μ g/mL. These results showed that L-Cys has antioxidant activity. Sánchez-Moreno [32] showed EC₅₀ values of a few other antioxidants, as gallic acid (26 μ g/mL), tannic (59 μ g/mL), caffeic acid (69 μ g/mL), ascorbic acid (76 μ g/mL),

queratin (84 $\mu\text{g/mL}$), BHA (93 $\mu\text{g/mL}$) and rutin (102 $\mu\text{g/mL}$). These values show that albeit L-Cys is antioxidant, its antioxidant power is low but can be increased by rising the L-Cys concentration, if necessary (customized).

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

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

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

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

5. Conclusions

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

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