Triazine Mediated Covalent Antibiotic Grafting on Cotton Fabrics as a Modular Approach for Developing Antimicrobial Barriers

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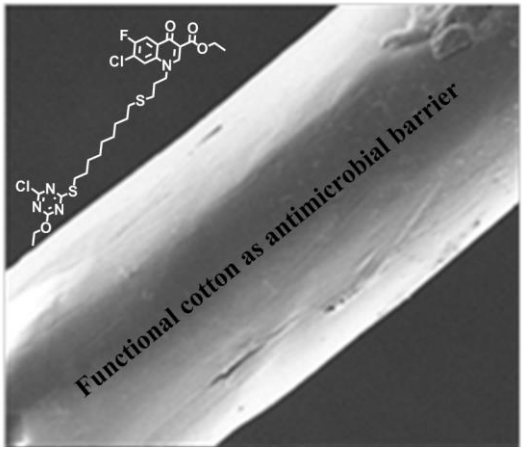
Abstract

New antimicrobial textiles were prepared through direct chemical linkage of bioactive molecules eugenol and fluoroquinolone derivatives, onto the surface of cotton fabrics. The attachment through a triazine moiety minimizes the leaching of the antimicrobial molecule into the surroundings of the material. Bacterial efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was studied. The treated textile with fluoroquinolone demonstrated

bacteriostatic antimicrobial effects having a tendency to decrease the population of *S. aureus* in the planktonic form. A significant effect was also observed in the prevention of *S. aureus* biofilm formation and in its ability to kill bacteria within a preformed biofilm. Eugenol-modified fabric was also active in the process of eradicating preformed *P. aeruginosa* biofilms. Further, *in vitro* assays using human dermal fibroblast cells indicate no effects on cell proliferation and viability, and *in vivo* tests in a murine skin wound model showed no increase of IL-6 for full-thickness wounds that were in contact with the fabrics.

Keywords: microbicidal; cotton fabrics; covalent functionalization; fluoroquinolone; biocompatibility; biofilm destruction



1. Introduction

The incorporation of bioactive molecules into the structure of textiles has gained increasing attention in the last decade (Shahidi and Wiener 2012, Pinho et. al. 2010, Koh and Hong 2014, Salama et. al. 2015). Antimicrobials are one of the most frequently used agents in bioactive textiles, since they effectively minimize the risk of microbial infection (Klivanov 2007, Ristić et. al. 2011, Hong 2014, Shahidi et. al. 2014). The bioactive agent may be either covalently bound or physio-adsorbed to the textile fiber. In the latter case, there is a burst release of the bioactive agent, which is detrimental for long term protection of the wound. Thus, chemical anchoring of bioactive molecule improves the lifetime of the material and also prevents the leaching of bioactive substances out of the textile, minimizing, for example, the risk of developing antibiotic resistant bacterial strains.

Naturally occurring fibrous structures, like cellulose, are particularly suitable for medical applications due to their mechanical properties, high hydrophilicity, and capability to absorb biofluids (*e.g.*, wound oozing). Cotton has been widely used for textiles because of its breathability, softness, and biodegradability (Xu et. al. 2010, Koga et. al. 2010). Thus, having the surface of cotton functionalized with antimicrobial agents will open the door to develop novel materials for controlling topical infections. Some strategies for bonding active substances to cellulosic fibers have been reported (Simoncic and Tomsic 2010), including antimicrobial agents such as quaternary ammonium salts (QAS) derivatives (Lin et. al. 2003, Hsu and Klibanov 2011, Gutarowska et. al. 2013, Song and Baney 2016), *N*-halamine derivatives (Sun et. al. 2001), and chitosan (Öktem 2003, Abramiuc et. al. 2013, Gargoubi et. al. 2016). Such methodologies are rather complex in nature, including for examples the use of polycarboxylic acids to chemically crosslink the chitosan to cellulose (Öktem 2003). Thus, there is still a need to develop synthetic routes for producing materials grafted with antimicrobial agents for wound dressing. For covalent anchoring on the textiles, cyanuric chloride, highly reactive towards nucleophiles through an aromatic nucleophilic substitution and of low cost, was selected as reactive group (Figure 1). This triazine unit has been previously used by our group for the dyeing of textiles by grafting. Triarylmethane (Montagut et. al. 2017), anthraquinone (Salabert et. al. 2015) and azo (Soler et. al. 2011) fluorinated dyes were anchored on textiles using a triazine moiety to obtain artificial repellent cotton fibers. Recently, other groups have used triazine to prepare covalently modified functional cotton fabrics, as for example triazine based reactive dyes for wool fabric ink printing (Yang et. al. 2018); monochlorotriazine triethylphosphite guanidine for flame retardant cotton fabrics (Dong et. al. 2018); and *N*-halamine triazine salts derivatives for antibacterial cellulose (Jiang et. al. 2019).

In this work, we present a novel approach for covalently tethering of antibiotics onto cotton fabrics through a nucleophilic aromatic substitution on a triazine moiety. The antibiotics were first modified with a linker (Figure 1). The long hydrocarbon chain in the linker will increase the hydrophobicity and the microbicide potency (Liu et. al. 2013) and will decrease the steric hindrance for the subsequent grafting onto the cotton surface. The ideal strategy should preserve the pharmacological efficacy of the active substance and be versatile to allow the anchoring of other active substances.

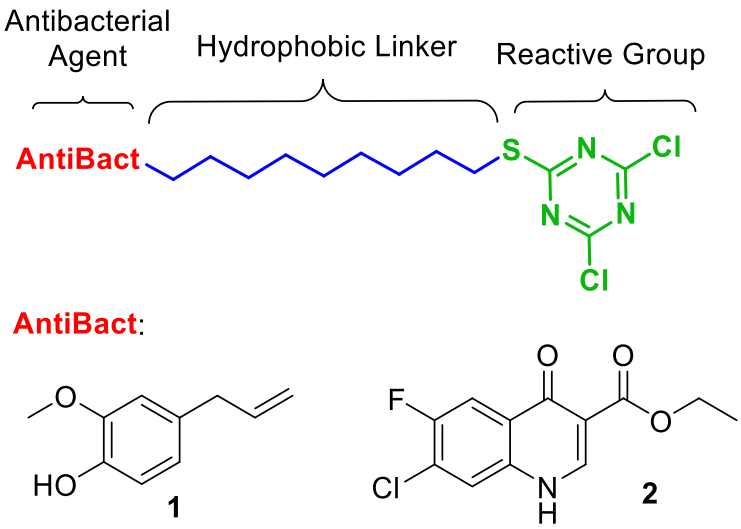


Fig. 1 Representation of our novel anchoring system for grafting antimicrobial agents to textiles. Structure of antibiotics **1** and **2**.

Two different families of antibiotics were selected to be grafted onto cotton fabrics: (i) 2-methoxy-4-(prop-2-en-1-yl)phenol (Eugenol, **1**) (Kalemba and Kunicka 2003, Rojo et. al. 2008), and (ii) ethyl 7-chloro-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (fluoroquinolone, **2**) (Koga et. al. 1980) (Figure 1). Eugenol, **1**, was first chosen due to its known antibacterial properties. It has been reported that **1** inhibits the growth of a range of microorganisms such as *Escherichia coli* (Blaszyk and Holley 1998), *Penicillium citrinium* (Vazquez et. al. 2001) and human herpes virus in vitro (Benecia and Courreges 2000). In medical field is mainly used in dentistry as a root canal sealer (Rojo et. al. 2008). Next, we selected quinolones another well-known family of antibiotics, and specifically one that is highly active the fluoroquinolone **2** (Leyva and Leyva 2008, Aldred et. al. 2014).

After the covalent tethering of Eugenol and fluoroquinolone to the surface of cotton textiles the *in vitro* antibacterial activity and cell toxicity of these functionalized fabrics were assessed together with *in vivo* assays in a full-thickness wound model in mice.

2. Experimental section

2.1. Materials

Eugenol, glycerol, diethyl ether, cyanuric chloride, *N*,*N*-Diisopropylethylamine, anhydrous tetrahydrofuran, anhydrous sodium sulfate, anhydrous acetone, sodium hydroxide, hexane, lysogeny (LB) broth, lysogeny (LB) broth agar, hydrochloric acid, acetic acid, tryptone, yeast

extract, DMSO-d_6, $CDCl_3$, potassium carbonate, and sodium chloride were purchased from Sigma Aldrich. All solutions were prepared using Milli-Q water. All cell culture media and reagents were purchased from Thermo Fisher Scientific Gibco unless otherwise specified.

2.2. General procedure for the thiol–ene reactions

In a 25 mL round bottom flask (see supporting information for details on structures), eugenol, **1**, (0.50 g, 3.0 mmol, 1 eq.), **2** (1.3 mL, 9.0 mmol, 3 eq.) and 6 mL of glycerol were added. The mixture was warmed to 80ºC and stirred for 3h. Extractions were done using Et_2O and water. Then, the organics were evaporated. The resulting crude product was purified by a chromatographic column with an eluent solution of 2:1 (Hex:Et_2O), affording 0.76 g of **3** as a white solid (85% yield) and 0.13 g of **4** (8% yield).

2.3. General procedure for the reaction with cyanuril chloride. Example of preparation of 9

In a 100 mL Schlenk flask under Ar atmosphere, cyanuric chloride (0.28 g, 1.4 mmol, 1.3 eq.), DIPEA (0.3 mL, 1.8 mmol, 1.7 eq.), and 30 mL of anhydrous THF were added. The mixture was cooled to -20ºC. Next, a solution of 4-(3-((9-mercaptononyl)thio)propyl)-2-methoxyphenol, **3**, (0.38 g, 1.1 mmol, 1 eq.) and 60 mL of anhydrous THF were added dropwise. The addition time was 5 h and the reaction was stirred for 1 h more. Afterwards, the THF was dried with anhydrous $NaSO_4$ and evaporated. The residue was purified by a chromatographic column using Hex:Et_2O as an eluent following a gradient from 3:1 to 1:1. The chromatographic column made long tails of the final product. A white solid was obtained, corresponding to **9**, 0.14 g, 36% yield.

2.4. General procedure for the attachment of the functional antimicrobial molecule on the cotton surface

A piece of cotton fabric (3 x 3 mm) was washed in a solution of soap and K_2CO_3 under reflux for 3 h. The cotton fabric was then dried and placed in a 1 M NaOH solution for 1 h. Next, the fabric was washed with anhydrous acetone and dried. Finally, the fabric was placed in a multi-reactor tube with a 0.23% w/v solution of the antibiotic molecule that was to be anchored to the fabric (the antibiotic derivative) in 10 mL of anhydrous THF. The set up was left under reflux for 3 days.

2.5. Characterization techniques

Melting points were measured in a *bloc Kofler* apparatus from *Reichert* or in a *B-545* apparatus from *Büchi* and are uncorrected. ^{1}H- (250 MHz) and ^{13}C- (62.5 MHz) NMR (Nuclear Magnetic Ressonance) spectra were obtained using a *Brucker AC-250* spectrometer. ^{1}H- (360 MHz) and ^{13}C- (90 MHz) NMR spectra were obtained using a *Brucker Avance 360* spectrometer. All chemical shifts are given in δ (ppm). ^{1}H-NMR and ^{13}C NMR are refereed with respect to TMS. IR (Infrared) spectra (neat) were performed in a *Bruker Tensor 27* using an ATR (Attenuated Total Reflectance) *Golden Gate* modulus provided with a diamond tip. All data are given in wave number υ and cm^{-1}. HR-ESI-MS (High Resolution-Electrospray Ionization Mass Spectrometry) experiments were performed using a *MicroTof-Q* from *Bruker daltronics* in either positive or negative ionization mode.

2.6. Antimicrobial assays

The antimicrobial properties of the fabrics were tested against *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (PAO1). Bacterial suspensions were freshly prepared by diluting overnight cultures in LB broth. Overnight cultures were prepared by resuspending one colony in 2.0 mL of LB agar and culturing in an orbital shaker for 18 h.

The antimicrobial effects of the fabrics were tested by incubating a 3 x 3 mm piece of antimicrobial fabric (**Fabric A** and **Fabric B**) in bacterial suspensions of 10^{7} CFU/mL at 37 ºC for 18 h. The number of surviving colonies was quantified by first diluting the treated solution to 10^{4} and 10^{5} CFU/mL, and then plating 10 µL of the diluted solution on an agar plate. The agar plates were then incubated at 37 ºC for 14 h, and the number of colonies was counted.

2.7. Biocompatibility and proliferation assay

To determine the biocompatibility of the fabrics, 5,000 human skin dermal fibroblasts/well were cultured in DMEM (Dulbecco's Modified Eagle Medium) in a 96-well plate. The cells were cultured for 2 days at 37 ºC with 5% CO_2. Next, a 3 x 3 mm piece of fabric was added to each well and cells were incubated for up to 15 days. An MTS assay was performed to determine the metabolic activity of the cells following the manufacturer's protocol (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). Briefly, 20 µL of the MTS solution was added to each well and incubated for 3 h. Then the absorbance of each well was measured at 490 nm.

2.8. In vivo IL-6 concentration

Animals

All animal studies were conducted with ethical approval from the University of Ottawa Animal Care Committee and in compliance with the National Institutes of Health Guide for the Use of Laboratory Animals. Female C57Bl/6 mice purchased from Charles River Laboratories were used for all experiments.

Eight week old female C57 mice were anesthetized with 2.5% isofluorane through a nose cone inhaler. Their backs were shaved and washed with 70% ethanol and two dorsal full thickness skin wounds were made with a circular punch, 6 mm in diameter. The left wound was used as a control while the right wound received the treatment, consisting of a 0.5 cm x 0.5 cm piece of fabric placed in the wound bed. The wounds were kept open with a silicone disk covered with Tegaderm™ and secured with 5 sutures. All animals were monitored for signs of inflammation, and pain was managed by Buprenorphine administered post-surgery. The animals were sacrificed 24h after the treatment. The wound area was collected and frozen at -80°C and the IL-6 concentration was measured by an enzyme-linked immunosorbent assay (ELISA; see below).

IL-6 concentration

The concentration of pro-inflammatory cytokine IL-6 was assessed in homogenized skin tissue harvested from the wound area after 24 hours of treatment using a mouse IL-6 ELISA kit (Thermo Fisher Scientific). Samples were homogenized in homogenization buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton-X, 0.5% sodium deoxycholate, 1 mM PMSF, 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific)) using 0.5 mm zirconium silicate beads (Next Advance) for 15 minutes in a Bullet Blender Tissue Homogenizer (Next Advance) followed by centrifugation at 10,000 xg for 10 minutes. The supernatant was then frozen at -80 °C until use. All samples were normalized to a starting concentration of 1 mg/mL of total protein, determined by bicinchoninic acid assay (BCA, Thermo Fisher scientific).

The ELISA assay was completed as per the manufacturer's instructions (see https://tools.thermofisher.com/content/sfs/manuals/KMC0061_KMC0062_Ms_IL6.pdf.). Briefly, a serial dilution of freshly prepared IL-6 standard (10,000 pg/mL) was completed to create a working range of 0 – 500 pg/mL. For this solid-phase sandwich ELISA, 100 µL of samples and standards were added to wells pre-layered with designated 1° antibodies and incubated for 2 h at room temperature. The plate was then washed 4 times with wash buffer. Next, 100 µL of mouse IL-6 biotin conjugate solution was added to the wells and incubated for 30 min at room temperature. The plate was then washed 4 times with wash buffer. Then 100 µL of streptavidin-HRP was added to the wells and incubated for 30 min at room temperature.

The plate was then washed 4 times with wash buffer. Next, 100 µL of chromogen was added to the wells and incubated for 30 min at room temperature in the dark. The reaction was halted by the addition of 100 µL of stop solution. The absorbance was then read at 450 nm. Controls were prepared with PBS without biotin conjugate and streptavidin-HRP. The concentration of IL-6 in samples was determined using a standard curve generated form the standard IL-6 solutions.

2.9. Anti-biofilm assay

Pseudomonas aeruginosa PAO1 biofilms were grown by incubating a bacterial suspension (10^7 cfu/ml) in M63 medium broth on glass surfaces arranged into the wells of a 12-well plate for 6h at 37 ºC. Once the biofilm was visibly formed, the remaining liquid was aspirated, and a piece of each fabric was placed on the formed biofilm and incubated for 12h at 37 ºC. Triplicate experiments were completed for each treatment. The fabrics were then removed, and the biofilm were visualized using crystal violet stain.

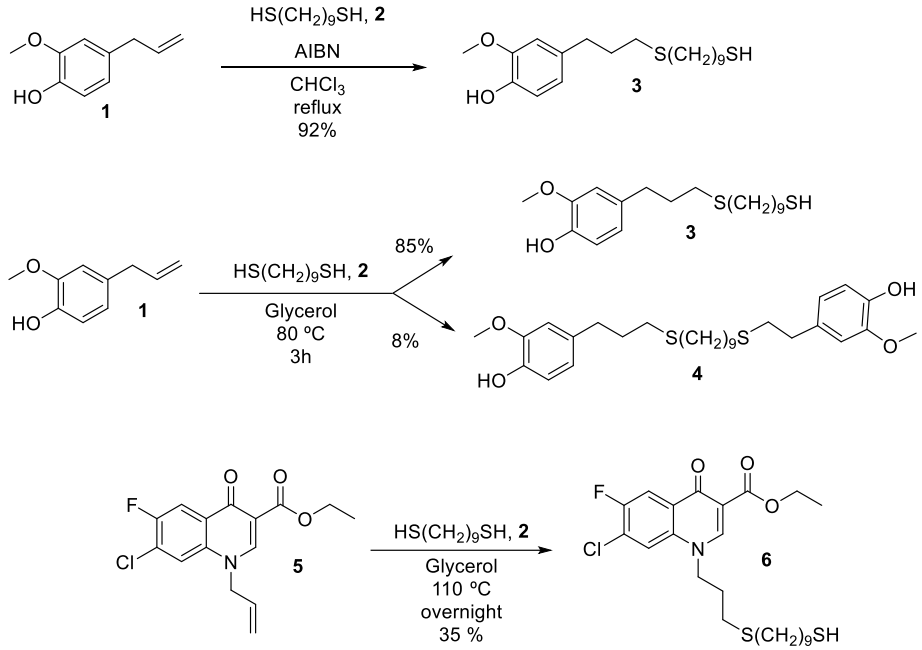
Antibiotic leaching was testing by incubating a 3 x 3 mm square piece of Fabric A with 100 µl of 25% LB broth overnight. The fabric was the removed and 10^7 CFU/mL of *P. aeruginosa* strain PAO1 was added to the solution and incubated at 37 ºC for 18 h. Bacterial growth was monitored to determine if leaching has occurred.

3. Results and discussion

3.1. Preparation of AntiBact-S-$(CH_2)_9$-SH

Given that the functional groups of antibiotics may play a crucial rule in their bioactivity, the point of attachment of the linker HS-$(CH_2)_9$-SH was carefully selected (**Scheme 1**). Thus, the double bound present on eugenol structure was deliberately picked to attach the linker. We tested several common thiol–ene coupling protocols between **1** and 1,9-nonandithiol. Moreover, we decided to incorporate a vinyl group on the fluoroquinolone that should allow the covalent attachment of the linker without affecting the functional groups involved in the bioactivity. Thus, derivative **5** was prepared following a methodology previously described by Koga (Koga et. al. 1980). Using the chemical anchoring system depicted in **Scheme 1**, we first linked both antibiotics (**1** and **5**) to a thioether hydrophobic arm to form the derivative of the formula AntiBact-S-$(CH_2)_9$-SH. The long hydrocarbon chain in the linker increases the hydrophobicity and the microbicide potency by acting as a barrier and controlling microorganism infiltration (Liu et. al. 2013). Furthermore, decreases the steric hindrance for subsequent chemical modifications. Then, the thiol group at the end of the AntiBact-S-$(CH_2)_9$-SH will be used to link the compound to the reactive group (**Figure 1**).

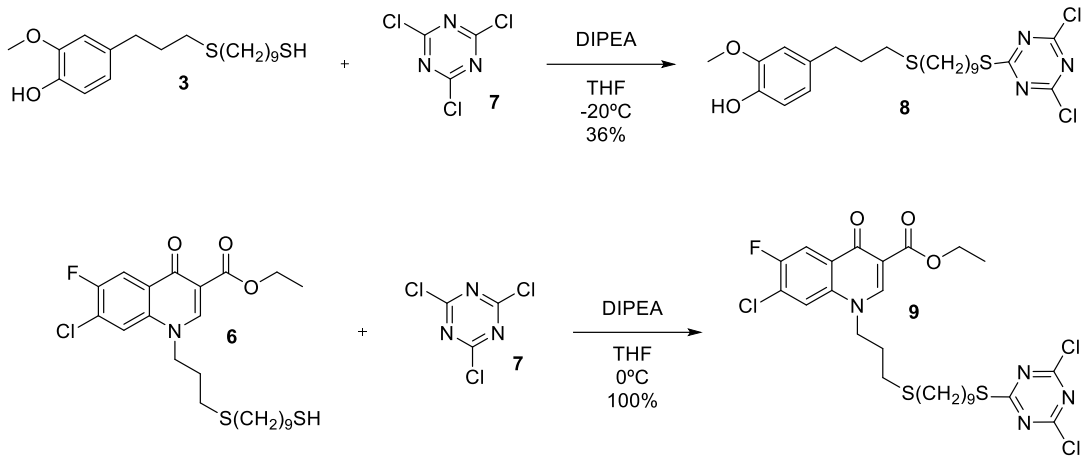
Both UV- and thermally-induced methods of the thiol–ene reactions were studied, with the best results achieved under thermal activation with AIBN as the radical source (Dondoni 2008). An excess of dithiol was used to minimize side reactions, giving a 92% yield of thioether **3** (**Scheme 1**). The high resolution mass-spectroscopy (HRMS (ESI^+)) analysis (m/z $[M+H]^+$ = 379.1733, Figure S6) and ^1H NMR of **3** (Figure S2) confirmed its structure. The signals present in the ^1H NMR of **3** were consistent with the presence of different methylene groups, among them CH_2-S-CH_2 and CH_2SH were identified (absorption at δ = 2.46-2.58). No signals corresponding to olefinic protons were detected. In addition, a multiplet at δ = 1.25-1.44 confirmed the presence of the SH group (see SI). However, under the optimized conditions, fluoroquinolone derivative **5** bearing a vinyl group (**Scheme 1**) did not react. Next, following the procedure from Perin *et al.*, we used a catalyst-free synthesis for linear thioethers (Lenardão et. al. 2013). First, eugenol, **1**, was reacted with nonane-1,9-dithiol, **2**, in glycerol at 80ºC (85% yield, **Scheme 1**). In this experiment, compound **4** was also isolated in 8% yield; its characterization confirmed the presence of the dithiol-bridged dimer. The HRMS (ESI^+) analysis (m/z $[M+H]^+$ = 543.2573, Figure S10) and ^1H NMR spectrum of **4** confirmed its structure. In the ^1H NMR spectrum the signals corresponding to the 10 methylene central protons of the bridge –$S(CH_2)_2(CH_2)_{10}(CH_2)_2S$ were observed (δ = 1.30-1.38 ppm, Figure S8). The same conditions were applied to obtain the thiol-bearing fluoroquinolone ester **6**. The reaction of **5** with 1,9-nonandithiol gave a moderate 35% yield of **6** (**Scheme 1**). The HRMS (ESI^+) analysis was recorded for compound **6** (m/z $[M+Na]^+$ = 524.1459, Figure S18). Its ^1H NMR spectrum (Figure S15) revealed a complex absorption at δ = 2.46-2.66 ppm characteristic of the methylene groups present in CH_2-S-CH_2 and CH_2SH and in addition, a multiplete at δ = 1.20-1.44 ppm corresponding to the SH free group was also observed.



Scheme 1. Synthesis of AntiBact-S-$(CH_2)_9$-SH

In the next step, the AntiBact-S-$(CH_2)_9$-SH compounds were attached to a reactive group to functionalize the antimicrobial molecules (**Figure 1**). Cyanuric chloride, **7**, (2,4,6-trichloro-1,3,5-triazine) was reacted with the thiol group of the AntiBact-S-$(CH_2)_9$-SH through an aromatic nucleophilic substitution (**Scheme 2**). This reaction was carried out in the presence of the weak and bulky base *N*,*N*-diisopropylethylamine (DIPEA) in tetrahydrofuran. The coupling reaction was completed at low temperature to avoid multiple substitutions on **7**, which has three available sites for substitution. A mono-substituted cyanuric chloride is preferred to avoid steric hindrance in the subsequent reaction of the reactive antibacterial molecule with the cellulose fabric. Moreover, tri-substitution would prevent the bonding of the functionalized antimicrobial molecule to the cellulose, since all the reactive sites of **7** would already be used. After some experimentation, reaction of **3** with **7** (1.25 equiv.) in the presence of 1.65 equiv. of DIPEA was carried out at -20ºC (**Scheme 2**). In these conditions, di- and tri-substitution reactions and the addition of the phenol to the cyanuric chloride were minimized, obtaining **8** in 36% yield. The ^{1}H NMR spectrum of **8** (Figure S20) showed different types of CH_2 groups, the most relevant being the triplet signal at δ = 3.18 ppm (CH_2-S-triazine). The HRMS $(ESI)^+$ m/z = 526.1112 corresponded to $[M+Na]^+$ (Figure S22). The ^{13}C NMR spectrum (Figure S21) presented a signal at δ = 170.0 ppm confirming the C_{ar}-Cl bond of the triazine moiety. In contrast of the moderate yield obtained in the synthesis of **8**, the reaction for compound **6** was quantitative at 0ºC (**Scheme 2**). The ^{1}H NMR spectrum (Figure S24) of **9** showed a triplet signal at δ = 3.13 ppm (CH_2-S-triazine) and the ^{13}C NMR spectrum (Figure 25) presented a signal at δ = 169.7

confirming the C_{ar}-Cl bond of the triazine moiety (see SI). In addition, the HRMS $(ESI)^+$ m/z = 671.0846 was consistent to $[M+Na]^+$ confirming the structure (figure S26).



Scheme 2. Synthesis of functional antimicrobial molecules

3.2. Preparation of the functionalized cotton fabrics

Once the functional antimicrobial molecules had been prepared, we proceeded to modify the cotton surface. First, each piece of cotton (piece of 3x3 cm) was washed under reflux in a water solution of soap and potassium carbonate for 3h. Then the cotton fabric was dried and soaked in a 1.0 M NaOH solution for 1h. Next, each piece of cotton fabric was washed with anhydrous acetone, dried, and added to a multi-reactor tube containing a solution 0.23% w/v of **8** or **9** in anhydrous THF. The mixture was left under reflux for a period of 3 days. After the anchorage process the fabrics were rinsed with THF and acetone. The obtained organic solvents were pure despite the high solubility of the functional antimicrobial molecules in both acetone and THF, proving the stability of the linkage of **8** (**Fabric A**) and **9** (**Fabric B**) through the covalent bond to the cotton surface (**Figure 2**).

SEM is the most widely used tool for morphological analysis. SEM micrographs of untreated cotton fabric, **Fabric A** and **Fabric B** were performed (**Figure 3**). After covalent anchoring of **8** and **9** the surface morphology has slightly changed and more significant differences are observed for **Fabric B**.

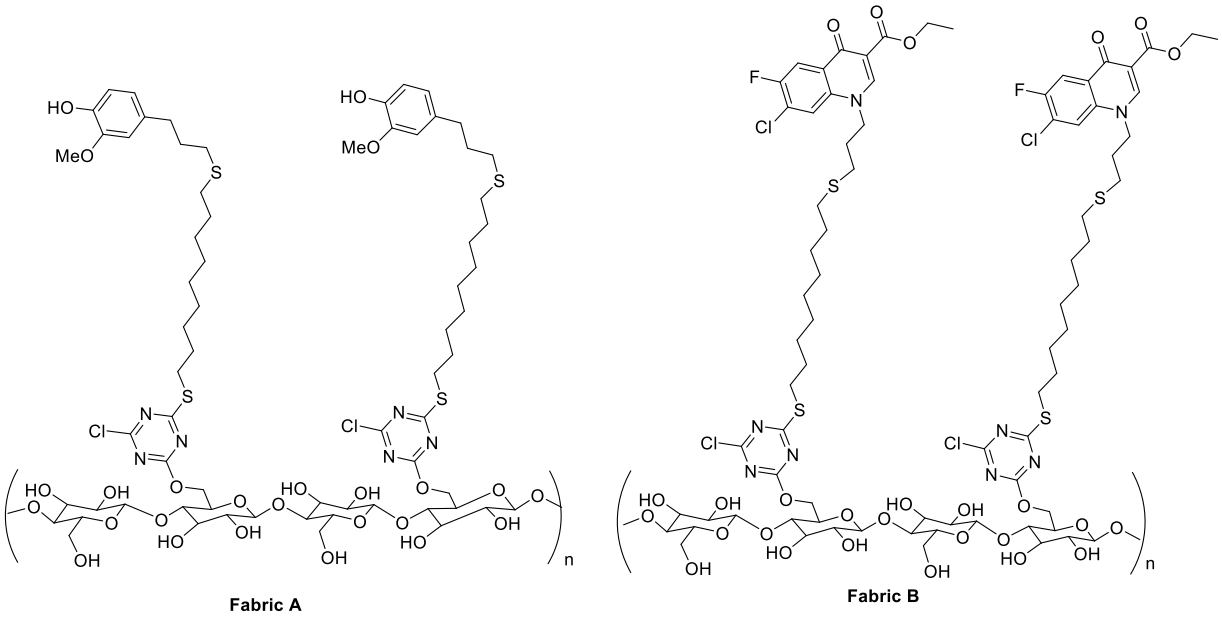


Fig. 2 Proposed structures for functionalized cotton fabrics.

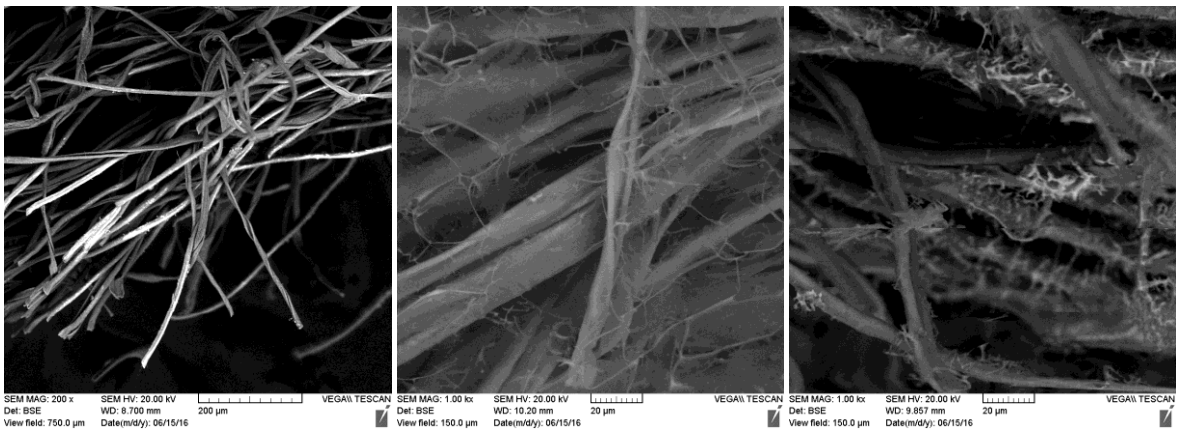


Fig. 3 Representative SEM images of cotton fibers: left image: untreated, central image: **Fabric A**, right image: **Fabric B**

3.3. Antimicrobial assays

The numbers of surviving colonies were quantified by plating the sample on agar plates (**Figure 4**). An additional set of experiments was carried out to test if antibiotic leaching from the fabric was occurring and could be responsible for the observed antimicrobial activity (**Figure 4**). Firstly, we compared the activity of both fabrics for *S. aeuriginosa* (**Figure 4**). Only a slight bactericidal effect was observed for **Fabric A**. In contrast, **Fabric B** presented an excellent bactericidal performance for *S. aureus*, with a decrease of 7 log units in the concentration of the bacteria. Moreover, as it can be seen for the most active antimicrobial agent, fluoroquinolone (**Fabric B**), the effect of preincubation with the supernatant from the fabrics

does not reach the extent of the antimicrobial activity seen for *S. aureus* (**Figure 4**). This result confirms the covalent attachment, which prevents leaching of the antibiotic from the textile, so free detached fluoroquinolone is not responsible for the observed antimicrobial activity. A plausible explanation for the antibacterial activity observed in our materials lies in the direct contact between the surface of the textile and the bacteria in suspension.

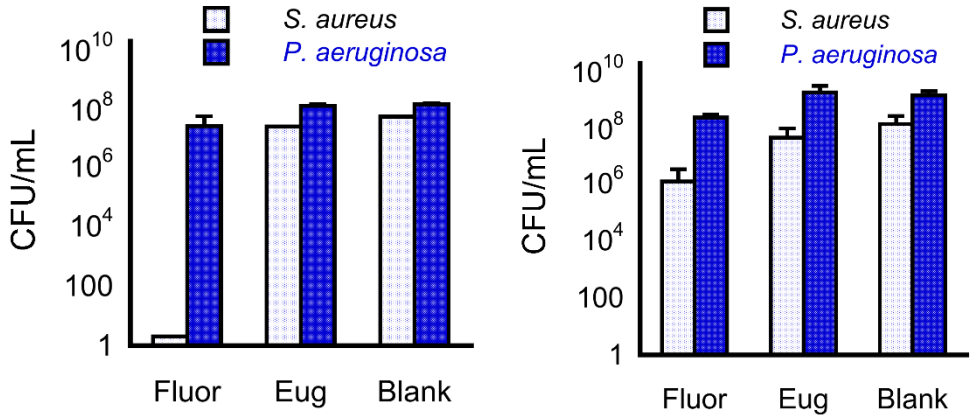


Fig. 4. Left: Surviving bacteria colonies after the incubation with *P. aeruginosa* and *S. aureus* for 18h, see experimental, for: fabrics without antibiotics (blank), or those modified with fluoroquinolone (Fluor) and eugenol (Eug). **Right:** Surviving bacteria colonies after incubation of the bacteria inoculum in supernatant of presoaked fabrics with and without the antibiotics. Values reported correspond to the standard deviation from the mean derived from 3 independent experiments.

3.4. Biocompatibility and cell proliferation studies

In vitro studies were performed using human dermal skin fibroblasts to evaluate biocompatibility and proliferation when cells are in contact with the fabrics. The results show that the fabrics did not affect the proliferation profile of the cells (**Figure 5**), suggesting that anchoring the antibiotic to the fabric does not lead to toxic side effects for cells in culture. Confirming this, a LIVE/DEAD™ assay indicated that cell viability after 72 hours of incubation in the presence of the fabrics was >95% in all cases. In addition, *in vivo* experiments carried out in a murine model for non-healing wounds indicate no increase in IL-6 levels when the fabric was put in contact with the wound (**Figure 5**).

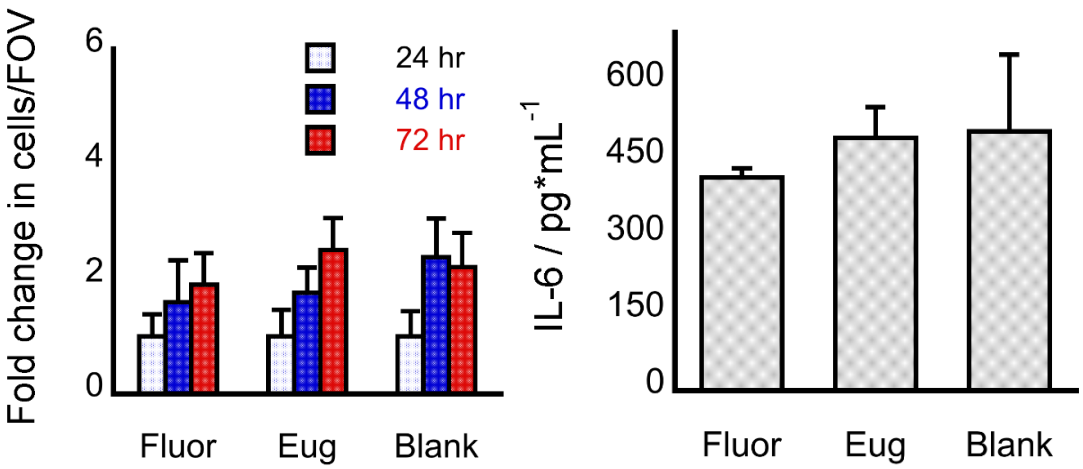


Fig. 5 Left: Number of human skin fibroblasts per field of view (FOV) measured at different time points after seeding. The fabrics were placed in contact with the cells and only removed for capturing the images. Numbers reported correspond to the average of 3 independent samples. **Right:** Interleukin-6 (IL-6) levels for murine skin tissue wound beds homogenized after implantation of the fabric for 24h (n=4). Error bars correspond to the standard deviation from the mean.

3.5. Antibiofilm assays

Formation of biofilm is a survival strategy for bacteria to adapt to their environment (Irie et al. 2012; Paharik et al. 2016). Under the protection of biofilm, microbial cells become tolerant and resistant to antibiotics and immune responses, which increase the difficulty for clinical treatments (Grupta et al. 2016; Katoona et al. 2018 (https://www.heliyon.com/article/e01067/)). Therefore, we studied the effectiveness of our treated fabrics on preventing and treating biofilms for *S. aureus* and *P. aeruginosa*. For *S. aureus* the fabrics containing eugenol showed a statistically lower number of survival bacteria in the prevention assay when compared to the fluoroquinolone group (**Figure 6A**). Meanwhile, the fluoroquinolone containing fabric was able to reduce the bacterial population in the biofilm form for *S. aureus* (**Figure 6B**). Thus, fluoroquinolone based **Fabric B** acts not only on the planktonic form of *S. aureus* strains but also on preformed *S. aureus* biofilms. As per the assays carried out for *P. aeruginosa*, no effect on preventing the biofilm formation was seen for any of the fabrics (**Figure 6C**), while only the eugenol containing fabric showed a lower bacterial population when acting on formed biofilm (**Figure 6D**).

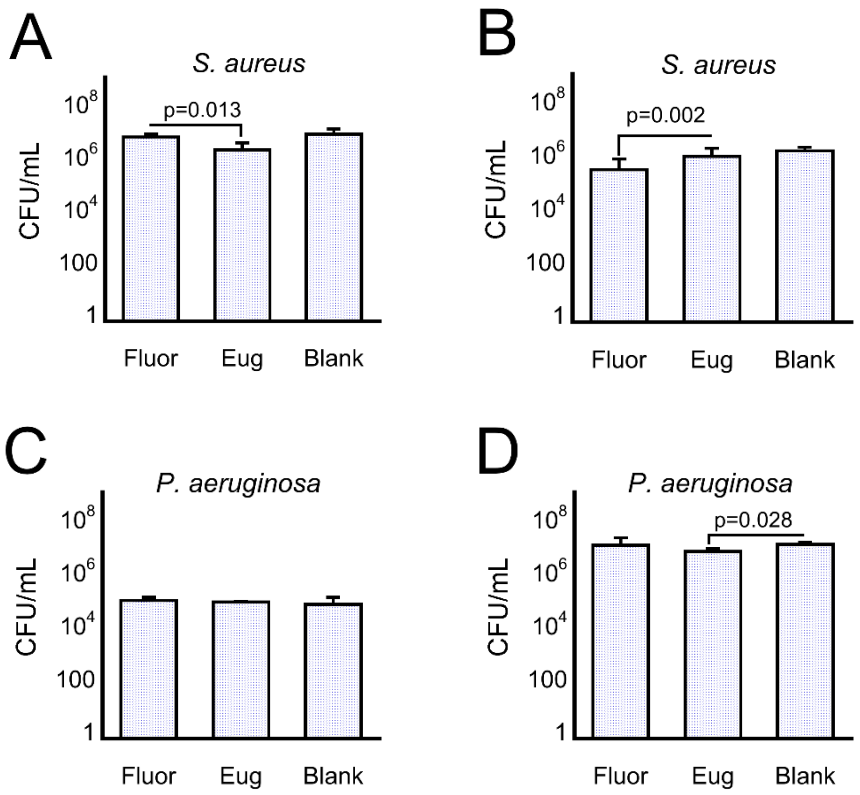


Fig. 6 Effect of antibiotic modification on the bacterial colony survival for *S. aureus* (A & B) and *P. aeruginosa* (C & D) biofilms. The assays were carried out measuring the potency of the fabrics to prevent the biofilm formation (A & C) as well as to assess their ability to kill bacteria within a preformed biofilm (B & D). Values reported correspond to the average of 3 independent measurements, and error bars are the standard deviation from the mean (y-axis is in log-scale). P values in the plots were calculated from t-Student test (two tailed).

4. Conclusion

In summary, new modified cotton fabrics were prepared through the covalent link of functional bioactive molecules, such as eugenol and fluoroquinolone derivatives, on the surface of cotton fabrics. Antibacterial tests performed with **Fabric A** coated with eugenol derivative showed no antibacterial activity against planktonic forms of *S. aureus* and *P. aeruginosa*. However, **Fabric B** coated with fluoroquinolone presented excellent antimicrobial activity with a decrease of 7 log units for the *S. aureus* strain. Similarly, the fluoroquinolone containing fabric was able to significantly reduce preformed *S. aureus* biofilms, while **Fabric A** was the most active in reducing *P. aeruginosa* biofilms. Moreover, **Fabric A** prevented to some extent *S. aureus* biofilm formation. Incubation of the bacteria inoculum in supernatant of presoaked **Fabric B** indicates no biocidal effect of the textile, which is indicative of no leaching of the

fluoroquinolone; similar results were observed for the less active eugenol modified fabric. These covalently linked microbicidal agents do not leach into the surroundings of the textile, so the probability of microorganisms developing resistance to them is small. Moreover, *in vitro* and *in vivo* assays showed good biocompatibility of the fabrics. Therefore, these antimicrobial textiles can be used, for example, for clothing, in hospital linen, in surgical masks, caps, and gowns, as well as for wound healing, particularly in bandages or gauzes.

Supplementary material

Supplementary material is available. Copies of NMR, IR, HRMS spectra and compound characterization are enclosed.

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Highlights

- Eugenol and fluoroquinolone derivatives have been covalently attached onto the surface of cotton fabrics.
- Fluoroquinolone containing presented antimicrobial activity decreasing 7 log units for the *S. aureus* strain and reduce significantly preformed biofilms.
- The evaluation of the *in vitro* biocompatibility showed no toxic effects in human skin cells.
- The modified fabrics did not increase the levels of IL-6 in a full-thickness wound model in mice.