COMMUNICATIONS

A general green method to link peptides to cotton through mild reactions in water is presented. A highly selective reaction allows rapid cotton modification and chemical ligation of any peptide. A mixture of different peptides may provide in a single step a multifunctional, biocompatible cotton. The new fabric material presented in this work shows interesting antibacterial properties resistant to washing and to sterilization processes.

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Sustainable, Site-Specific Linkage of Antimicrobial Peptides to Cotton Textiles

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A new general method to covalently link a peptide to cotton via thiazolidine ring formation is developed. Three different analogues of an ultrashort antibacterial peptide are synthesized to create an antibacterial fabric. The chemical ligation approach to the heterogeneous phase made up of insoluble cellulose fibers and a peptide solution in water is adapted. The selective click reaction occurs between an N-terminal cysteine on the peptide and an aldehyde on the cotton matrix. The aldehyde is generated on the primary alcohol of glucose by means of the enzyme laccase and the cocatalyst 2,2,6,6-tetramethylpiperidine-1-oxyl. This keeps the pyranose rings intact and may bring a benefit to the mechanical properties of the fabric. The presence of the peptide on cotton is demonstrated through instant colorimetric tests, UV spectroscopy, IR spectroscopy, and X-ray photoelectron spectroscopy analysis. The antibacterial activity of the peptides is maintained even after their covalent attachment to cotton fibers.

Most bacterial infections occur by surface contact between people and objects, especially in hospital environments.[1] For this reason, the development of safe textiles is of fundamental importance to protect from infections healthcare workers and immunosuppressed or debilitated people.

In general, antibacterial textiles are prepared by simply impregnating the tissue with antimicrobial agents. However, we aim at forming a covalent link between the antimicrobial peptide and the cotton, in order to create an antibacterial tissue able to resist against repetitive washings and frictions.

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In our previous contributions on this topic,[2] we exploited a literature approach[3] to firmly anchor a peptide to cotton tissues. In this work, we describe a new general method to covalently bind peptides to cotton through a green, mild reaction in water.

Antimicrobial peptides (AMPs) are a class of peptides, present in innate immune system.[4] They are active on an wide range of microorganisms including bacteria, viruses, and fungi.[5] They demonstrated excellent potential as new therapeutic agents[6] because they are effective against antibiotic-resistant bacteria, thanks to their mechanism of action.[7] Indeed, whereas common antibiotics interact with enzymes and/or receptors, AMPs and many other natural peptides act by physically disrupting or making permeable the membrane of the targets.[8] In addition, AMPs rapidly decompose when dispersed in the environment as they consist exclusively of α-amino acids.[9]

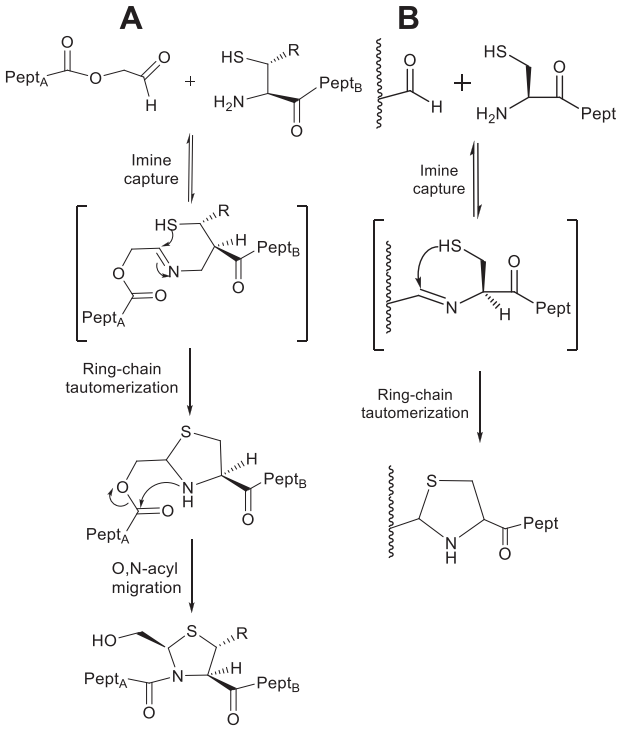
Cotton is an abundant natural fiber.[10] It is widely used in the medical field because it is breathable and resistant. It is mainly formed by cellulose, a linear polysaccharide consisting of β-1,4-glycosidic linked D-glucose units (called anhydroglucose unit).[11]

In this work, we adapted the native chemical ligation (NCL) approach,[12] introduced in 1992 by Kent,[13] to anchor a peptide to the cotton in a heterogeneous system. The NCL involves two reactions that occur spontaneously one after the other: a capture step that selectively connects the two moieties to be linked, followed by an intramolecular rearrangement.

An electrophile (i.e., an aldehyde or a thioester) and a nucleophile (i.e., cysteine, serine, threonine) are involved in the capture step. This greatly simplifies the synthetic strategy and, in the case of peptides, unprotected segments can be used as the very efficient ligation reaction that has excellent selectivity and reactivity.[12]

Over the years, a number of additional chemoselective reactions were identified as capture steps. Examples include the formation of hydrazone,[14] oxime,[15] thioether,[13,16] thiazolidine,[17] and oxazolidine.[18] Some of them lead to the formation of an amide bond while others involve unnatural peptide bonds.

For this work, we chose the pseudoproline ligation conceived by Tam in 1994.[17a] The initial capture product, an intermediate imine, quickly tautomerizes to a stable thiazolidine ring.

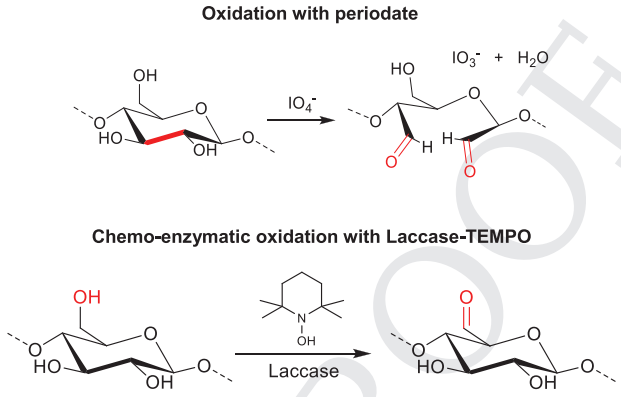


Scheme 1. A) Tam's pseudoproline ligation starting from glycolaldehyde ether as a peptide C-terminal electrophile and from an N-terminal cysteine as a nucleophile. B) Our ligation between the cotton aldehyde and the N-terminal 1,2-amino thiol group of cysteine.

The reaction then continues with an intramolecular acyl rearrangement to form the non-natural amino acid pseudoproline (**Scheme 1**A).[18,19] In our case, the reaction stops at the thiazolidine ring level because it cannot rearrange as a carbonyl is lacking on the pyranose ring of cellulose (Scheme 1B).

The major novelty of this work is the application of the reaction to a heterogeneous phase in water. Indeed, the reaction occurs between an insoluble cotton fabric and a water-soluble peptide. A comparable procedure was already reported.[20] However, in that case the cellulose needs to undergo a surface modification (adsorption of carboxymethyl cellulose) before being ready for a click (azide–alkyne) reaction in water.

To generate aldehydes on cotton, we did not exploit the commonly used periodate oxidation.[21] Although not affected by side reactions, this oxidation implies the breaking of a



Scheme 2. Top: Oxidation of cellulose with periodate that causes the opening of the glucose ring. Down: The chemoenzymatic oxidation using TEMPO and laccase preserves the integrity of the glucose monomer.

glucose carbon–carbon bond and a consequent worsening of the mechanical properties of the fiber (**Scheme 2**). Thus, we recurred to the less aggressive chemoenzymatic, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation with laccase in aqueous medium, under mild conditions (Scheme 2). TEMPO is a stable and water-soluble nitrosyl radical, which preferentially oxidizes the primary alcohols of a polysaccharide. In the case of cellulose, the C6 hydroxyl group is oxidized without affecting the integrity of the glucose ring.[22] Laccase is an enzyme able to reoxidize the reduced TEMPO using oxygen as the primary oxidant.

This reaction is interesting from an ecological point of view because it takes place in water, under mild conditions, it does not involve the use of chlorinated compounds such as NaClO and $NaClO_2$,[23] it uses only atmospheric oxygen, and the only by-product formed is water.[24]

The oxidation to aldehyde was then achieved by dipping the cotton fabric (100 mg) in a 50×10^{-3} M acetate buffer (pH 5), containing about 1 mg mL^{-1} of laccase (from *Trametes villosa*) and 0.8 mL^{-1} of TEMPO. After 24 h at 30 °C, under gentle stirring, the cotton was taken out, washed with water and methanol, and dried in a desiccator. To qualitatively check the presence of aldehydes, a small piece of cotton was soaked into a Schiff's reagent solution (**Figure 1**A). Fourier transform infrared (FTIR) absorption is often used to detect aldehydes on cotton,[25] but in this case it is not sensitive enough to detect the scarce C=O occurrence attained by laccase oxidation.

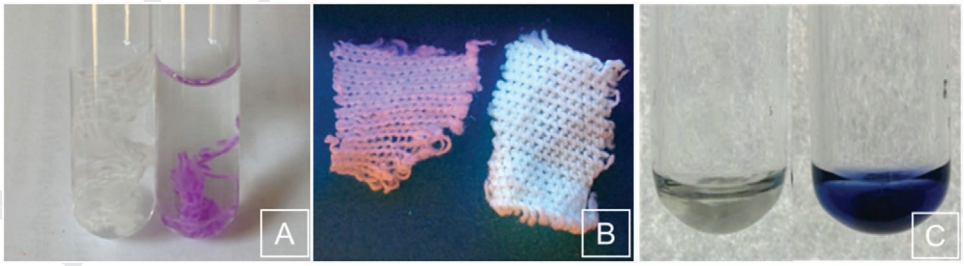


Figure 1. Colorimetric tests on treated cotton samples. A) Schiff test for aldehydes: test tubes with cotton samples before (left, uncolored) and after oxidation (right, purple color, originated from aldehydes). B) Oxidized cotton (left) and peptide-functionalized cotton (right) under UV irradiation (365 nm); the white color is due to the presence of tryptophan. C) Kaiser test for amines: oxidized cotton (left) and peptide functionalized cotton (right); the blue color originates from the amines on the Orn/Lys side chains.

Table 1. Amino acid sequence of the peptides investigated, their antibacterial activity and loading on the cotton.

Sample	Amino acid sequence	Antibacterial activity, MIC [μM]				Loading on cotton [mmol g^{-1}]
		S. aureus ATCC 25923	*S. epidermidis* ATCC 12228	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	
Pept 1	H-Cys-Lys(Laur)-Lys-Lys-Trp-Trp-NH_2	12.5–25	0.080	50	50–100	0.080
Pept 2	H-Cys-Orn-Orn-Trp-Trp-NH_2	100–200	0.097	100	200 to >200	0.097
Pept 3	H-Cys-Lys(Laur)-Orn-Orn-Trp-Trp-NH_2	50	0.085	100	100	0.085

An ultrashort antimicrobial peptide (USP)[26] was chosen to create the antibacterial tissue. We synthesized three analogues (**Table 1**) of H-Orn-Orn-Trp-Trp-NH_2, a peptide known for its antifungal and antimicrobial activity.[27] We appended a cysteine at the N-terminus of the peptides to introduce the 1,2-amino thiol group required for the selective reaction with the aldehyde on cotton.

In **Pept 1** [H-Cys-Lys(Laur)-Lys-Lys-Trp-Trp-NH_2], the overall positive charge of the parent peptide is maintained (Orn replaces Lys) but an additional Lys was added to incorporate a lipophilic chain. Lauric acid enhances the hydrophobicity of the peptide. Consequently, its affinity for bacterial membranes is expected to increase, as well as its resistance to enzymatic degradation.[28]

In **Pept 2** [H-Cys-Orn-Orn-Trp-Trp-NH_2], we only added a Cys residue, with respect to the parent peptide. In **Pept 3** [H-Cys-Lys(Laur)-Orn-Orn-Trp-Trp-NH_2], we also inserted the Lys(Laur) hydrophobic moiety.

The peptides were synthesized by SPPS, using a Rink amide resin and *tert*-butyloxycarbonyl (Boc) and trityl (Trt) protections for the side chain protection of Lys, Trp, and Cys. All amino acids were introduced using the [2-(1H)-1,2,3-benzotriazolyl]-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxy-1,2,3-benzotriazole (HOBt) C-activation method.[29] The peptides were purified by low pressure, reverse-phase chromatography.

The reaction between peptide and cotton occurred simply by dipping a piece of oxidized cotton into an aqueous solution containing the peptide. Tris(2-carboxyethyl)phosphine (TCEP) was also used to prevent the formation of disulfide bridges between cysteines. Peptides and TCEP were dissolved in the minimum volume of acetate buffer (200×10^{-3} M, pH 4.5) in the ratio of 2.4 and 3.6 mmol, respectively, per gram of cotton. The reaction proceeded under gentle stirring for 1 d at room temperature. Then, the cotton was washed with water and methanol and dried in a desiccator.

We assessed the presence of the peptide on cotton through the UV absorption of tryptophan (Figure 1B) and the Kaiser test[30] essay that reveals the free amine belonging to lysine or ornithine (Figure 1C).

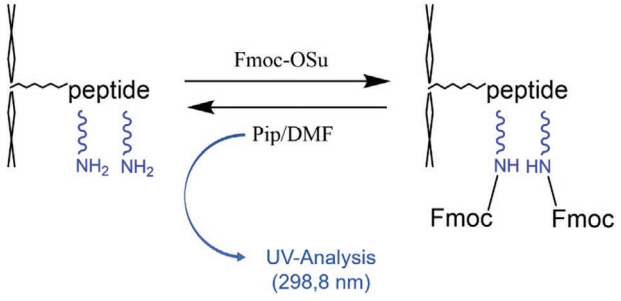
To quantify the peptide loading on the cotton, we devised the procedure summarized in **Scheme 3**. We introduced the fluorenylmethyloxycarbonyl (Fmoc) group on the free amines of the peptide by soaking the functionalized cotton in a 0.28 M $NaHCO_3$ solution and adding over 4 h aliquots of a Fmoc-OSu solution in a 3:1 mixture of acetonitrile/1,4-dioxane. Then, we carefully rinsed the cotton with N,N-dimethylformamide (DMF) and removed the Fmoc group by treatment with 20% (v/v) piperidine solution in DMF. From the UV absorption of this solution we quantified the dibenzofulvene-piperidine adduct (ε = 6089 cm^{-1} mol^{-1} at 298.8 nm) release upon Fmoc cleavage.[31] Based on the weight of the starting cotton, we estimated the peptide loadings on the cotton (Table 1).

To detect the nitrogen on the surface of the peptide-functionalized cotton, we performed an X-ray photoelectron spectroscopy (XPS) analysis (**Figure 2**, left). The weak signal intensity did not allow us to discriminate between amines and amides, but the binding energy values clearly indicate that the nitrogen is bound to C and H atoms, thus confirming that the peptide is definitely linked to the cotton surface.

The presence of the peptide was corroborated also by an IR absorption analysis that was run by shredding the cotton inside a KBr pellet (Figure 2, right). The absorption of the Amide A (amide N—H stretching), which falls around 3300 cm^{-1}, is completely hidden by the absorption of the numerous O—H functions of cellulose. However, the Amide I (C=O stretching) and Amide II (combination of CN stretching and N—H bending) are clearly observed. Amide I falls at 1656 cm^{-1} in our cotton-peptide samples (Figure 2 right). The very close band at 1632 cm^{-1}, also present in the untreated cotton, is attributable to the normal mode of bending of water molecules adsorbed on the surface of cotton.[32] The band centered at 1530 cm^{-1} is due to the peptide Amide II.

The antibacterial tests were performed on the free peptides and on the peptide-cotton samples. Table 1 reports the results of the tests on the peptides, carried out with the broth microdilution susceptibility test, according to the guidelines suggested by the Clinical and Laboratory Standards Institute.[33]

The three peptides are more active against the Gram-positive bacterium *Straphylococcus epidermidis*, a coagulase-negative *Staphylococcus* present in human skin and mucous membranes. *S. epidermidis* can cause severe infections by forming biofilms on indwelling medical devices. Indeed, it is the most frequent cause of nosocomial sepsis.[34] **Pept 1** (MIC = 12.5×10^{-6} M) was



Scheme 3. Method for quantifying the peptide on cotton. First, the Fmoc group is linked to the free amines of the cotton-bound peptide. Then, the Fmoc group is removed and the dibenzofulvene-piperidine adduct in the cleavage solution is quantified by its UV absorption intensity at 298.8 nm.

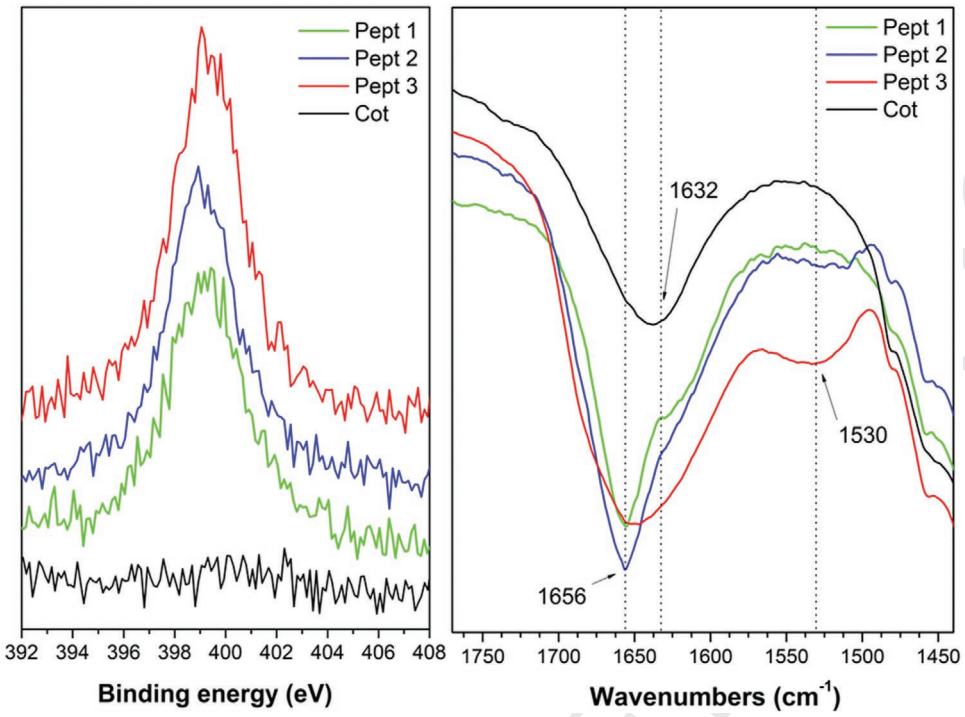


Figure 2. Left: XPS spectra of cotton and peptide-bound cotton, in the binding energy region of nitrogen. Right: IR absorption spectra of cotton and peptide-cotton samples in the Amide I and Amide II regions.

the most active compound is followed by **Pept 3** and **Pept 2**. This ranking suggests that the presence of an aliphatic chain may increase the antibiotic activity.

To assess the antibacterial activity of the peptide-functionalized cotton, we used a method for solid media described in the AATCC Technical Manual (American Association of Textile Chemists and Colorists). The bacterial inoculum was prepared by adding 1 mL of a culture of *S. epidermidis*, grown overnight at 37 °C in Mueller-Hinton (MH) broth, to 9 mL of a phosphate-buffered saline (PBS) solution (pH 7.4). The inoculum was then transferred to the surface of an MH agar plate by means of a sterile soaked swab. The cotton samples were placed on the surface with sterile tweezers by exerting a slight pressure. The plates thus prepared were incubated at 37 °C for 24 h.

While the oxidized cotton had very weak effect on the bacterial growth, the peptide-functionalized samples clearly inhibited the growth of bacteria (e.g., **Pept 1** in **Figure 3**A). The inhibition zone corresponded to the size of cotton samples without a visible ring of diffusion. Interestingly, peptide-bound cotton continued to inhibit bacterial growth also after a 30-min sterilization by UV-light treatment (Figure 3B). Overall these observations support the hypothesis that the peptides are not released from the textile to perform their activity and that they remain bound to the cotton which maintains its antibacterial properties.

To summarize, our simple, two-step method allows to selectively link a peptide to cotton tissues (**Figure 4**). Both starting materials are biodegradable. In addition, cotton oxidation and peptide anchoring take place in environmentally sustainable conditions.

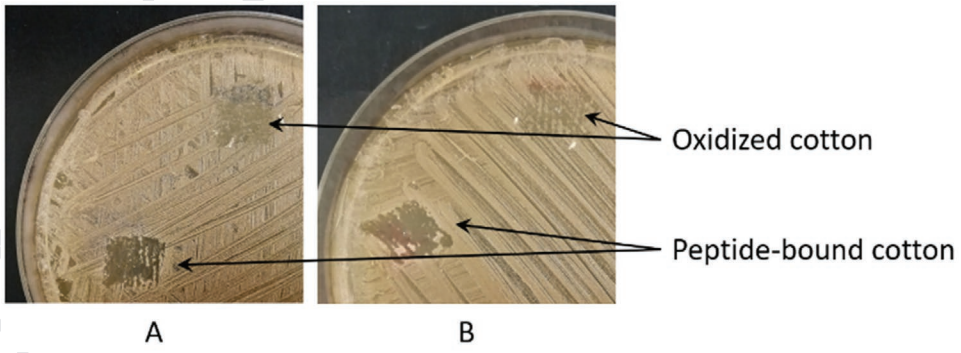


Figure 3. A,B) Antibacterial activity of peptide-functionalized cotton against *Straphylococcus epidermidis* before and after sterilization. The arrows indicate the glossy rectangles where pieces of oxidized cotton and of **Pept 1**-cotton have been placed.

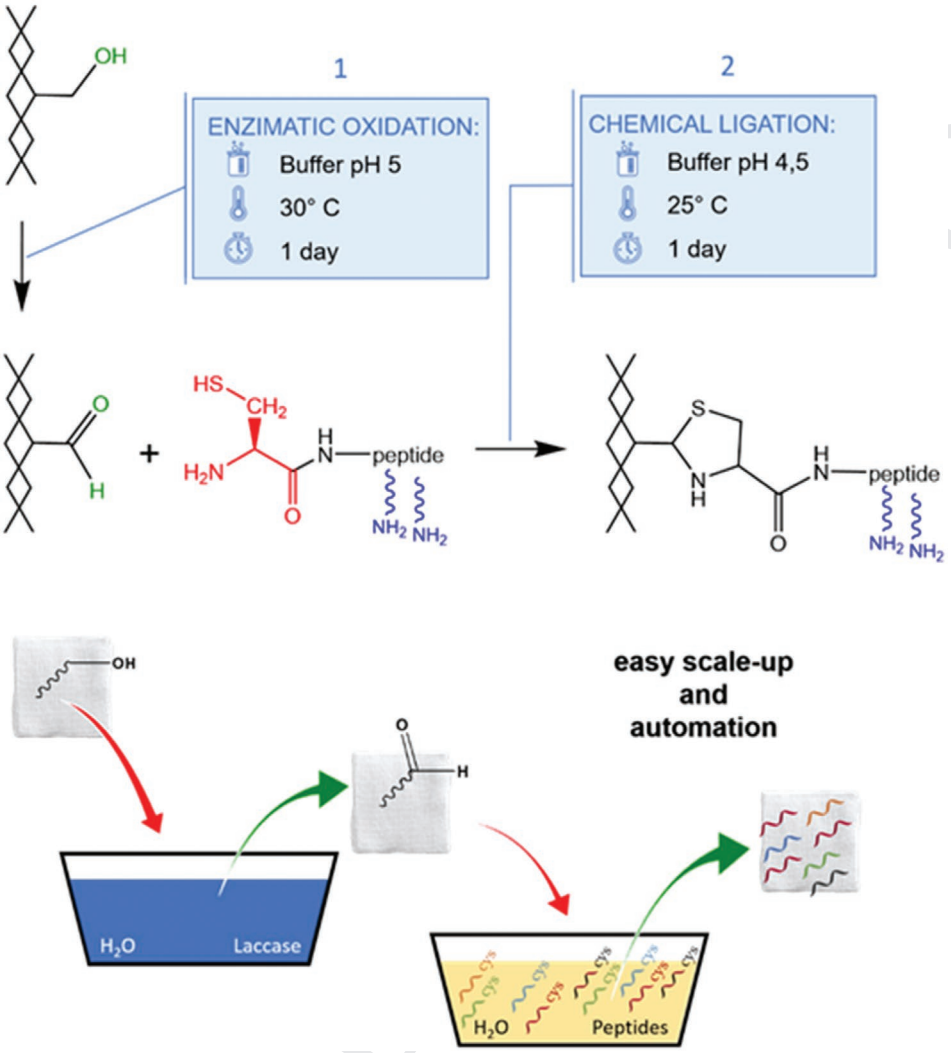


Figure 4. Schematic representation of the chemoenzymatic oxidation of cotton with TEMPO and laccase (**1**) and of its chemical ligation with the peptide (**2**). Both reactions take place in water under mild conditions. The process can be automated by dipping the cotton first in the oxidation and then in the ligation reservoir.

The highly selective reaction mechanism allows the ligation of any unprotected peptide bearing a N-terminal cysteine. Mixture of different peptides may also be considered to create a multifunctional cotton. Short peptides like those described in this work are economically appealing in view of large-scale applications. Finally, we envisage an easy automation and scale-up of the process by dipping the cotton first in the oxidation vessel and then in the container with the peptide(s).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

antibacterial tissue, antimicrobial peptides, chemical ligation, green chemistry, thiazolidine bond

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