Covalent grafting of antimicrobial peptides onto microcrystalline cellulose

Paola Sperandeo,[1] Fabrizio Bosco,[2] Francesca Clerici,[2] Alessandra Polissi,[1] Maria Luisa Gelmi,[2] Alessandra Romanelli[2]*

[1] Department of Pharmacological and Biomolecular Sciences, University of Milan, via Balzaretti 9, 20133 Milan
[2] Department of Pharmaceutical Sciences, University of Milan, via Venezian 21, 20133 Milan.

*: corresponding author
e-mail: alessandra.romanelli@unimi.it

Abstract
The purpose of this work is to set up a general protocol for the production of antimicrobial materials based on cellulose and peptides. We exploited the chemical ligation reaction to achieve conjugation of peptides to cellulose; to this aim we produced thioester peptides and a cysteine modified cellulose. As the thioester handle can be inserted at any position of the peptide, the peptide can be immobilized onto the cellulose through its N or C terminal end or through any other position within the sequence. Our experiments performed on *Escherichia coli* cultures show that the cellulose conjugated to the peptides lasioglossin III and TBKKG6A causes a significant reduction in the concentration of viable cells, as compared to unmodified cellulose. In conclusion, antimicrobial peptides bound to cellulose through a covalent bond retain their activity and therefore have potential to be used as active ingredients in antimicrobial materials.

Keywords: peptide, cellulose, conjugation, chemical ligation, antimicrobial, *E. coli*.

Introduction

Cellulose is the most abundant naturally occurring polymer obtained from renewable sources, composed of repeating units of poly (β-1,4-D-anhydroglucopyranose); it can be isolated by plant cell walls, or it can be produced by bacteria from the genera *Gluconacetobacter*, *Sarcina*, and *Agrobacterium* or by tunicates, algae and fungi. Importantly it can also be derived from agro-industrial waste.[1]
The cellulose macromolecular structure presents both crystalline and amorphous regions; physical properties of cellulose such as crystallinity and polymerization degree are different depending on its origin and processing.[2-4]
Due to its biocompatibility and biodegradability, cellulose finds application in different fields; as an example, in the food industry it is used as gelling or stabilizing agent, whereas in cosmetics it is employed as a scrub, or as a water retainer, emulsifier, suspension stabilizer in pastes and cream. Other applications are in the paper, fuel and leather industry.[5] Composites made of bacterial cellulose (BC) and polymeric or non-polymeric compounds have been exploited to produce artificial bones or cartilages.[6-8]
The use of cellulose addressed to the preparation of antimicrobial materials is reported in an increasing number of publications. The combination of Ag nanoparticles and cellulose matrices results in nanocomposites, endowed with antimicrobial activity against Gram-positive and Gram-negative bacteria. [9-11] Immobilization of peptides on cellulose to produce antimicrobial materials is little explored. Antimicrobial peptides (AMPs) are natural molecules, produced by organisms of all domains of life, that represent the weapon by which organisms defend themselves by external pathogens.[12] They show wide spectrum of activity and are selective towards bacterial cells. Being natural molecules, they are well tolerated by the body and importantly they are less prone to trigger resistance as compared to common antibiotics. For this reason they are considered as a valid alternative to small molecules to combat infections caused by resistant bacteria; use of AMPs to coat biodevices, such as implants, is widely explored.[13-16]
In a recent paper, Gonzalez et al. report the immobilization of an antimicrobial peptide on cellulose nanopapers; the peptide is adsorbed on nanopapers previously oxidized by 2,2,6,6-Tetramethylpiperidinyloxy radical (TEMPO) and treated with alkyl ketene dimer to increase hydrophobicity.[17] Release of the peptide upon contact with the culture medium results in antimicrobial activity.
Based on the observation that antimicrobial peptides retain their activity upon immobilization on a solid surface [14,15,18], it may be envisaged that covalent immobilization of antimicrobial peptides on cellulose may result in some advantages with respect to absorption. The main one is that peptides covalently bound to a surface are not released

in culture media, that in turn implies the possibility to re-use the surface. This aspect is of particular relevance if we think of an application of peptide-cellulose conjugates to the production of textiles. For the covalent immobilization, the free hydroxyl of cellulose can be exploited.

Protocols for the grafting of peptides on cellulose have been described by Orlandin et al. [19,20] In these papers, cellulose was used as a solid support for the synthesis of peptides, yielding peptides connected to the cellulose through their C-terminal end.

In addition, Edwards et al. reported the conjugation of peptides to cellulose to produce sensors for elastase and antibacterial cotton fibers.[21-23] The immobilization of peptides was performed using a glycine modified cellulose. The reaction between the glycine amino group and a tripeptide bearing at the N-terminal a succinic acid linker results in the formation of an amide bond and immobilization of the peptide on the cellulose through its N-terminal. This reaction affords a single conjugate product only when the peptide has one carboxylic acid function to react with the amine of glycine on cellulose. As a matter of fact, coupling of unprotected mixed sequence peptides containing lysine or serine or threonine residues in combination with aspartic and/or glutamic acid will result in a complicate mixture of products, that will lower the yield of immobilization.

It appears that a general protocol for the covalent immobilization of unprotected peptides on cellulose is lacking. Considering the strong potential in the use of this bioconjugated material with antimicrobial activity, we explored the chemical ligation reaction to achieve peptide immobilization. This reaction can be applied to conjugate peptides of any length and also proteins. Moreover, it allows to bind the peptide through its C-and/or its N-terminal end and also at any desired position within the peptide chain, therefore enabling the control of the orientation of the peptide with respect to the cellulose. It has been demonstrated that the activity of antimicrobial peptides grafted on solid supports may depend on the site of anchoring. For example, Bac2A derivatives exhibit stronger activity when the hydrophilic residues are placed close to the cellulose surface, whereas melimine shows the best antibacterial activity when hydrophobic residues at the N-terminal are linked to glass surfaces. [24,25] Moreover, temporin SH maximizes its antimicrobial potential when tethered to titanium surfaces through an amino acid inserted in the middle of the sequence.[26] In the present study we modified Avicel PH101, a microcrystalline cellulose suitable for pharmaceutical applications.[4] We accomplished functionalization of Avicel PH101 with two antimicrobial peptides, namely TBKKG6A and lasioglossin III (Table 1). TBKKG6A is an analogue of the natural peptide Temporin B, a peptide secreted by the skin of the *Rana temporaria* frog; lasioglossin III was isolated from the venom of *Lasioglossum laticeps*.[27-30] Both peptides show activity at very low concentration against Gram-positive and Gram- negative bacteria. The antibacterial activity of the new generated biopolymers was investigated.

Results and discussion

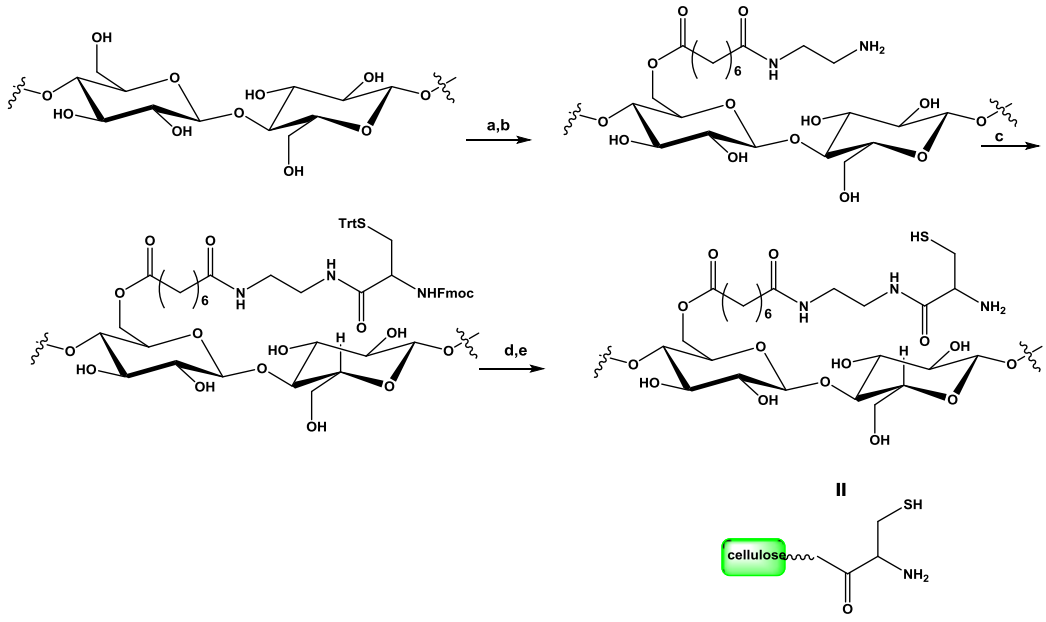
Peptide synthesis and conjugation to cellulose

The immobilization of peptides on cellulose is achieved by a chemoselective reaction, the native chemical ligation, affording highly homogeneous products. The native chemical ligation reaction, described by Dawson et al., involves the reaction of a N-terminal cysteine with a thioester function and results in the formation of an amide bond between the N-terminal of the cysteine and the activated carboxyl group.[31] Importantly, no other residue participates to the reaction, granting therefore the formation of a single product. As reported above, two antimicrobial peptides have been chosen for immobilization purposes, TBKKG6A and lasioglossin III (Table 1).

Table 1: Name and sequences of the antimicrobial peptides immobilized on cellulose.

Name (number)	Sequence
lasioglossin III (1)	VNWKKILGKIIKVVK
TBKKG6A (2)	KKLLPIVANLLKSLL
TBKKG6A-K (3)	KKLLPIVANLLKSLLK

To achieve the conjugation, both cellulose and peptides need to be modified. We installed the cysteine on the cellulose and the thioester on the peptides.
First, the cellulose scaffold to be used for tethering peptides was prepared (Scheme 1).
To preserve the antimicrobial activity of the peptide and prevent adhesion of the peptide to the cellulose, we immobilized on the cellulose a long linker composed of suberic acid and ethylendiamine. A Fmoc-cysteine, protected at sulfur with a trityl group was successively reacted with the free amine. The main advantage of linking Fmoc-cysteine on cellulose is that it is possible to calculate the loading of cellulose with the amino acid by quantifying the Fmoc group released by cysteine. We produced cellulose with a final loading of cysteine ranging from 0.050 to 0.075 mmol/g. Protecting group on cysteine were removed by standard protocols, to afford a scaffold ready for the chemical ligation reaction with thioester peptides.



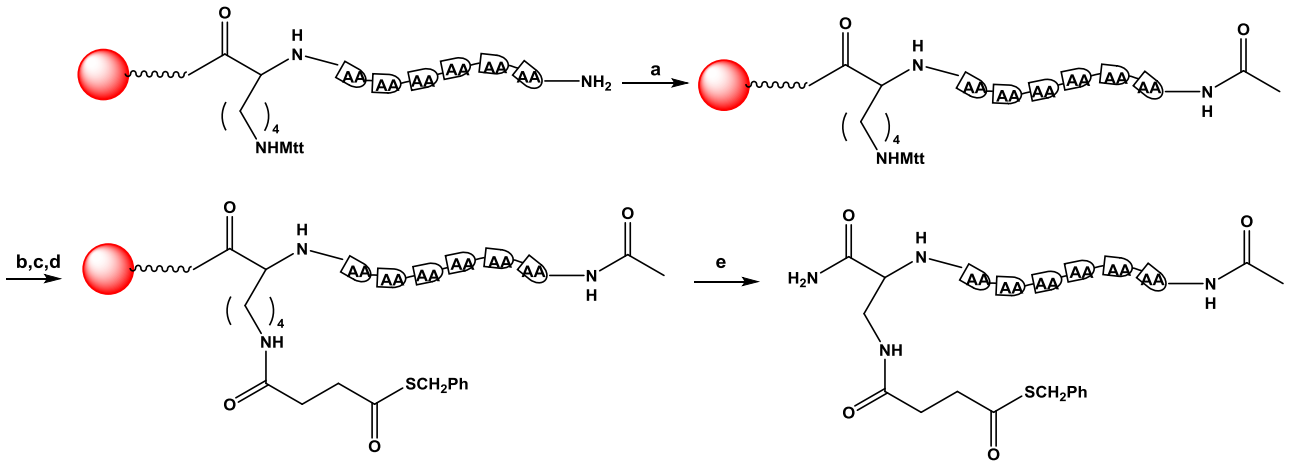
Scheme 1: Synthesis of cysteine-cellulose conjugate. Reagents and conditions: a: Suberic acid bis (hydroxysuccinimide), DIPEA, DMF, 2 hours, r.t.; b: ethylediamine, DMF, 2 hours, r.t.; c: Fmoc-Cys(Trt)-OH, HOBT/HBTU, N-methlmorpholine, DMF , overnight, r.t.; d: TFA (30%),DCM, 30 minutes (x2); e: piperidine 30%, DMF, 5 minutes (x2).

Next, we obtained thioester peptides. The thioester handle that is needed to perform ligation may be linked either at the C and at the N-terminal end of the peptide, and also at any desired position within the peptide chain, enabling all possible orientations of the peptide with respect to the cellulose to be tested.

We produced by solid phase synthesis two different derivatives of TBKKG6A: one with a thioester linker, *i.e.* benzyl thiosuccinic moiety, at the C-terminal, the other with the thioester linker at the N-terminal end. In case of lasioglossin III we produced the N-terminal modified peptide only, as it has been demonstrated that, when immobilized in this orientation, the peptide keeps its antimicrobial activity. [16]

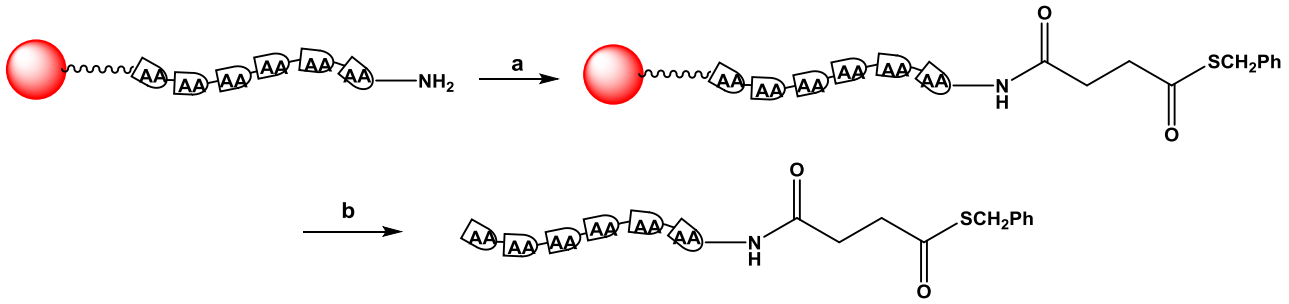
C-terminal derivatization (Scheme 2):

To obtain the TBKKG6A peptide modified at the C-terminal, we prepared by solid phase synthesis peptide **3**.This peptide bears as C-terminal amino- acid a lysine, protected on the epsilon amine with a methyltrityl group that can be selectively removed when the peptide is still anchored to the resin. After acylation of the N-terminal residue, the methyltrityl on lysine is removed; then the benzyl thiosuccinic linker is covalently linked, affording the C-terminal thioester peptide. Cleavage from the resin and deprotection of the peptide occur simultaneously, treating the resin with a concentrated TFA solution. The peptide is obtained as a single product and is then purified by reverse phase high pressure liquid chromatography (RP-HPLC) and characterized by mass spectrometry (see Supporting information for HPLC profile and mass spectrum).



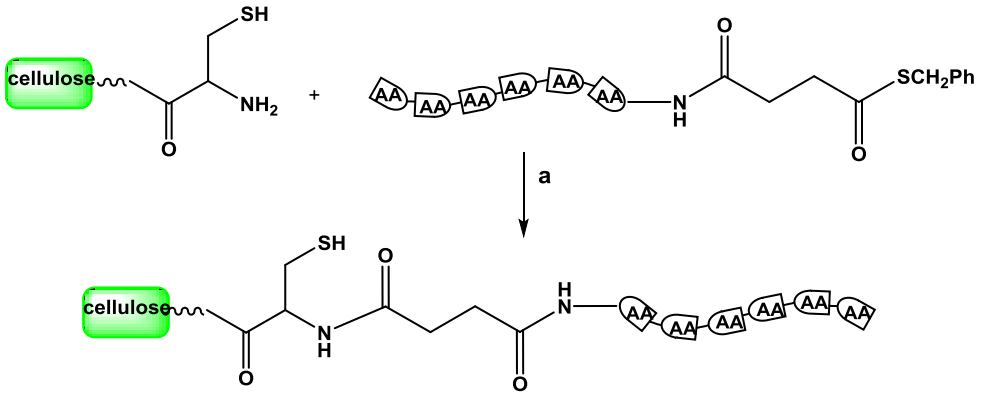
Scheme 2: Derivatization of the peptide at the C-terminal. ●: Rink amide resin; AA block chain represents a peptide, in this case TBKKG6A_K. Reagents and conditions: a: acetic anhydride/DIPEA/DMF (15/15/70) v/v/v, 10 min, r.t.; b: TFA (1%), TIS (5%),DCM, 2 min. (x10); c: DIPEA (10%), DMF, 10 min., r.t.; d: benzylthiosuccinic acid, HATU, DIPEA, DMF, 2 hours, r.t.; e: TFA, benzylmercaptan, phenol, H_2O (90/5/2.5/2.5, v/v/v/v), 2 hours, r.t..

N-terminal functionalization (Scheme 3): Peptides **1** and **2,** obtained by solid phase synthesis, were reacted with the benzyl thiosuccinic linker, to give N-terminal modified peptides. Cleavage from the resin and deprotection of the peptide occur simultaneously, as described earlier. The peptides are obtained as single products and are then purified by RP-HPLC (see Supporting information for HPLC profiles and mass spectra).



Scheme 3: Derivatization of peptides at the N-terminal end. ●: Rink Amide resin; AA block chain represents a peptide, in this case TBKKG6A or lasioglossin-III. Reagents and conditions: a: benzylthiosuccinic acid, HATU, DIPEA, DMF, 2 hours, r.t.; b: TFA, benzylmercaptan, phenol, H_2O (90/5/2.5/2.5, v/v/v/v), 2 hours, r.t..

Grafting of the peptides on the cellulose was achieved by chemical ligation reaction; to this aim peptides were dissolved in buffer at pH 7.5, and incubated with the functionalized cellulose. (Scheme 4)



Scheme 4: Immobilization of a thioester peptide on the cellulose support by chemical ligation reaction. AA block chain represents any peptide modified by a thioester linker. Reagents and conditions: a: 100 mM NaH_2PO_4, 50 mM Na ascorbate, 20 mM TCEP, pH 7.5, 48h, r.t..

Progress of the reaction was monitored by checking the disappearance of the peptide from the solution incubated with the cellulose by (HPLC). As the peptide peak disappears, we assume that peptides quantitatively react with the cysteine on cellulose, therefore the loading of the peptide on the cellulose is identical to the loading of the cysteine.
The peptide-cellulose conjugates were characterized by Fourier Transform InfraRed spectroscopy FT-IR (Figure 1 and Supporting information). Appearance of the band around 1650 nm is diagnostic of amide bonds and therefore confirms the conjugation of peptides to cellulose. Interestingly, the spectra recorded for lasioglossin-cellulose conjugates is very similar, in the amide region, to that reported in the literature for the same peptide conjugated to a silicon surface. [32] Dry peptide-cellulose conjugates are stored at -20°C.

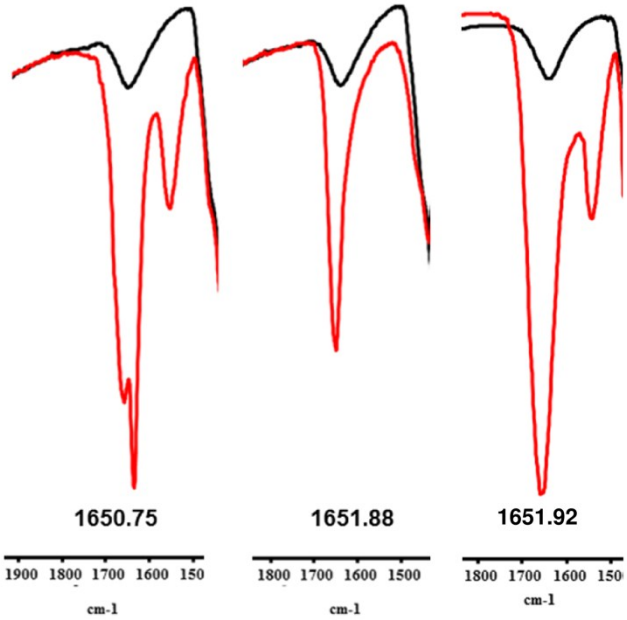


Figure 1: Detail of the FT-IR spectra recorded for cellulose (black) and peptide- cellulose conjugates (red). On the left the lasioglossin III-cellulose conjugate, in the middle the TBKKG6A-K- Link-cellulose conjugate and on the right the Link-TBKKG6A-cellulose conjugate

Antimicrobial activity

The MIC (Minimal Inhibitory Concentration) of the TBKKG6A-K peptide against *Escherichia coli* was evaluated to be 50 μg/mL; the MIC of TBKKG6A and lasioglossin III is reported in the literature to be 5 μg/mL and 2.5 μg/mL, respectively.[27,29,30,33] The antimicrobial properties of the functionalized crystalline cellulose were evaluated against *Escherichia coli* MG1655, a reference strain for Gram-negative bacteria, as described in the Experimental section. The antibacterial activity was determined by calculating the percent reduction of bacterial concentration (%R) after 6 h of contact of *E. coli* cells with different concentrations of functionalized or non-functionalized crystalline cellulose. The results representative of at least two independent experiments are reported in Table 2.
Cellulose functionalized with peptides **1-3** showed a significant reduction (%R=100%) in the concentration of viable cells relative to the initial inoculum. The %R values displayed by peptide-functionalized cellulose samples correspond to a $\log_{10}$ reduction ranging approximately from 3 to 4. The highest potency is observed when peptides are immobilized through the N-terminal end, at a concentration of 20 mg/mL in case of lasioglossin-III and 50 mg/mL in case of TBKKG6A.

Table 2
Antibacterial properties of functionalized crystalline cellulose

Sample[a]	**CFU/ mL t=0**[b]	**CFU/ mL t=6h**[c]	**%R**[d]
Bacteria only	$4x10^6$	$1.6x10^{10}$	nr[e]
Cellulose only 50 mg/mL	$2x10^6$	$3x10^9$	nr[e]
Cellulose only 100 mg/mL	$8x10^6$	$8x10^{10}$	nr[e]
Cellulose+Link TBKKG6A 10 mg/mL	$2x10^6$	$1x10^9$	nr[e]
Cellulose+Link TBKKG6A 20 mg/mL	$1.6x10^6$	$4x10^8$	nr[e]
Cellulose+Link TBKKG6A 50 mg/mL	$2x10^6$	$<1x10^3$	100
Cellulose+Link TBKKG6A 100 mg/mL	$4x10^6$	$<1x10^3$	100
Cellulose + TBKKG6A_K Link 10 mg/mL	$1.6\ x10^6$	$2.5x10^9$	nr[e]
Cellulose + TBKKG6A_K Link 20 mg/mL	$4x10^6$	$2.9x10^9$	nr[e]
Cellulose + TBKKG6A_K Link 40 mg/mL	$4x10^6$	$4x10^8$	nr[e]
Cellulose + TBKKG6A_K Link 80 mg/mL	$3x10^6$	$<1x10^3$	100
Cellulose+TBKKG6A_K Link 100 mg/mL	$4x10^6$	$<1x10^3$	100
Cellulose +Link lasioglossin-III 10 mg/mL	$4x10^6$	$3.1x10^9$	nr[e]
Cellulose +Link lasioglossin-III 20 mg/mL	$8x10^6$	$<1x10^3$	100
Cellulose +Link lasioglossin-III 100 mg/mL	$8x10^6$	$<1x10^3$	100

[a] *E. coli* MG1655 cells alone (Bacteria only) or in contact with non-functionalized (Cellulose only) or functionalized crystalline cellulose.
[b] t=0: bacterial concentration at the time of contact.
[c] t=6h bacterial concentration after 6h of contact with the indicated sample.
[d] % of bacterial reduction (%R) calculated as described in Experimental section.
[e] no reduction

These results confirm that both peptides maintain their antimicrobial activity after immobilization as cellulose functionalized with peptides not only prevents bacterial growth relative to control cellulose at the same (or even higher) concentration, but also determines a significant decrease in viable cell number with respect to the initial inoculum. This result suggests that conjugation with cellulose does not interfere with the mechanism of action of the peptides, providing them with enough flexibility to interact with bacterial membranes. The peptide TBKKG6A exhibits a stronger antimicrobial activity when immobilized to the cellulose through its N-terminal.

Conclusions
A protocol based on chemical ligation for the immobilization of peptides on cellulose through the C or the N-terminal end has been set-up. Following this general protocol, the amount of peptide immobilized on cellulose may be modulated. In fact, the loading of cellulose with cysteine can be increased or decreased, depending on the need. Antimicrobial activities suggest that peptides maintain their activity upon immobilization, opening the way to studies with different peptides. The cellulose-peptide conjugates could have potential application in the formulation of antibacterial cream and pastes or as preservative agents to protect cosmetic or pharmacological formulation by microbial contamination.

Experimental section

Peptide synthesis

Peptides were obtained by solid phase synthesis on an automated synthesizer, Liberty Blue CEM on the Rink Amide resin (loading 0.69 mmol/g). Derivatization of peptides **1** and **2** at the N-terminal was performed on the resin, by coupling the linker benzylthiosuccinic acid to the N-terminal amino acid, following procedures reported in the literature.[34] Briefly, the linker is activated with HATU in DMF in the presence of DIPEA and reacted with the free amine. The synthesis of the TBKKG6A modified by the thioester linker at the C-terminal was performed as follows. Resin bound Peptide **3** was acetylated at the N-terminal by treatment with a acetic anhydride/DIPEA/DMF 15/15/70 v/v/v for 5 minutes; after selective deprotection of the epsilon amino group on the C-terminal lysine by treatment with 1% TFA, 5% TIS in DCM for 2 minutes, ten times, the resin was treated with a solution of DIPEA (10%) in DMF for 10 minutes. Then the linker was reacted to this amino group as described above. The peptide was cleaved off the resin and deprotected by treatment of the resin as already reported.[34]
Peptides were purified by RP-HPLC (Phenomenex Jupiter 10µ Proteo 90Å 100x21.2 mm) and analyzed by mass spectrometry on a Thermo Scientific LCQ Fleet ion trap LC/MS. Follows the sequences and data for the peptide characterization. Link indicates the benzylthiosuccinic linker. HPLC profiles of the peptides are provided in the Supporting information.

Link-TBKKG6A: Sequence: Link-KKLLPIVANLLKSLL. Calculated mass: (Da) 1868.16 found $[M+H]^+$ 1869.76; $[M+2H]^{2+}$ 935.67; $[M+3H]^{3+}$ 623.99
Purification gradient: from 30 to 80% of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) in 20 minutes

TBKKG6A-K Link. Sequence: KKLLPIVANLLKSLLK(Link) Calculated mass: (Da) 2037.28; found:$[M+2H]^{2+}$ 1019.71; $[M+3H]^{3+}$ 680.39; $[M+4H]^{4+}$ 510.24
Purification gradient: from 15 to 50% of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) in 20 minutes

Link-lasioglossin III Sequence: Link- VNWKKILGKIIKVVK Calculated mass: 1971.34 found: $[M+H]^+$ 1972.77; $[M+2H]^{2+}$ 987.03; $[M+3H]^{3+}$ 658.68
Purification gradient: from 30 to 80% of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) in 20 minutes

Cellulose derivatization

In a reaction vessel for peptide synthesis, microcristalline cellulose (150 mg, Avicel.PH101.) was suspended in DMF. Suberic acid bis (hydroxysuccinimide) (0.06 mmol), dissolved in DMF (1.0 mL) and DIPEA (0.6 mmol) were added. The solution was incubated for 2 hours at r.t., then reagents were filtered out and cellulose was rinsed with DMF. An excess of ethylediamine (2.0 mmol) was dissolved in DMF (860 µL) and reacted with the cellulose for 2 hours. After filtering reagents and washing the cellulose with DMF, a solution of Fmoc-Cys(Trt) (0.3 mmol), HOBT/HBTU (0.27 mmol), and N-methlmorpholine (0.48 mmol) in DMF (0.8 mL) was added. Reaction was performed overnight at r.t..
The cellulose was rinsed. The cysteine residue was then deprotected either on the side chain (by treatment with a solution of TFA/DCM/TIS 30/79/1 v/v/v for 30 minutes two times) and on the amino terminal, by using a solution of piperidine in DMF (30/70 v/v).

Resin loading was calculated by the Fmoc test, and resulted around 0.075 mmol/g for Cellulose + TBKKG6A_K Link and Cellulose+Link TBKKG6A and 0.05 mmol/g for Cellulose +Link lasioglossin-III.[35]
For the chemical ligation reaction, 1 eq. of peptide was dissolved at a $4x10^{-4}$M concentration in a buffer composed of NaH_2PO_4 (100 mM), sodium ascorbate (50 mM) e TCEP (20 mM), pH 7.5. 6 M Guanidinium hydrochloride is added to the buffer to dissolve Link TBKKG6A. The peptides were incubated with the functionalized cellulose. Aliquots of the supernatant were collected and analyzed by RP-HPLC to follow the immobilization reaction. After 48 hours of incubation the reaction was complete. The cellulose was further rinsed with buffer, DMF, DCM and dried under vacuum. The conjugated cellulose was analyzed by ATR FT-IR on a Perkin Elmer Spectrum One FT-IR spectrometer equipped with a Universal ATR sampling accessory..

Antimicrobial activity
We determined the MIC of the peptides according to the standard broth microdilution method by preparing twofold serial dilutions in Mueller-Hinton Broth (MHB) in 96-well microtiter plates.[27] A stationary phase culture of the *Escherichia coli* K12 MG1655 strain grown over-night at 37°C with aeration was diluted to an optical density at 600 nm (OD_{600}) of 0.05 in the same medium. A stock solution of antibacterial peptides was made in water at the initial concentration of 5 mg/mL and used to perform serial dilutions in a ratio of 1:2 from 200 µg/mL in the final volume of 100 µL of water in the wells of the microtiter plate. 100 µL of bacterial inoculum at OD_{600} 0.05 was added in each well. Inoculated microtiter plates were incubated at 37°C for 16–20 h. The MIC was calculated as the minimal concentration of peptide that inhibits bacterial growth, as assessed by measuring the absorbance at 600 nm in a microtiter plate reader (EnSpire Multimode Plate reader, PerkinElmer). Lasioglossin III was used as positive control for MIC determination of TBKKG6A-K. Untreated culture was used as negative control.

To assess the antimicrobial activity of peptide-functionalized cellulose powder in a concentrated bacterial suspension under a fixed contact time (6 hours), we used the standard method ASTM E2149-10 (Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions) with slight modifications. Briefly, bacterial cultures of MG1655 strain were grown in LB broth at 37°C under aeration, until the optical density OD_{600} reached approximately 0.2 and adjusted to an OD_{600} of 0.003 (corresponding approximately to a concentration of 1-5 x 10^6 CFU/mL) in the same medium.[36] We resuspended bacteria in medium, instead of buffer, to explore not only bactericidal but also bacteriostatic activity of peptide-functionalized cellulose powder. The bacterial concentration of the diluted suspension was determined by serial dilution and considered as contact time “0” (t=0) concentration. Peptide-functionalized cellulose powder was mixed with bacterial cultures at concentration ranging from 10 to 100 mg/mL and the suspensions were incubated in Petri dishes at 37°C for 6 hours under static conditions. As negative controls, the bacterial suspensions incubated with microcrystalline cellulose at a concentration of 100 mg/mL (untreated sample control) or alone (inoculum only control) were used. To enumerate bacterial cells treated with cellulose or not treated, 10 volumes of sterile 0.9% NaCl solution were added to the Petri dishes and bacterial were resuspended by pipetting. The resulting suspensions were used for the determination of CFU/mL using serial dilutions prepared in sterile 0.9% NaCl solution and plated on LB-agar plates. The plates were incubated at 37°C for 18 h. The antibacterial activity was expressed by the percentage of reduction of bacterial concentration (%R), according to the formula:

%R = (A – B) /A x 100
where A = CFU / mL of the sample at t=0, and B = CFU / mL of the treated sample at t=6 h.
The antibacterial activity of the tested sample is considered significant if %R is higher than 90%.

Supporting information

HPLC profiles and mass spectra of peptides; FT-IT spectra of cellulose-peptides conjugates in full.

Acknowledgments

P.S. was supported by Italian Ministry of Education, University and Research, (FABBR)-MIUR 2018, funding for the financing of basic research activities. A.R., M.L.G. F.C., A.P., P.S. were supported by Regione Lombardia- 2016 (Smart fashion and design, “ORANGE LEATHER” N°187084).

References

(1) Lee, H. V.; Hamid, S. B. A.; Zain, S. K. *Sci World J* **2014**.

(2) Halib, N.; Perrone, F.; Cemazar, M.; Dapas, B.; Farra, R.; Abrami, M.; Chiarappa, G.; Forte, G.; Zanconati, F.; Pozzato, G.; Murena, L.; Fiotti, N.; Lapasin, R.; Cansolino, L.; Grassi, G.; Grassi, M. *Materials* **2017**, *10*.

(3) Phanthong, P.; Reubroycharoen, P.; Kongparakul, S.; Samart, C.; Wang, Z. D.; Hao, X. G.; Abudula, A.; Guan, G. Q. *Carbohydr Polym* **2018**, *190*, 184.

(4) Habibi, Y.; Lucia, L. A.; Rojas, O. J. *Chem Rev* **2010**, *110*, 3479.

(5) Tamilselvi, A.; Jayakumar, G. C.; Charan, K. S.; Sahu, B.; Deepa, P. R.; Kanth, S. V.; Kanagaraj, J. *J Clean Prod* **2019**, *230*, 694.

(6) Ullah, H.; Wahid, F.; Santos, H. A.; Khan, T. *Carbohydr Polym* **2016**, *150*, 330.

(7) Hong, L.; Wang, Y. L.; Jia, S. R.; Huang, Y.; Gao, C.; Wan, Y. Z. *Mater Lett* **2006**, *60*, 1710.

(8) Ul-Islam, M.; Khan, S.; Ullah, M. W.; Park, J. K. *Biotechnol J* **2015**, *10*, 1847.

(9) Singla, R.; Soni, S.; Patial, V.; Kulurkar, P. M.; Kumari, A.; S, M.; Padwad, Y. S.; Yadav, S. K. *Sci Rep* **2017**, *7*, 10457.

(10) Dacrory, S.; Abou-Yousef, H.; Abouzeid, R. E.; Kamel, S.; Abdel-aziz, M. S.; El-badry, M. *Int J Biol Macromol* **2018**, *117*, 179.

(11) Tran, C. D.; Prosenc, F.; Franko, M.; Benzi, G. *Acs Appl Mater Inter* **2016**, *8*, 34791.

(12) Yount, N. Y.; Yeaman, M. R. *Annu Rev Pharmacol Toxicol* **2012**, *52*, 337.

(13) Yu, K.; Lo, J. C. Y.; Yan, M.; Yang, X. Q.; Brooks, D. E.; Hancock, R. E. W.; Lange, D.; Kizhakkedathu, J. N. *Biomaterials* **2017**, *116*, 69.

(14) Li, T.; Wang, N.; Chen, S.; Lu, R.; Li, H. Y.; Zhang, Z. T. *Int J Nanomed* **2017**, *12*, 2995.

(15) Liu, Z. H.; Ma, S. Q.; Duan, S.; Deng, X. L.; Sun, Y. C.; Zhang, X.; Xu, X. H.; Guan, B. B.; Wang, C.; Hu, M. L.; Qi, X. Y.; Zhang, X.; Gao, P. *Acs Appl Mater Inter* **2016**, *8*, 5124.

(16) Mishra, B.; Basu, A.; Saravanan, R.; Xiang, L.; Yang, L. K.; Leong, S. S. J. *Rsc Adv* **2013**, *3*, 9534.

(17) Gonzalez, I.; Oliver-Ortega, H.; Tarres, Q.; Delgado-Aguilar, M.; Mutje, P.; Andreu, D. *Int J Biol Macromol* **2017**, *105*, 741.

(18) Mishra, B.; Wang, G. *Biofouling* **2017**, *33*, 544.

(19) Orlandin, A.; Dolcet, P.; Biondi, B.; Hilma, G.; Coman, D.; Oancea, S.; Formaggio, F.; Peggion, C. *Coatings* **2019**, *9*.

(20) Orlandin, A.; Formaggio, F.; Toffoletti, A.; Peggion, C. *J Pept Sci* **2014**, *20*, 547.

(21) Edwards, J. V.; Prevost, N.; Sethumadhavan, K.; Ullah, A.; Condon, B. *Cellulose* **2013**, *20*, 1223.

(22) Liu, Y.; Edwards, J. V.; Prevost, N.; Huang, Y. X.; Chen, J. Y. *Mat Sci Eng C-Mater* **2018**, *91*, 389.

(23) Edwards, J. V.; Fontenot, K. R.; Liebner, F.; Condon, B. D. *Sensors-Basel* **2018**, *18*.

(24) Hilpert, K.; Elliott, M.; Jenssen, H.; Kindrachuk, J.; Fjell, C. D.; Korner, J.; Winkler, D. F.; Weaver, L. L.; Henklein, P.; Ulrich, A. S.; Chiang, S. H.; Farmer, S. W.; Pante, N.; Volkmer, R.; Hancock, R. E. *Chem Biol* **2009**, *16*, 58.

(25) Chen, R.; Willcox, M. D.; Cole, N.; Ho, K. K.; Rasul, R.; Denman, J. A.; Kumar, N. *Acta Biomater* **2012**, *8*, 4371.

(26) Masurier, N.; Tissot, J. B.; Boukhriss, D.; Jebors, S.; Pinese, C.; Verdie, P.; Amblard, M.; Mehdi, A.; Martinez, J.; Humblot, V.; Subra, G. *J Mater Chem B* **2018**, *6*, 1782.

(27) . CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA, 2012

(28) Malgieri, G.; Avitabile, C.; Palmieri, M.; D'Andrea, L. D.; Isernia, C.; Romanelli, A.; Fattorusso, R. *ACS Chem Biol* **2015**, *10*, 965.

(29) Cerovsky, V.; Budesinsky, M.; Hovorka, O.; Cvacka, J.; Voburka, Z.; Slaninova, J.; Borovickova, L.; Fucik, V.; Bednarova, L.; Votruba, I.; Straka, J. *Chembiochem* **2009**, *10*, 2089.

(30) Slaninova, J.; Mlsova, V.; Kroupova, H.; Alan, L.; Tumova, T.; Monincova, L.; Borovickova, L.; Fucik, V.; Cerovsky, V. *Peptides* **2012**, *33*, 18.

(31) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.

(32) Mishra, B.; Basu, A.; Chua, R. R. Y.; Saravanan, R.; Tambyah, P. A.; Ho, B.; Chang, M. W.; Leong, S. S. J. *J Mater Chem B* **2014**, *2*, 1706.

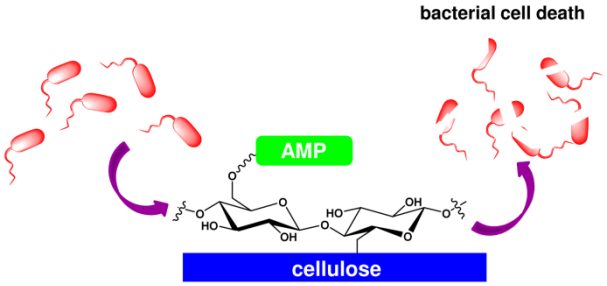
(33) Bezzerri, V.; Avitabile, C.; Dechecchi, M. C.; Lampronti, I.; Borgatti, M.; Montagner, G.; Cabrini, G.; Gambari, R.; Romanelli, A. *J Pept Sci* **2014**, *20*, 822.

(34) Pensato, S.; Renda, M.; Leccia, F.; Saviano, M.; D'Andrea, L. D.; Pedone, C.; Pedone, P. V.; Romanelli, A. *Biopolymers* **2010**, *93*, 434.

(35) Eissler, S.; Kley, M.; Bachle, D.; Loidl, G.; Meier, T.; Samson, D. *J Pept Sci* **2017**, *23*, 757.

(36) Blattner, F. R.; Plunkett, G., 3rd; Bloch, C. A.; Perna, N. T.; Burland, V.; Riley, M.; Collado-Vides, J.; Glasner, J. D.; Rode, C. K.; Mayhew, G. F.; Gregor, J.; Davis, N. W.; Kirkpatrick, H. A.; Goeden, M. A.; Rose, D. J.; Mau, B.; Shao, Y. *Science* **1997**, *277*, 1453.

For table of contents only



Supporting information

Covalent grafting of antimicrobial peptides onto microcrystalline cellulose

Paola Sperandeo,[1] Fabrizio Bosco,[2] Francesca Clerici,[2] Alessandra Polissi,[1] Maria Luisa Gelmi,[2] Alessandra Romanelli[2]*

[1] Department of Pharmacological and Biomolecular Sciences, University of Milan, via Balzaretti 9, 20133 Milan

[2] Department of Pharmaceutical Sciences, University of Milan, via Venezian 21, 20133 Milan.

*: corresponding author

e-mail: alessandra.romanelli@unimi.it

FT-IR spectra of cellulose and cellulose loaded with peptides.

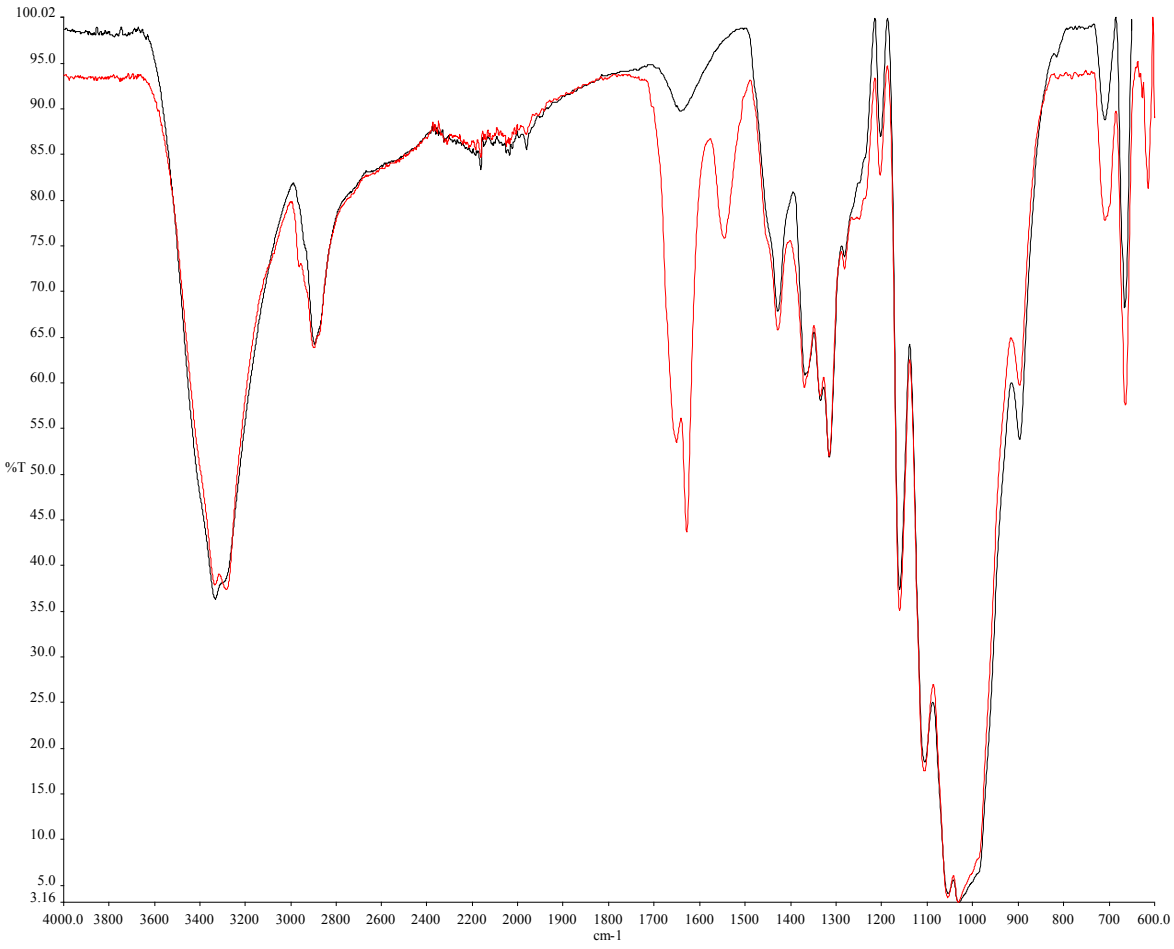


Figure S1: FT-IR spectra of cellulose Avicel PH 101 (black) and Avicel PH101conjugated to Link-Lasioglossin III (red)

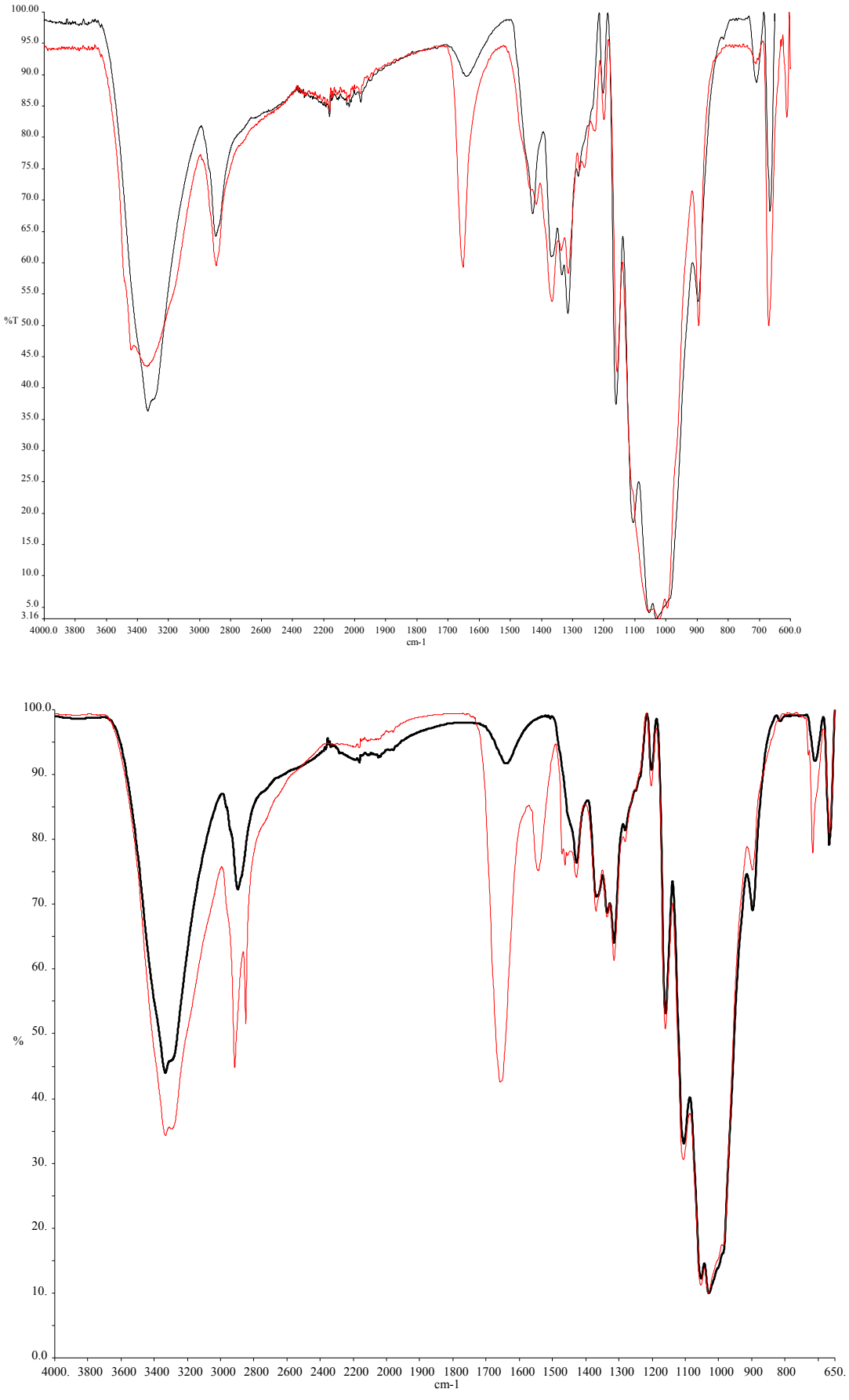


Figure S2: FT-IR spectra of cellulose Avicel PH 101 (black) and Avicel PH101 conjugated to TBKKG6A_K Link (red) Top and Link TBKKG6A (red) bottom.

HPLC profiles and mass spectra of peptides

Peptide Link-TBKKG6A

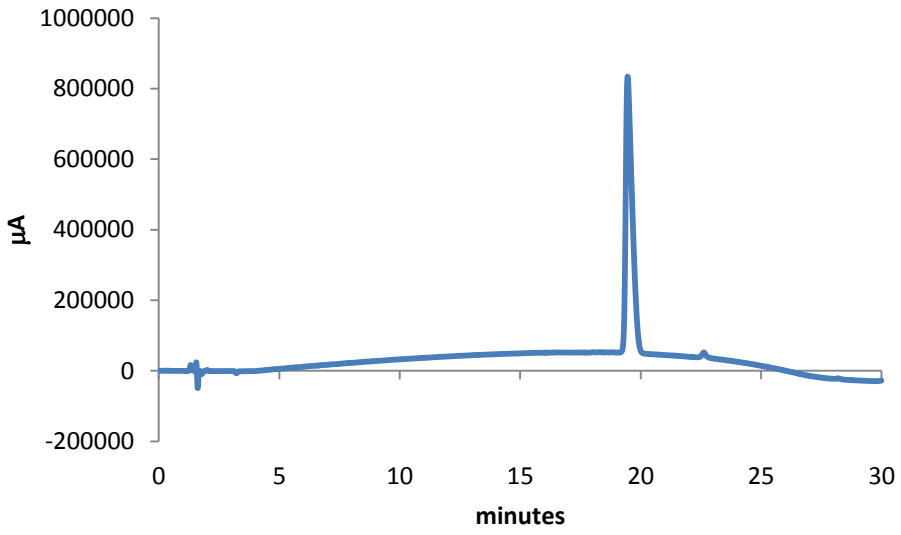


Figure S3: HPLC profile at 210 nm; column: VYdamass C18, 100Å 150x4.6 mm, gradient : acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 80% in 20 minutes.

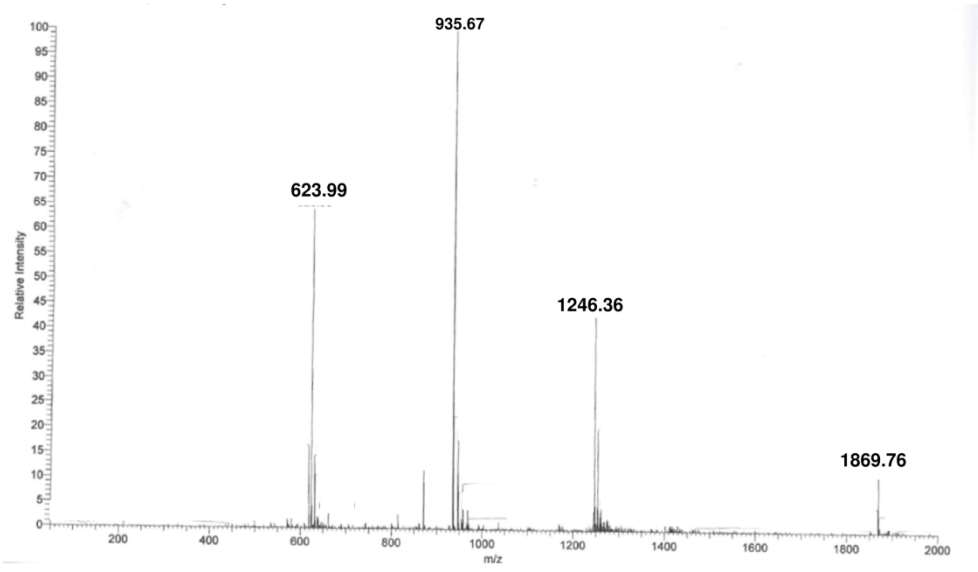


Figure S4: Mass spectrum of purified peptide

Peptide Link-Lasioglossin III

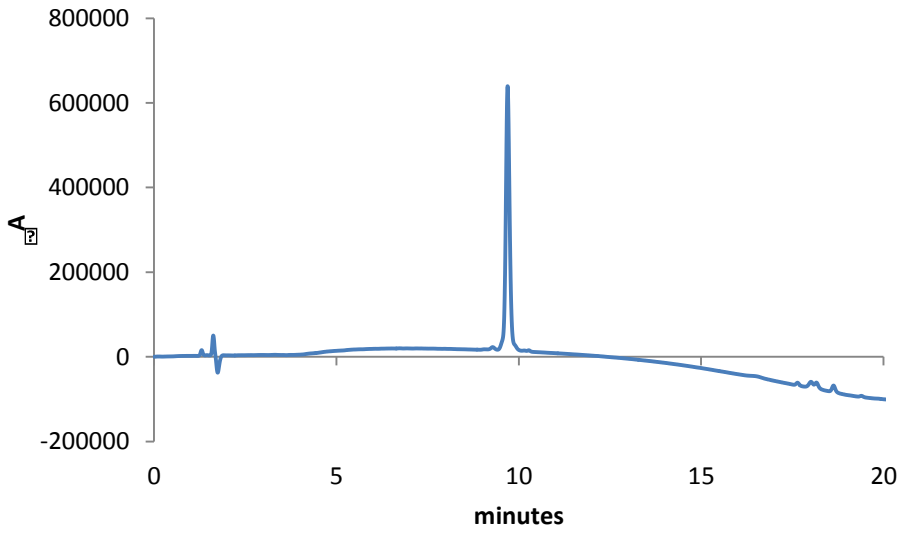


Figure S5: HPLC profile at 210 nm; column:VYdamass C18, 100Å 150x4.6 mm; gradient : acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 80% in 20 minutes.

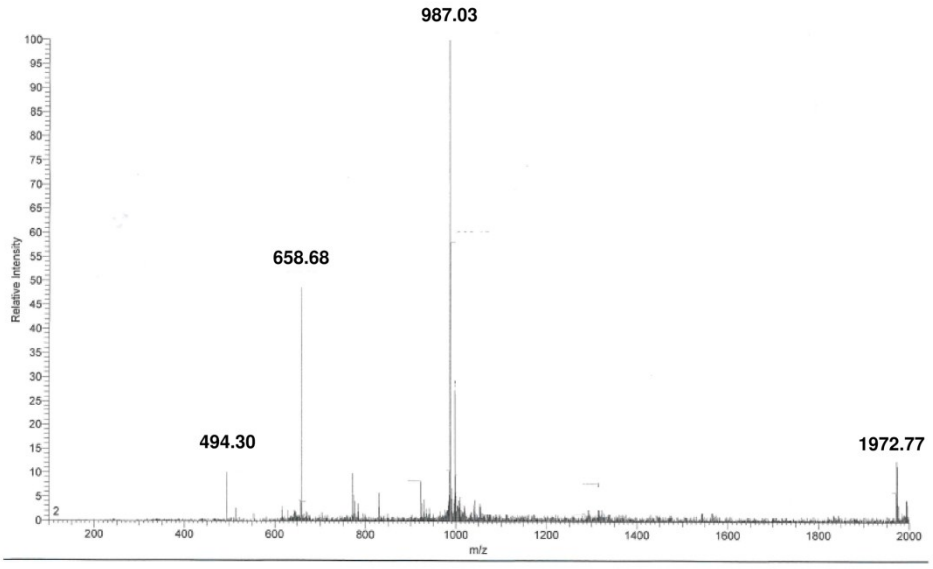


Figure S6: Mass spectrum of purified peptide

Peptide TBKKG6A K Link

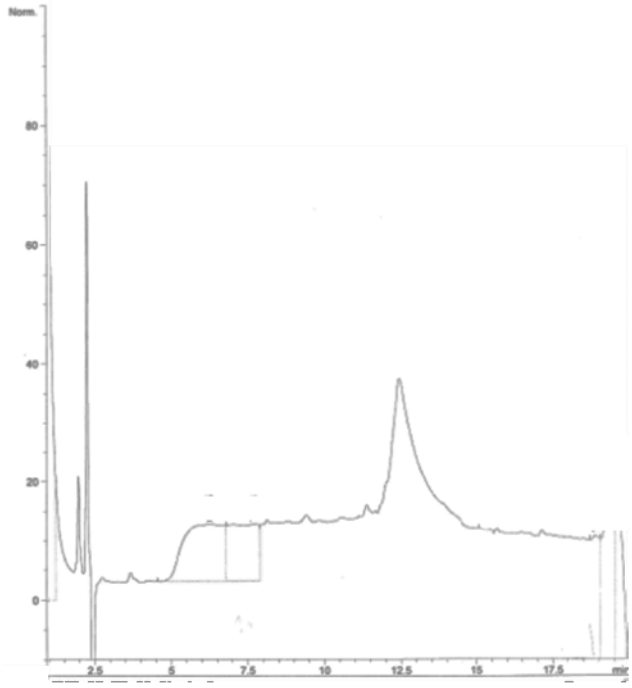


Figure S7: HPLC profile at 210 nm; column: Phenomenex Jupiter 4µ Proteo 90Å 150x4.6 mm, gradient : acetonitrile (0.1% TFA) in water (0.1% TFA) from 15 to 50% in 20 minutes.

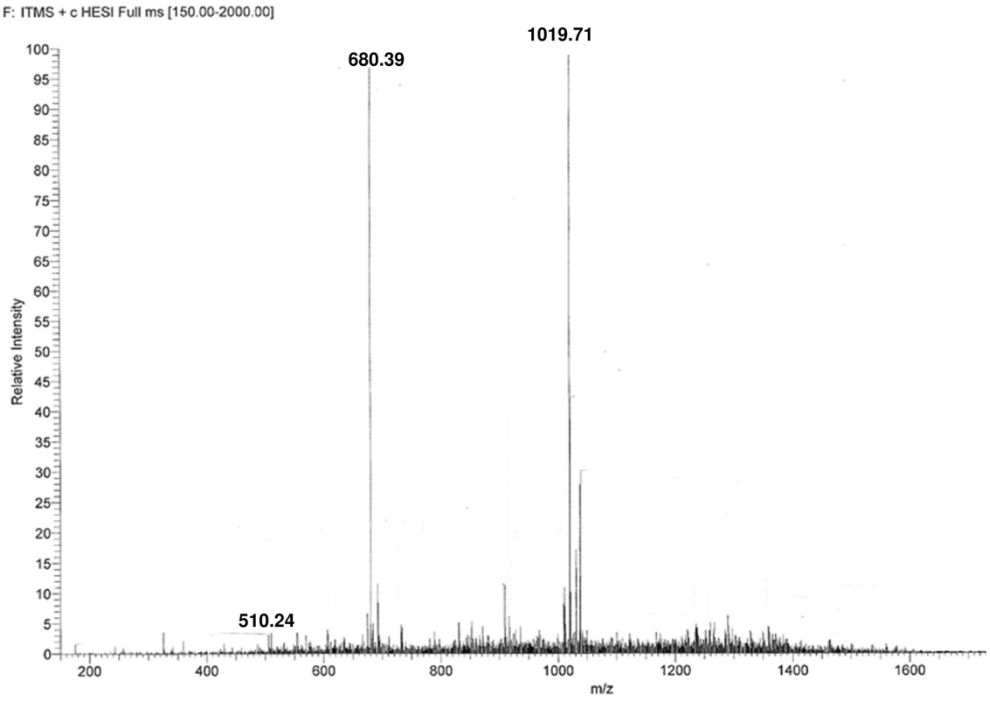


Figure S8: Mass spectrum of purified peptide