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Self-defensive biomaterial coating against bacteria and yeasts: polysaccharide multilayer film with embedded antimicrobial peptide

By *G. Cado⁺, R. Aslam⁺, L. Séon⁺, T. Garnier, R. Fabre, A. Parat, A. Chassepot, J.-C. Voegel, B. Senger, F. Schneider, Y. Frère, L. Jierry, P. Schaaf, H. Kerdjoudj, M.-H. Metz-Boutigue and F. Boulmedais**

+ These authors contributed equally

[*] Dr. F. Boulmedais and Prof. P. Schaaf, Corresponding-Authors, Dr. G. Cado, L. Séon, Dr. T. Garnier, Dr. A. Parat, Dr. Y. Frère, Dr. L. Jierry
Centre National de la Recherche Scientifique, Institut Charles Sadron, UPR 22, 23 rue du Loess, 67034 Strasbourg Cedex 2, France. E-mail: fouzia.boulmedais@ics-cnrs.unistra.fr and pierre.schaaf@unistra.fr

Prof. P. Schaaf, R. Aslam, Dr. R. Fabre, A. Chassepot, Dr. J.-C. Voegel, Dr. B. Senger, Dr M.-H. Metz-Boutigue
Institut National de la Santé et de la Recherche Médicale, INSERM UMR 1121, 11 rue Humann, 67085 Strasbourg Cedex, France
Université de Strasbourg, Faculté de Chirurgie Dentaire, 1 place de l'Hôpital, 67000 Strasbourg, France.

Prof. F. Schneider
Service de Réanimation Médicale, Hôpital de Hautepierre, Hôpitaux Universitaires de Strasbourg, 1 avenue Molière, 67098 Strasbourg Cedex, France.

Dr. H. Kerdjoudj
EA 4691, Université de Reims Champagne Ardenne, SFR-CapSanté, 1 avenue du Maréchal Juin, 51100 Reims.

Dr. L. Jierry, Prof. P. Schaaf
Université de Strasbourg, Ecole Européenne de Chimie, Polymères et Matériaux, 25 rue Becquerel, 67087 Strasbourg Cedex 2, France.

Dr. L. Jierry, Prof. P. Schaaf, Dr. F. Boulmedais
International Center for Frontier Research in Chemistry, 8 allée Gaspard Monge, 67083 Strasbourg, France.

Prof. P. Schaaf
Institut Universitaire de France, 103 boulevard Saint-Michel, 75005 Paris, France.

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40 **Abstract**

Prevention of pathogen colonization of medical implants is a major medical and financial issue since infection by microorganisms constitutes one of the most serious complications after surgery or critical care. Immobilization of antimicrobial molecules on biomaterials surfaces is an efficient approach to prevent biofilm formation. To the best of our knowledge, we developed herein the first self-defensive coating against both bacteria and yeasts where the release of the antimicrobial peptide is triggered by enzymatic degradation of the film due to the pathogens themselves. Biocompatible and biodegradable polysaccharide multilayer films based on functionalized hyaluronic acid by cateslytin (CTL), an endogenous host-defensive antimicrobial peptide, and chitosan (HA-CTL-C/CHI) were deposited on a planar surface with the aim of designing both antibacterial and antifungal coating. After 24 h of incubation, HA-CTL-C/CHI films fully inhibit the development of Gram-positive *Staphylococcus aureus* bacteria and *Candida albicans* yeasts, which are common and virulent pathogens agents encountered in care-associated diseases. Hyaluronidase, secreted by the pathogens, leads to the film degradation and the antimicrobial action of the peptide. Furthermore, the limited fibroblasts adhesion on HA-CTL-C/CHI films, without cytotoxicity, highlights a medically relevant application to prevent infections on catheters or tracheal tubes where fibrous tissue encapsulation is undesirable.

1. Introduction

Implantable medical devices are widely used in surgery not only to replace altered or lost tissues but also in critical care for fluid or gas administration using catheters or tracheal tube, respectively. These devices constitute an open gate for pathogens invasion.^[1] Prevention of pathogen colonization of medical implants constitutes a major medical and financial issue since nosocomial infection represents one of the most serious complications after surgery or critical care. Indeed each year in Europe, 5% of patients admitted to hospitals suffer from hospital-acquired infections leading to a mortality of 10%.^[2] *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium, is responsible for hospital-acquired infections especially in immunocompromized patients. It is one of the most virulent bacteria leading to high rates of device-related systemic infections and mortality.^[3] A recent study has genetically characterized the strains of *S. aureus* responsible of catheter-related infections and demonstrated that 82% of these strains are methicillin resistant and contain numerous genes involved in biofilm formation and bacterial dispersion.^[4] *Candida albicans* (*C. albicans*), the most common human yeast pathogen, possesses the ability to form biofilms that are sources of local and systemic infection. Moreover *C. albicans* biofilms allow the formation of *S. aureus* microcolonies on their surface and even enhanced *S. aureus* resistance to antibiotics.^[5] When associated with bacterial infections, fungal proliferation induces an increased frequency or severity of diseases.^[6-8] The recent resistance of *C. albicans* to antifungal therapies^[9, 10] and of *S. aureus* to antibiotics points out the need of multifunctional coatings that prevent infections of both yeast and bacteria.

Various approaches based on immobilization or release of bactericidal substances, using self-assembled monolayers or grafting of polymers have been explored and extensively reviewed.^[11, 12] Polyelectrolyte multilayer (PEM) films, based on an alternated deposition of polycations and polyanions onto a solid surface, emerged as a simple and efficient approach

to functionalize surfaces in a controlled way.^[13, 14] The first antibacterial PEM films were
 85 designed by insertion of silver nanoparticles.^[15-20] Later on, hydrophobic bactericide using
 dendritic block copolymer^[21] and silver ion containing liposomes^[22] were inserted in PEM
 films to obtain efficient bactericidal coatings. Chitosan based PEM films were demonstrated
 to be antibacterial against *Escherichia coli* and *Enterococcus faecalis*.^[16, 23, 24] Antibiotics,
 like gentamicin, have been inserted in hydrolysable^[25] or crosslinked PEM films^[26] to be later
 90 released. Despite of an improvement of the antibacterial activity of the films, the use of
 antibiotics or silver particles have serious drawbacks because of their limited efficiency, their
 toxicity or their role in the emergence of multi-resisting pathogens.^[27, 28]

Natural antimicrobial peptides (AMPs), secreted by numerous living organisms
 against pathogens, gain increased attention due to their broad spectrum of antimicrobial
 95 activity and their low cytotoxicity^[12]. They predominantly cause disruption of the membrane
 integrity of pathogen agents and thus unlikely initiate the development of resistance.^[12]
 Positively charged AMPs were already used as a part of the PEM architecture to obtain
 antibacterial effect by contact^[29] or by release.^[26, 30] Guyomard et al. succeeded in embedding
 in PEM films a complex of poor water soluble AMPs and amphiphilic polyelectrolytes. They
 100 obtained antibacterial activity against Gram-positive bacteria.^[31] Active PEM films with
 embedded antifungal peptides were also reported.^[32, 33] Yet, it would be of high interest to
 design coatings bearing both antibacterial and antifungal properties. Up to now, only few
 systems share both properties. They are mainly based on silver coating^[34] or quaternary
 ammonium cationic molecules as surfactant,^[35] synthetic polymer^[36] or silane.^[37]

105 To our knowledge, no coating based on AMP peptides possessing both properties has
 been reported so far. To achieve this goal, we used bovine cateslytin (CTL), a Chromogranin
 A (CGA) derived peptide, an endogenous protein, secreted with its numerous natural derived
 peptides by nervous, endocrine and immune cells during infection^[38] acting in the innate

immunity system^[39]. CTL, an AMP corresponding to CGA344-358, acts in the micromolar

110 range with a wide spectrum of antimicrobial activities against Gram-positive bacteria, yeasts and filamentous fungi, without cytotoxic effect on mammalian cells.^[40, 41] Moreover, it is very stable against bacterial proteases.^[42] We used polysaccharide multilayer films based on CTL-C-functionalized hyaluronic acid as polyanion and chitosan as polycation, (HA-CTL-C/CHI), that were deposited on a planar surface with the aim of designing a self-defensive coating

115 against both bacteria and yeasts (**Scheme 1**). A cystein residue (C) was added at the C-terminal end of the CTL sequence to allow its grafting to HA. HA and CHI are biodegradable by enzymatic hydrolysis with hyaluronidase^[43] and chitosanase^[44] respectively. Both polysaccharides are already widely used in biomedical applications due to their interesting intrinsic properties.^[45, 46] The ability of *Staphylococcus*,^[47] *Candida* species^[48] and *M. luteus*

120 to degrade HA, by producing hyaluronidase, allows the CTL-C to be released from PEM films only in the presence of the pathogens. Release of antibacterial compounds (AMPs or classical antibiotics) are usually obtained by passive diffusion from the films at physiological pH^[31, 32] or by pH-induced degradation of the films^[25, 30]. Pavlukhina *et al.* reported the release of antimicrobial agents using pH variations associated with growth of bacteria as an

125 internal trigger to release.^[26] This coating can thus be named as self-defensive as it is related to a local change of the environment of the coating due to the pathogens themselves. This was the first system developed based on this idea. We developed herein a new self-defensive coating where the release of the antimicrobial peptide is triggered by enzymatic degradation of the film due to the pathogens themselves. Polysaccharides adsorbed mass was determined

130 by Surface Plasmon Resonance (SPR). The buildup and the topography of the films were characterized by Atomic Force Microscopy (AFM). Antibacterial and antifungal activities of HA-CTL-C in solution and HA-CTL-C/CHI films were tested against two strains Gram-positive bacteria, i.e. *S. aureus* and *M. luteus* and one strain of yeast strain *C. albicans*,

respectively, by using microdilution assays.^[49] Confocal laser scanning microscopy (CLSM)

135 allowed following the penetration of the fluorescently labeled HA^{FITC}-CTL-C, diluted in solution or embedded in a PEM film, into the cell membrane of *C. albicans*. Finally, the cytotoxicity of HA-CTL-C/CHI films was tested through Human gingival fibroblasts (HGFs) viability.

2. Result and discussion

140 2.1 Physical-chemical characterization of HA-CTL-C/CHI films

CTL-C peptide was covalently coupled to HA in two steps using successively the carbodiimide chemistry to graft maleimide functions on HA and the thiol-maleimide coupling reaction to graft CTL-C on the modified HA. Synthetic procedures of CTL-C peptide and its conjugation to HA are described in Supporting Information (SI). After dialysis and freeze-
145 drying, a coupling ratio of 5% was determined by ¹H-NMR, corresponding to the grafting of approximately 5 CTL-C peptides for 100 HA dimer units (Figure 1a). The antimicrobial and antifungal activities of CTL and modified CTL were tested in solution against two bacterial strains *M. luteus* and *S. aureus* (ATCC 25923) and a yeast *C. albicans*. Minimal inhibitory concentration (MIC) in peptide of CTL, CTL-C and HA-CTL-C were determined using
150 bacterial and fungal assays described in SI. CTL-C remains antimicrobial at micromolar concentration (< 100 μM) and displays an even better activity against *M. luteus* compared to the cystein free CTL peptide. In comparison with the non-grafted CTL-C peptide, MIC value of HA-CTL-C increases from 35 to 45 μM for *S. aureus* and from 20 to 25 μM for *C. albicans* (**Table 1**). In the case of *M. luteus*, the MIC of HA-CTL-C (5 μM) is five times
155 higher than that of free CTL-C (1 μM). In spite of this decrease in efficiency, the CTL-C peptide covalently linked to HA polymer can still be considered as antimicrobial, acting in the micromolar range. The buildup of HA-CTL-C/CHI film was monitored by SPR. A linear increase of the mass adsorbed is observed at each deposited layer of polysaccharide indicating

the buildup of the film (Figure 1b). We investigated the topography and the roughness of HA-

CTL-C/CHI films at different numbers of deposition steps by means of AFM in dry state
(**Figure S-1 in SI**). It was difficult to obtain good quality AFM images in the wet state due to
the viscoelasticity of the films as we found for HA/CHI films in our previous work.^[50] AFM
imaging allows measuring film thickness after scratching. Table S-1 in SI summarizes the
thicknesses and roughnesses measured by AFM. With 5 bilayers, the surface is already
entirely covered with a 5 nm thick film with a roughness of 1.6 nm. As the buildup process
goes on, the film thickness increases up to 52 nm at 30 bilayers. The film grows linearly up to
30 bilayers (**Figure S-2 in SI**) and the film roughness increases up to 16.5 nm (for 30 pairs of
layers).

2.2 Bacterial and fungal assays of HA-CTL-C/CHI films

After characterization of HA-CTL-C/CHI multilayers buildup, the antibacterial and antifungal
activities of the functionalized films were evaluated against two strains of bacteria *M. luteus*
and *S. aureus* and one of yeast strain *C. albicans*, respectively. The influence of the number of
embedded functionalized layers was studied by monitoring pathogen growth for different
films with increasing number of HA-CTL-C/CHI bilayers. To this aim, PEI-(HA/CHI)_{15-n}-
(HA-CTL-C/CHI)_n films, with $n = 0, 5, 10$ and 15 , and PEI-(HA-CTL-C/CHI)₃₀ films were
built. Pathogens were incubated for 24 h in contact with HA-CTL-C/CHI films at 37°C for
bacteria strains and at 30°C for *C. albicans*. For each pathogen, the microbial growth was
measured at different times (1 h, 4 h, 6 h and 24 h) by determination of the optical density at
620 nm (OD₆₂₀) of the bacterial suspension in contact with the film. The data were normalized
to OD₆₂₀ value obtained in similar conditions in the absence of films and are expressed as a
percentage of growth (**Figure 2a-c**). For each tested pathogen, we observed that by increasing
the number of HA-CTL-C/CHI bilayers the normalized microbial growth decreases. An
important decrease of microbial growth is obtained for at least 5 bilayers for *M. luteus* and *C.*

albicans and 15 bilayers for *S. aureus*. After 6 h of incubation, at least 70% of inhibition is

obtained with 15 bilayers for all tested pathogens. After 24 h of incubation, the growth of *M.*

luteus, *C. albicans* and *S. aureus* are fully inhibited with 5, 15 and 30 HA-CTL-C/CHI

bilayers, respectively. These results are in agreement with the MIC values of HA-CTL-C in

solution (Table 1) that follow the same trend towards the different pathogens. In order to

check if the films can be reused several times as antimicrobial coatings, the growth of the

three pathogens was monitored when incubated for 24 h in contact with reused PEI-(HA-

CTL-C/CHI)₁₅ films for *M. luteus* and *C. albicans* and PEI-(HA-CTL-C/CHI)₃₀ film for *S.*

aureus. To this aim, a fresh pathogen suspension was brought in contact with the

functionalized film. After an incubation of 24 h, the supernatant was withdrawn and replaced

by a fresh pathogen suspension. After each withdrawal, the OD₆₂₀ of the supernatant was

measured to determine pathogen growth after 24h of incubation. When *M. luteus* and *C.*

albicans suspensions in contact with (HA-CTL-C/CHI)₁₅ film are renewed every 24 h, a

complete inhibition was observed at least for two and three cycles of use respectively. A

significant decrease in efficiency of (HA-CTL-C/CHI)₁₅ film is detectable after the following

renewal of pathogens suspension (**Figure 2d**). *S. aureus* growth is only inhibited by 40% for

the second use of the (HA-CTL-C/CHI)₃₀ film and becomes fully inefficient in the third cycle.

In parallel, the supernatants withdrawn were incubated with fresh pathogen suspensions for

24 h and the OD₆₂₀ was also measured (**Figure S-3 in SI**). No inhibition of growth was

observed showing that there is no or a weak amount (quite less than the MIC) of CTL-C

released in solution.

2.3 Mechanism of pathogen growth inhibition of HA-CTL-C/CHI

To clarify the mechanism of HA-CTL-C inhibition, we synthesized fluorescently labelled

HA^{FITC}-CTL-C and HA^{FITC} to analyze the films after contact with the pathogens. *S. aureus*

and *C. albicans* are known to secrete hyaluronidase, a class of enzymes able to hydrolyze

HA.^[47, 48] The cutting of one constitutive partner of the film should lead to its degradation as

210 it was observed by Etienne *et al.*^[51] We were then interested in imaging PEI-(HA^{FITC}/CHI)₁₅ films, by CLSM, before and after 24 h of contact with the pathogens. The three of pathogens induce the degradation of HA/CHI films with, however, some differences in the resulting film morphologies (**Figure 3**). After 24h of incubation with *S. aureus*, HA^{FITC}/CHI films were almost totally degraded (**Figure 3a**). The film appears inhomogeneous with fluorescent dots
215 after incubation with *M. luteus* (**Figure 3b**). *C. albicans* induces the formation of honeycombs in the film due to the degradation of HA (**Figure 3c**). Degradation of HA should release CTL-C in the supernatant and promote the contact between CTL-C peptides and the pathogens. To check this hypothesis, we built hyaluronidase resistant films functionalized by CTL-C. CTL-C grafted on poly(allylamine hydrochloride) was thus synthesized and used to build
220 poly(acrylic acid)/CTL-C functionalized poly(allylamine hydrochloride) (PAA/PAH-CTL-C) film. After 24h of incubation, (PAA/PAH-CTL-C)₁₅ films show no inhibition against *C. albicans* (data not shown). This emphasizes the fact that the antimicrobial activity of the HA-CTL-C/CHI film is due to its degradation by the pathogens. This property renders the film specifically active in the presence of hyaluronidase secreted by the pathogens. Pathogens thus
225 initiate their own death when brought in contact with the HA-CTL-C/CHI film. Even though the film is degraded with time in the presence of pathogens, it can be reused at least two and three times without losing its activity against *M. luteus* and *C. albicans*.

Using fluorescently labelled CTL-C, a previous study showed that the peptides penetrate into cell membranes and accumulate inside yeasts.^[40, 41] The interactions of HA^{FITC}-CTL-C and
230 HA^{FITC} with *C. albicans* was thus studied when solubilized in solution or embedded in multilayer films. After 45 min of incubation at 30°C with HA^{FITC} or HA^{FITC}-CTL-C in solution, *C. albicans* were observed by CLSM. The fluorescent HA^{FITC}-CTL-C was detectable in cytoplasm without inducing cell lysis (**Figure 4a**). On the contrary, HA^{FITC} is clearly

observed all around the yeast cells, stacking probably on the membranes and leading to a
 235 honeycomb structure (**Figure 4b**). This suggests that CTL-C can cross the cell membrane,
 even when it is coupled to HA, leading to an accumulation of HA inside the cytoplasm. To
 image them by CLSM, the films were treated by paraformaldehyde (PFA) to fix the pathogen
 in contact. This treatment was first applied on the films to evaluate its effect. PFA treatment
 induces no change in the case of HA^{FITC}/CHI films in contrary to HA^{FITC}-CTL-C/CHI films
 240 (**Figure S-4 in SI**) where heterogeneities appear. *C. albicans* were incubated for 45 min at
 30°C in contact with PEI-(HA^{FITC}-CTL-C/CHI)₁₅ film and then observed by CLSM. Among
 the heterogeneities due to PFA treatment, a strong green fluorescence is observed mainly
 inside the yeast (**Figure 4c**). In the case of HA^{FITC}/CHI films, a little fluorescence is localized
 inside the yeasts and only few of them are strongly fluorescent (**Figure 4d**). In spite of its
 245 insertion into the PEM films, CTL-C allows the penetration of HA^{FITC}-CTL-C inside the
 yeasts explaining the activity of the films.

2.4 Biocompatibility tests of HA-CTL-C/CHI films

Finally, it is important to ensure that the film is not cytotoxic to healthy wound healing cells.

250 Fibroblasts are one of the first anchorage-dependent cells to come at an implant surface
 during the wound healing process. The viability of human gingival fibroblasts (HGFs)
 cultivated on PEI-(HA-CTL-C/CHI)₁₅ films, compared to PEI-(HA/CHI)₁₅ films and glass
 substrate, has been evaluated through their mitochondrial activity, monitored by Alamar
 blue™ assays. Already after one day, the metabolic cell activity measured on HA-CTL-
 255 C/CHI films is lower compared to HA/CHI films and non-coated glass substrate (**Figure 5a**).
 The good biocompatibility of HA/CHI films towards HGFs, mediated *via* CD44 receptor, was
 already reported in our previous work.^[50] After 7 days of culture, the number of viable HGFs

on HA-CTL-C/CHI films represents 25% of viable HGFs on HA/CHI films. The functionalization of HA by CTL-C peptide induces a lower adhesion of HGFs which seems to slow down the proliferation of cells. Knowing that HA-CTL-C in solution at 100 μ M did not show any cytotoxicity (data not shown), such behavior would suggest that the multilayers are not cytotoxic. To discriminate between cytotoxic or low initial adhesion properties of HA-CTL-C/CHI, we performed two experiments. First after 24 h of contact, suspended cells harvested from HA-CTL-C/CHI film supernatant were passed to fresh culture plates. After 24 h of culture, many cells readily attached and spread as fresh cells. Second, we analyzed the biocompatibility of films by a complementary assay based on cell spreading *via* cytoskeleton arrangement when the cells are seeded on a glass substrate half coated by (HA-CTL-C/CHI)₁₅. After 24 h, HGFs adhere on the glass substrate (**Figure 5b**, zone II), but to a lesser degree on (HA-CTL-C/CHI)₁₅ film (**Figure 5b**, zone I). After 24 h of culture, a confluent layer with a typical fibroblastic cell shape and with polymerized F-actin fibers is observed on the glass substrate (**Figure S-5a in SI**). On the HA-CTL-C/CHI film, cells appear less elongated and have a peripheral actin distribution (**Figure S-5b in SI**). This indicates that (HA-CTL-C/CHI)₁₅ films are not cytotoxic but seem rather anti-adherent towards HGFs. It is known that persistent excessive functions of fibroblasts have been linked to detrimental fibrous tissue formation which may cause implant failure. The present results of decreased fibroblast adhesion on functionalized substrate with HA-CTL-C/CHI films shows promise for implant applications.

3. Conclusions

In conclusion, we designed a new surface coating based on polysaccharide multilayer films containing a functionalized HA with 5% of CTL-C, a peptide possessing both antibacterial and antifungal properties. Antimicrobial properties of CTL-C were preserved when grafted on HA either in solution or when embedded into PEM films. After 24 h of incubation, HA-CTL-

C/CHI films fully inhibit the development of *S. aureus* and *C. albicans*, which are common and virulent pathogens agents encountered in care-associated diseases. The presence of CTL-C peptides on HA allows the penetration of the modified polysaccharide inside *C. albicans* after 45 min of contact. The secretion of hyaluronidase by all tested pathogens seems to be responsible for HA-CTL-C release from the film and for its activity. The film can keep its activity during 3 cycles of use against fresh incubated *C. albicans* suspension. Furthermore, the limited fibroblasts adhesion, without cytotoxicity, on HA-CTL-C/CHI films highlights a medically relevant application to prevent infections on catheters or tracheal tubes where fibrous tissue encapsulation is undesirable.

4. Experimental

Polysaccharide solutions. Chitosan (CHI, PROTASAN Ultrapure Chitosan CL213, 260 000 g/mol, DA 83 %) was purchased by Novamatrix (Sandvika, Norway). Dried Sodium Hyaluronate (HA, 420 000 g/mol) was purchased by Lifecore (Chaska, USA). Poly(ethylene imine) (PEI, 60 000 g/mol, 50% in water), sodium chloride, sodium dodecyl sulfate (SDS), HCl and NaOH were purchased by Sigma-Aldrich (Quentin-Fallavier, France). All products were used without further purification. 150 mM NaCl solution was prepared with Ultrapure Milli-Q® water having a resistivity of 18.2 MΩ.cm. CHI and HA solutions were prepared at 0.3 mg/mL in 150 mM NaCl adjusted at pH 4 with NaOH or HCl solutions. HA-CTL-C and HA^{FITC}-CTL-C solutions were prepared at 0.2 mg/mL and 0.1 mg/mL, respectively. PEI solution was prepared at 0.5 mg/mL in 150 mM NaCl solution adjusted at pH 7.5 with NaOH or HCl solutions.

Film buildup. Before PEM buildup, glass coverslips (Ø = 14 mm; VWR, Strasbourg, France) were cleaned in a SDS solution at 0.01 M, ultrasonicated for 5 min and then submitted to a hot 0.1 M HCl solution for 10 min and finally rinsed with ultrapure Milli-Q® water. The precursor PEI layer and CHI/HA films were deposited using an automated

spraying device described in a previous work^[50]. CHI/HA-CTL-C and CHI/HA^{FITC}-CTL-C

films have been prepared in 24-well plates on PEI precoated glass substrates. 300 μ L of the

310 polycation solution were deposited for 5 min on glass coverslips followed by a rinsing step

(500 μ L) with ultra Milli Q water. Then, 300 μ L of polyanion solution were deposited for 5

min followed by a rinsing step (500 μ L) with ultra Milli Q water. To test the antimicrobial

activity of PEM films versus the number of HA-CTL-C layers, PEI-[HA/CHI]_{15-n}[HA-CTL-

C/CHI]_n with n equal to 0, 5, 10 and 15 and PEI-[HA-CTL-C/CHI]₃₀ films were built.

315 *Surface Plasmon Resonance.* Surface plasmon resonance (SPR)^[53, 54] as some other

detection techniques (optical waveguide lightmode spectroscopy, quartz crystal

microbalance), provides a label-free, *in situ* method to monitor the buildup of e.g. a polymer

film on a solid surface. When a beam of light passes from a material of refractive index, n_1 ,

into a material with a lower refractive index, n_2 , some light is reflected from the interface.

320 When the angle of incidence of the light, θ , on the interface is greater than $\theta_{TIR} =$

$\arcsin(n_2 / n_1)$, the light is completely reflected (total internal reflection). In the case of the

SPR instrument used here (SPR NaviTM 200, Bionavis, Finland), the most refractive medium

is a BK7-glass prism. Because the bottom surface of the prism is coated with a thin film of a

noble metal (gold), this reflection is not total; some of the light is 'lost' into the metallic film.

325 There then exists a second angle, $\theta_{SPR} > \theta_{TIR}$, at which the intensity of the reflected light goes

through a minimum. In addition, an evanescent electrical field travels for a short distance into

the medium (e.g. film) from the metallic film. The probing distance reaches generally a few

hundreds of nm depending on the refractive index of the probed medium and θ_{SPR} is sensitive

to the optical characteristics of the deposited film. The reflection curves, i.e. the variation of

330 the intensity of the reflected light with θ , corresponding to the successive polymer deposition

steps have been analyzed with a home-made software based on Maxwell's equations. The

parameters involved are the refractive indices of the prism, the chromium and gold layers, the

polymer film (n_{film}) and the buffer solution (n_{sol}), as well as the thickness of the chromium and gold layers and of the film (d_{film}). From n_{film} , n_{sol} and d_{film} , we derive the mass of film per unit area:

$$m = \frac{(n_{\text{film}} - n_{\text{sol}})d_{\text{film}}}{dn/dc} \quad (1)$$

where dn/dc is the change in refractive index with concentration and is equal to $0.197 \text{ cm}^3/\text{g}$ for polyelectrolytes^[55]. The SPR experiment was performed under a continuous flow rate of $100 \mu\text{L}/\text{min}$ of 150 mM NaCl solution adjusted at pH 4. The time of deposition and of rinsing of polyelectrolytes was fixed at 5 min.

Atomic Force Microscopy (AFM). Atomic force microscopy (AFM) images were obtained in contact mode in dry state with the Nanoscope IV from Veeco (Santa Barbara, CA). The images were carried out with silicon nitride cantilevers, spring constant 0.03 N/m (model MSCTAUHW, Veeco, CA). Several scans were performed over a given surface area. These scans had to give reproducible images to ascertain that there is no sample damage induced by the tip. Deflection and height mode images are scanned simultaneously at a fixed scan rate (2 Hz) with a resolution of 512×512 pixels. Data evaluation was performed with the NanoScope software version 5.31r1 (Digital Instruments, Veeco). The buildup of the film was made on glass substrate and the film thickness was measured by using the “scratch” method. Profilometric section analyses of a scratched film allowed us to determine precisely the quality of the film and its thickness over the scanned area. The scratches were achieved with a plastic cone tip and were always imaged perpendicular to the fast scan axis. The profiles correspond to a cross section along this axis. The mean thickness of the scratched film was determined by measuring the thickness at least on three areas.

Confocal Laser Scanning Microscopy (CLSM). Confocal laser scanning microscopy (CLSM) observations were carried out with a Zeiss LSM-510 microscope using a $40 \times / 1.31$ oil

immersion objective and a 0.43 μm z-section interval. FITC fluorescence was detected upon excitation at 488 nm, through a cut-off dichroic mirror and an emission bandpass filter of 505–530 nm (green).

360 *Bacterial and fungal growth.* To determine the antibacterial activity, microdilution assay was used on two bacterial strains *M. luteus* (A270) and *S. aureus* (ATCC25923). The antifungal activity was tested against one yeast strain *C. albicans*. Bacteria were precultured aerobically in Mueller-Hinton (MH) broth (Merck, Darmstadt, Germany) with agitation at 37°C for 18 h. *C. albicans* preculture was carried out on a Sabouraud broth (SB) medium 365 (BioMérieux S.A., Marcy l'Etoile, France) with agitation at 30°C for 24 h. The antimicrobial activity was tested using a mid-logarithmic-phase culture of bacteria or yeast with an initial optical density at 620 nm (OD_{620}) of 0.001. For the tests performed in solution, aqueous solutions of CTL, CTL-C and HA-CTL-C were prepared in bacterial or yeast medium. Aliquots of CTL-C or HA-CTL-C (10 μL) were incubated in 96-well microplates (Falcon, 370 Becton Dickinson, USA) with 90 μL of bacteria or yeast at final concentrations of 100 μM to 2 μM of peptide. After 24 h of incubation at 37°C under gentle stirring, the OD_{620} of the 96-well plate was measured by a microplate reader.^[56, 57] MIC_{100} , the minimal inhibitory concentration, is the lowest concentration that is able to completely inhibit the growth of bacterial or fungi after 24 h of contact. To test the antibacterial and antifungal properties of 375 the multilayer films, $\text{PEI}-(\text{HA}/\text{CHI})_{15-n}-(\text{HA-CTL-C}/\text{CHI})_n$ with $n = 0, 5, 10$ and 15 and $\text{PEI}-(\text{HA-CTL-C}/\text{CHI})_{30}$ films were prepared. 400 μL of a mid-logarithmic-phase culture of bacteria or yeast with OD_{620} of 0.001 were placed in 24-well plate containing multilayer films. For each tested film, 100 μL of the supernatant was taken to measure its OD_{620} by a microplate reader. The withdrawn volume was compensated with the appropriated fresh 380 bacteria or yeast solution of the same measured OD_{620} . Intermittent controls were performed by inoculating the culture medium on MH (resp. SB) agar plates by spreading method and

counting colonies for verification at different steps. Several controls were used: a fresh medium without inoculation of pathogens was used to ensure sterility, a mixture of Tetracycline (10 µg/mL) and Cefotaxime (0.1 µg/mL) was used as positive control (90 µL of culture and 10 µL of antibiotics) and a fresh inoculated culture medium without any addition was taken as negative control. For the bacterial and yeast quantification, a conversion factor was devised to convert OD into bacterial and yeast counting, respectively. Briefly, OD of fresh bacteria (resp. yeast) culture was measured at 620 nm and was plated to MH (resp. SB) agar for 24 h at 37°C (resp. 30°C). Colony count was performed and plotted versus the OD₆₂₀ and the slope of the linear curve was taken as conversion factor. Pathogen quantification (colony forming unit (CFU) per mL) was performed at time zero and then at each hour for 6 h and then finally at 24 h. For quantification, conversion factors were determined for each strain used separately.^[58] Each assay was performed in triplicate and the experiments were repeated at least three times. The normalized growth of pathogens (in %) was estimated by comparing the OD₆₂₀ values in the presence of multilayer films and the positive and the negative controls. The OD₆₂₀ value of control cultures growing in the absence of films and antibiotics was taken as 100% growth (negative control) and the OD₆₂₀ value of cultures growing in the presence of antibiotics (Tetracycline and Cefotaxime) was taken as 0% growth (positive control). To this aim, the following equation was used:

$$Normalized\ pathogen\ growth\ (\%) = \frac{(OD_{620, sample} - OD_{620, positive\ control})}{(OD_{620, negative\ control} - OD_{620, positive\ control})} \times 100$$

To follow the interaction of fluorescently labeled HA^{FITC}-CTL-C in solution and HA^{FITC}-CTL-C/CHI films with *C. albicans*, CLSM was used based on a protocol previously described with few modifications.^[59] Briefly, for experiments done with HA^{FITC}-CTL-C in solution, poly-L-Lysine coated microscopic slides were covered with fresh medium containing *C. albicans* (OD₆₂₀ = 0.001) in SB culture medium and incubated for 24 h at 30°C without

agitation. The medium was then removed and replaced by 50 μM (in peptide) of HA^{FITC}-CTL-C. After an incubation period of 45 min, the slides were washed with fresh culture medium and subsequently treated for 30 min with 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.3. After several rinsing steps with PBS, slides were covered with Moviol
410 4-88 (Aldrich, Steinheim, Germany). For experiments performed with HA^{FITC}-CTL-C/CHI, fresh *C. albicans* medium, previously incubated at OD₆₂₀ = 0.001 in SB culture medium for 24 h at 30°C without agitation, was put in contact with PEI-(HA^{FITC}-CTL-C/CHI)₁₅ films for 45 min at 30°C without agitation. HA^{FITC} in solution and HA^{FITC}/CHI films were used as control. *C. albicans* were subjected to optical serial sectioning (0.2 - 0.3 μm) to produce
415 images in the x-y plane. Each optical section was scanned several times to obtain an average image. Images were recorded digitally in a 768 \times 576 pixel format.

Human gingival fibroblasts viability assays. The biocompatibility of films was tested using human gingival fibroblasts (HGFs). HGFs were extracted from human gingival connective tissue of healthy individuals according to a protocol approved by the ethics
420 committee for patient protection of CPP Strasbourg Hospitals. Cells were grown in DMEM containing 1 g/L of glucose and supplemented with 10% fetal bovine serum and 100 IU/mL penicillin-streptomycin (all from Gibco®). Cells were cultured at 37°C in 5% CO₂ in 75 cm² flasks. HGFs were used between the 6th and the 9th passage. Before cell seeding, glass coverslips (\varnothing = 14 mm) coated with different architectures were irradiated by UV for 15 min.
425 Then HGFs were seeded at 3×10^4 cells per cm² and cultivated at 37°C under a 5% CO₂ humidified atmosphere for different culture times (Day 1, Day 2 and Day 7). Cell viability was assessed by Alamar BlueTM assay (Biosource International). This assay is based on the reduction of the blue, non-fluorescent resazurin dye to the pink and fluorescent resorufin dye by living cells.^[60] The overall conversion rate is proportional to the metabolic activity of
430 living cells.^[61] Viability was assessed for different times 1, 2 and 7 days. After rinsing with

PBS, cells were incubated with 10% reagent in complete medium for 2 h. After incubation optical density (OD) at 570 nm and 630 nm were determined with a microplate reader. The percentage of reduction of Alamar Blue™ was calculated according to the procedure provided by the manufacturer. The experiments were performed in triplicate. Actin filament staining with tetramethylrhodamine B isothiocyanate coupled phalloidin® (Sigma-Aldrich) and DAPI (Invitrogen) nuclei counterstaining were performed as follows: cells were fixed with 3.7% paraformaldehyde for 10 min at 4°C, permeabilized in 0.25% Triton X-100 in PBS for 10 min, and blocked in 1% BSA-PBS for 30 min. Thereafter, cells were incubated for 30 min at room temperature with 5×10^{-5} mg/mL mL phalloidin® followed by nuclear counterstaining with DAPI (50 ng/mL) incubated for 2 min at room temperature. Washed slides were mounted on blades with DAKO fluorescent mounting medium and fluorescence distribution was examined by means of an inverse fluorescence microscope (Axiovert, Zeiss).

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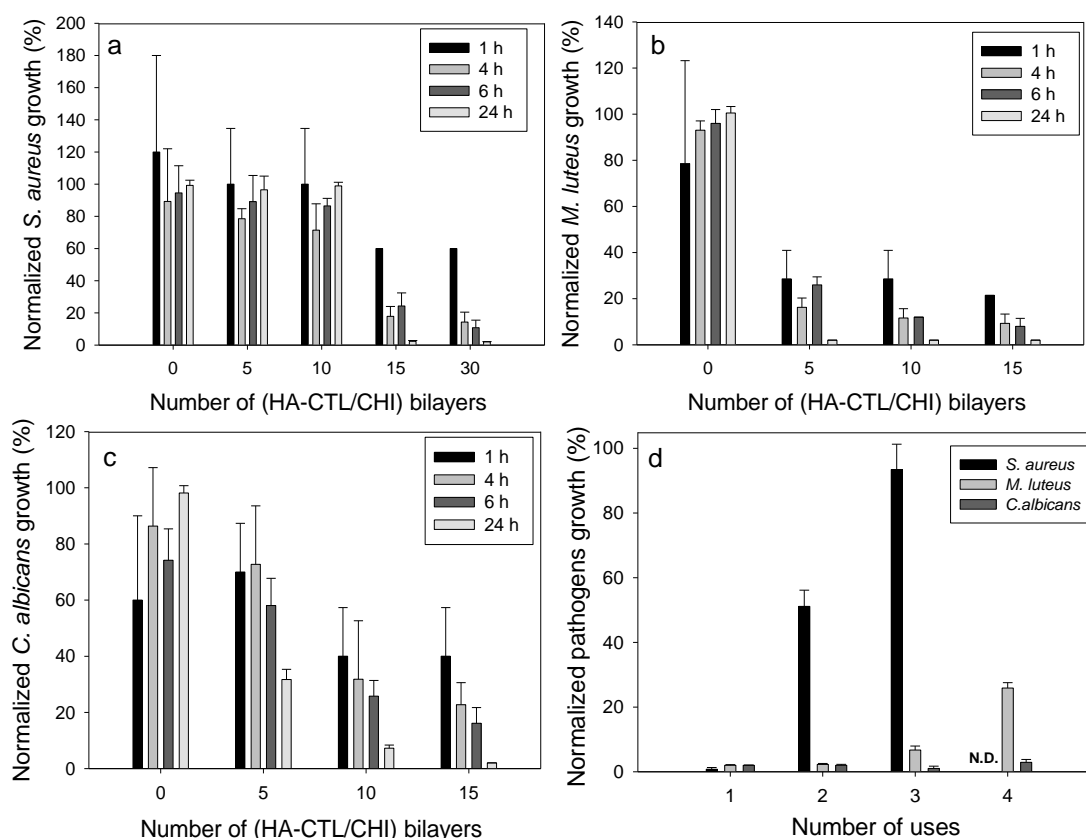


Figure 2. Normalized growth of (a) *S. aureus*, (b) *M. luteus* and (c) *C. albicans* incubated for 1 to 24 h in contact with PEI-(HA/CHI)_{15-n}-(HA-CTL-C/CHI)_n with $n = 0$ to 15 and (HA-CTL-C/CHI)₃₀ multilayer films. (d) Normalized growth of *S. aureus* incubated on PEI-(HA-CTL-C/CHI)₃₀ and *M. luteus* and *C. albicans* incubated on PEI-(HA-CTL-C/CHI)₁₅ films, as a function of the number of uses. The film was brought in contact with a fresh pathogen suspension for 24 h. Every 24 h, the supernatant is removed and replaced by a fresh suspension and its OD₆₂₀ is measured. The films were built in 150 mM NaCl at pH 4. The normalization was performed with respect to OD₆₂₀ measured in the absence of film and antibiotics taken as 100% growth and in the presence of antibiotic taken as 0% growth. ND means not determined.

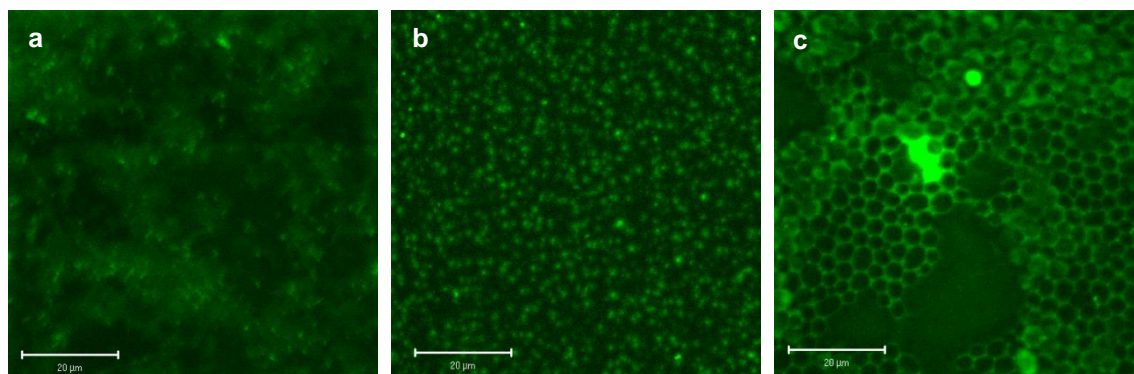


Figure 3. CLSM images of PEI-(HA^{FITC}/CHI)₁₅ after 24 h of incubation with (a) *S. aureus*, (b) *M. luteus* and (c) *C. albicans*. All the films underwent a PFA treatment. The scale bars represent 20 μm.

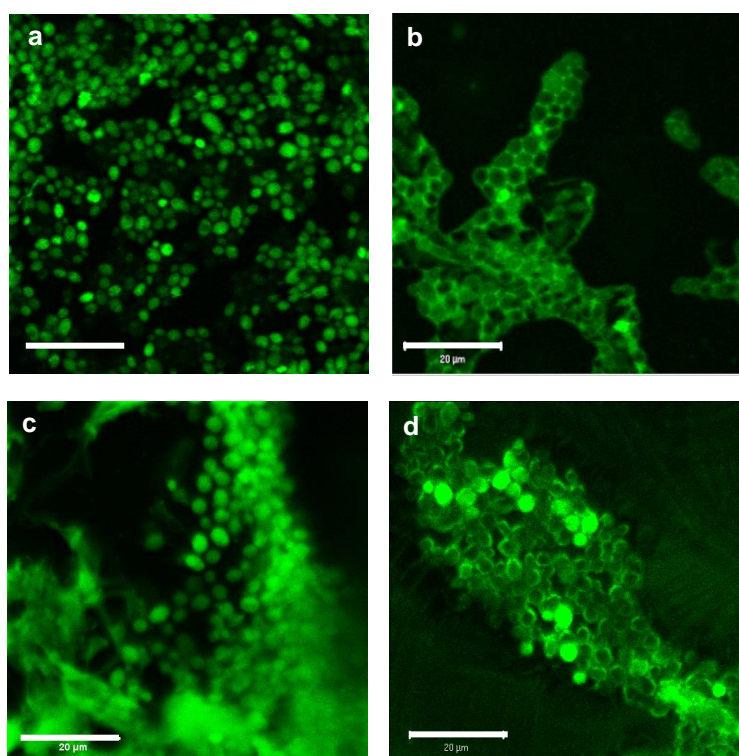


Figure 4. CLSM images of *C. albicans*, after 45 min of incubation, (a) in the presence of HA^{FITC}-CTL-C in solution (50 μM in peptide), (b) in the presence of HA^{FITC} in solution, (c) in contact with PEI-(HA^{FITC}-CTL-C/CHI)₁₅ and (d) in contact with PEI-(HA^{FITC}/CHI)₁₅ multilayer films. The scale bars represent 20 μm.

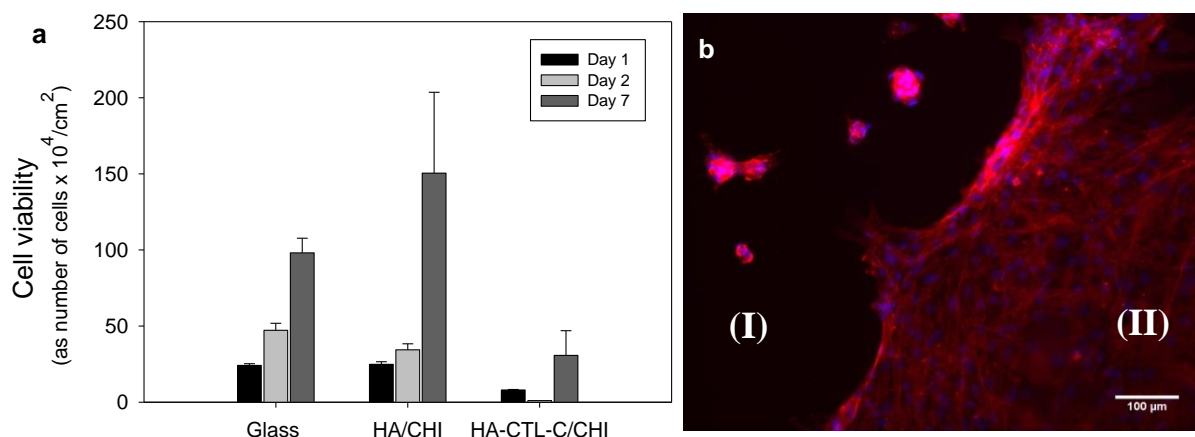


Figure 5. (a) Viability of HGFs, evaluated by metabolic activity of cells, cultured on PEI-(HA/CHI)₁₅, named HA/CHI and PEI-(HA-CTL-C/CHI)₁₅, named HA-CTL-C/CHI, films built on glass substrates. Cell viability was determined by Alamar Blue™ assays after 1, 2 and 7 days of culture. The values represent the mean and the standard deviation of three films. (b) Cytoskeleton visualization by actin filament immunofluorescent staining with phalloidin® (red labeling) and DAPI nuclei counterstaining (blue labeling) of HGFs after 24 h of culture on half coated glass substrate by PEI-(HA-CTL-C/CHI)₁₅: the areas represent (I) the HA-CTL-C/CHI film and (II) the bare glass substrate, respectively.

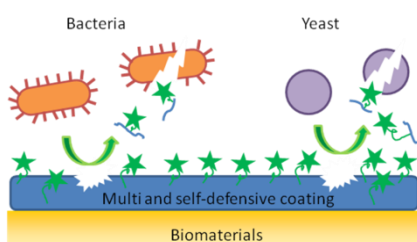
The table of contents entry:

Polysaccharide multilayer films based on antimicrobial peptide functionalized hyaluronic acid as polyanion and chitosan as polycation, is deposited on a planar surface with the aim of designing a self-defensive coating against both bacteria and yeasts.

Keyword: Biomedical Applications

G. Cado, R. Aslam, L. Séon, T. Garnier, R. Fabre, A. Parat, A. Chassepot, J.-C. Voegel, B. Senger, F. Schneider, Y. Frère, L. Jierry, P. Schaaf^{*}, H. Kerdjoudj, M.-H. Metz-Boutigue and F. Boulmedais^{*}

Self-defensive biomaterial coating against bacteria and yeasts: polysaccharide multilayer film with embedded antimicrobial peptide



Supporting Information

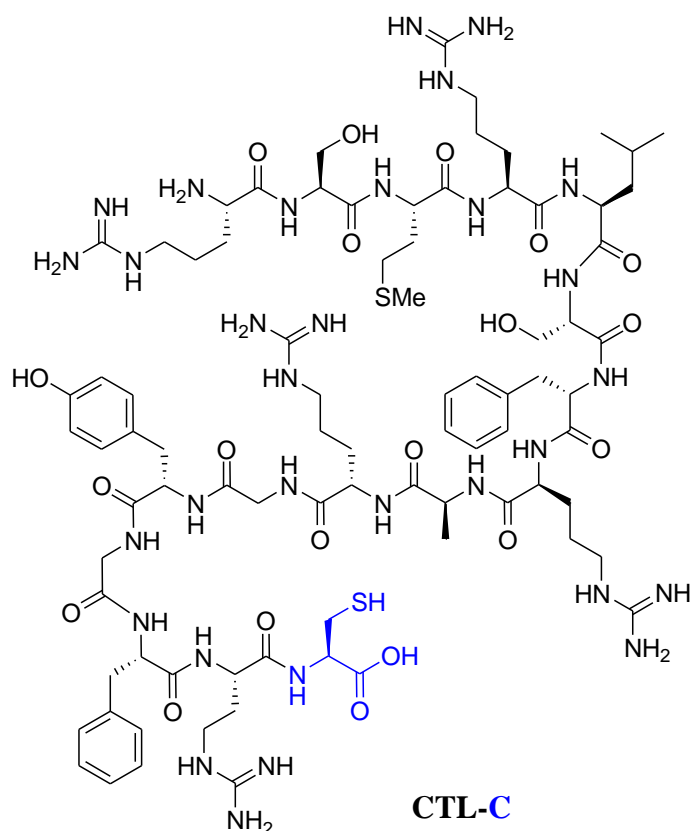
685 **Self-defensive biomaterial coating against bacteria and yeasts: polysaccharide
multilayer film with embedded antimicrobial peptide**

*G. Cado, R. Aslam, L. Séon, T. Garnier, R. Fabre, A. Parat, A. Chassepot, J.-C. Voegel, B.
Senger, F. Schneider, Y. Frère, L. Jierry, P. Schaaf*, H. Kerdjoudj, M.-H. Metz-Boutigue
690 and F. Boulmedais**

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Abbreviations: AMP = antimicrobial peptides, CGA₃₄₄₋₃₅₈ = chromogranin A 344-358; CTL = cateslytin; CHI = chitosan; CTL-C = cateslytin with a cystein in C terminal; HA = hyaluronic acid; EDC = N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; sulfo-NHS = N-Hydroxysulfosuccinimide sodium salt; HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; TCEP = tris(2-carboxyethyl)phosphine hydrochloride; Da = Dalton; tBuOH = tertio-butanol; DS = degree of substitution; NMR = nuclear magnetic resonance; MHz = megahertz; ppm = parts per million ; br = broad; s = singlet; d = doublet; m = multiplet.

1. Preparation and analysis of synthetic antimicrobial peptides.



The synthetic peptide corresponding to bovine CGA (Uniprot P05059), named CTL, (CGA₃₄₄₋₃₅₈: RSMRLSFRARGYGFR) was synthesized with a cystein at the C-terminal end (CTL-C) on a phase approach with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry.^[1] After purification by a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Macherey Nagel Nucleosil RP 300-5C18 column (10 × 250 mm; particle size 5 μm and pore size 100 nm), the peptide was analyzed by mass spectrometry (MALDI-TOF) and automated Edman sequencing on an Applied Sequencing System Procise (Applied Biosystems, Foster City, USA).^[2] MALDI mass measurements were carried out on an Ultraflex™ TOF/TOF (BrukerDaltonics, USA) to perform a rapid control of synthetic peptides according to the procedure previously reported.^[3]

2. Preparation of modified HA and PAH.

715 Peptide and FITC functionalized HA, *i.e.* HA-CTL-C, HA^{FITC} and HA^{FITC}-CTL-C, and peptide modified PAH have been prepared according to the general synthetic pathway described below.

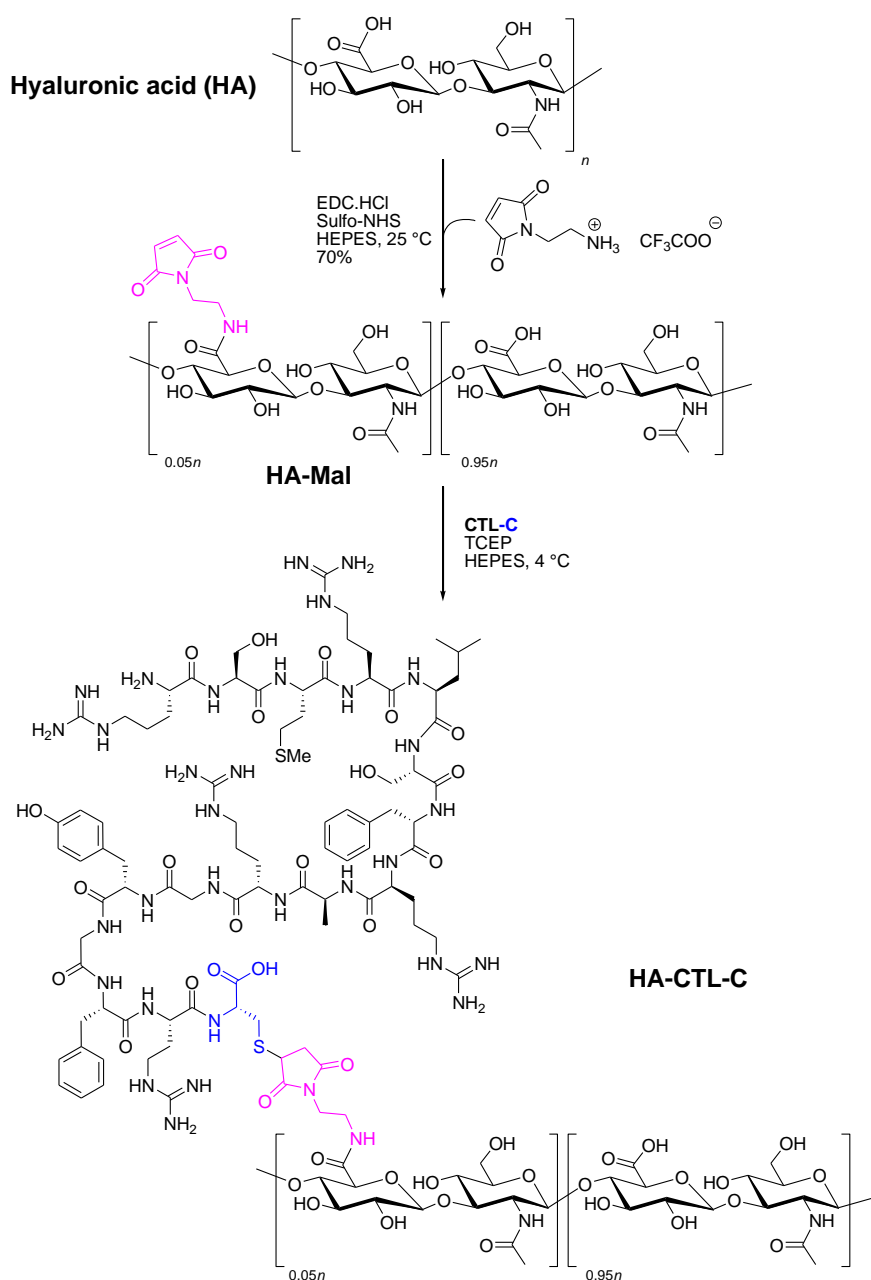
2.1. Preparation of HA-CTL-C

720 Hyaluronic acid (HA) 420 000 Da, was functionalized with maleimide group according to previously reported method.^[4] HA (100.9 mg) was dissolved in 15 mL of 0.01M HEPES buffer (pH 6). EDC (35.2 mg) and sulfo-NHS (10.2 mg) were added to this solution and the resulting mixture was stirred 2 h at room temperature. Afterwards, N-(2-aminoethyl) maleimide trifluoroacetate salt (6.7 mg) was added to the reaction mixture and let stirred for 20 h. The mixture was dialyzed (cut-off 12 000-14 000 Da) first against 0.5 M NaCl for one day and MilliQ water for 6 days. Water was changed every day. HA-Mal (86 mg) was 725 obtained as a white solid after freeze-drying. The degree of substitution (DS) of HA-Mal, defined as the number of maleimide groups per 100 disaccharide unit of HA, was determined by ¹H NMR (D₂O, 400 MHz). All spectra were realized in D₂O containing 5% of *t*BuOH: the singlet of the *t*-butyl group of *t*BuOH was calibrated at δ 1.24 ppm and thus used as internal 730 reference. The singlet at δ 7.1 ppm was assigned to the two symmetric protons on the double bond of the maleimide group. By comparing the integration of this signal with the singlet at δ 2.0 ppm, assigned to the methyl protons of the acetamide group, the DS was determined equivalent to 5%.

735 **¹H NMR** (D₂O, 400MHz, δ ppm): δ 6.9 (s, maleimide), 4.5 (br d, HA), 3.5 (m), 2.0 (s, acetyl group of HA).

The coupling reaction between HA-Mal 5% (80 mg) and CTL-C (10.1 mg) was performed at 4°C for 20°h in 16mL of 0.01M HEPES buffer (pH 6) and 50 μ M of TCEP. Then, the mixture was dialyzed (cut-off 50 000Da) against 0.5 M NaCl for one day and MilliQ water for 6 days. Water was changed every day. Peptide-conjugated HA, named HA- 740 CTL-C, was freeze-dried to provide a white solid with 80-85% of overall yield (\approx 79 mg). The DS, defined as the number of CTL-C peptide per 100 disaccharide units of HA, was determined by ¹H NMR (D₂O, 400 MHz). All aromatic signals between δ 7.0 and 7.5 ppm corresponding to the protons of the aminoacids Phe and Tyr were compared to the singlet at δ 2.0 ppm (assigned as the methyl group of the acetamide). A DS of 5% is determined.

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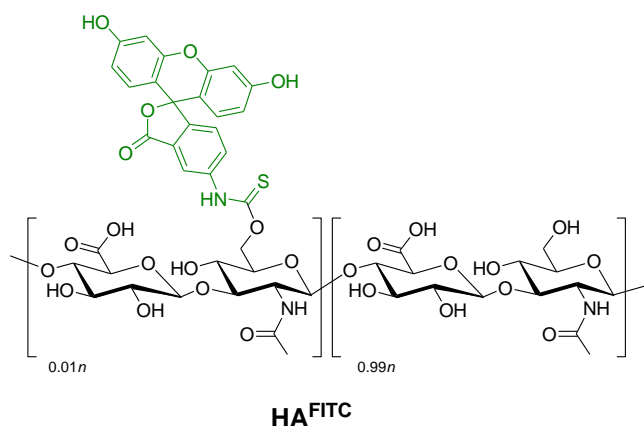


^1H NMR (D_2O , 400MHz, δ ppm): 7.35 (br s, Ar aminoacid of CTL-C), 7.25 (br s, Ar aminoacid of CTL-C), 7.10 (br s, Ar aminoacid of CTL-C), 6.85 (br s, Ar aminoacid of CTL-C), 4.50 (br s, HA), 4.40 (br s, CTL-C), 3.10 (br s, CTL-C), 2.50 (br s, CTL-C), 2.00 (s, methyl from acetyl group of HA), 1.75 (br s, CTL-C), 0.95 (br s, CTL-C).

2.2. Preparation of HA^{FITC}

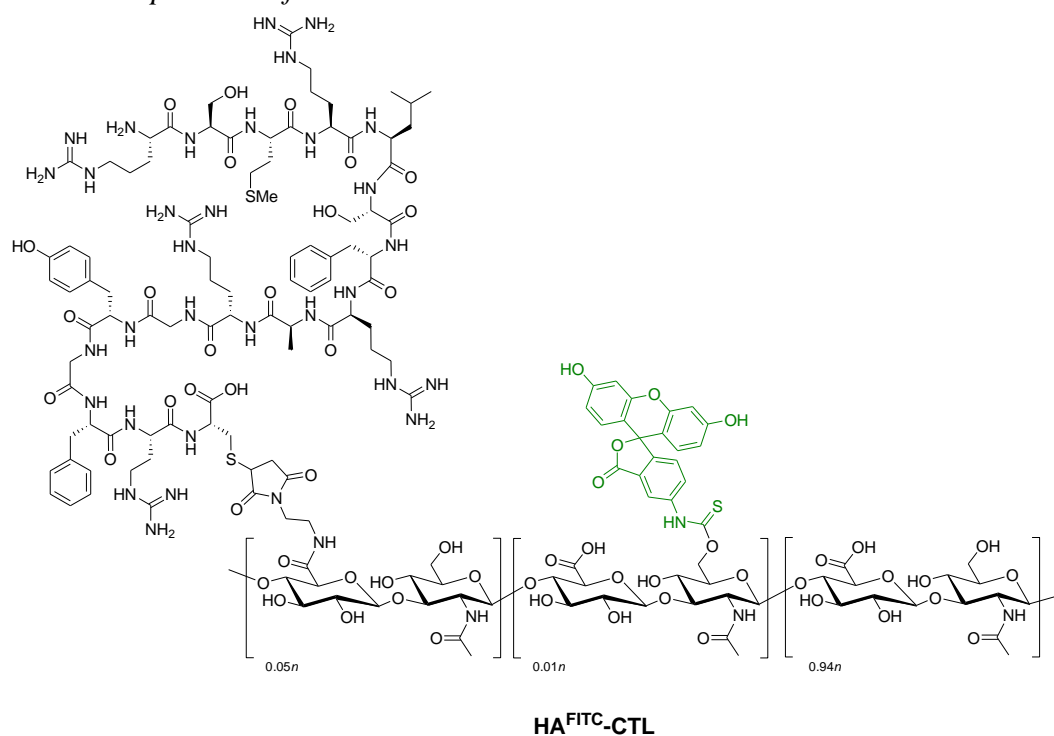
Fluorescein Isothiocyanate (FITC) has been covalently attached to HA according to the following procedure: a solution of FITC (41 μmol dissolved in 2 mL of DMSO) and a solution of HA (0.31 μmol dissolved in 18 mL of deionized water) were mixed. The resulting solution was then adjusted at pH 9 by using a 0.01M NaOH solution. The reaction mixture

was stirred for 12 h at room temperature. Then, 40 mL of deionized water was added and this
final mixture was dialyzed (cut-off: 12 000 – 14 000 Da) against deionized water until no
absorbance was detected in the water ($\lambda = 494$ nm). The obtained compound is a yellowish
solid and corresponds to functionalized HA by 1% of fluorescein. The ^1H NMR (D_2O)
spectra of HA^{FITC} is identical to the non-modified HA. Because of the very low loading of
fluorescein on HA, the ^1H NMR (D_2O) spectra of HA^{FITC} is identical to the non-modified HA.



The fluorescein moiety may also be linked through the secondary alcohol groups.

2.3. Preparation of HA^{FITC} -CTL-C



The fluorescein moiety may also be linked through the secondary alcohol groups.

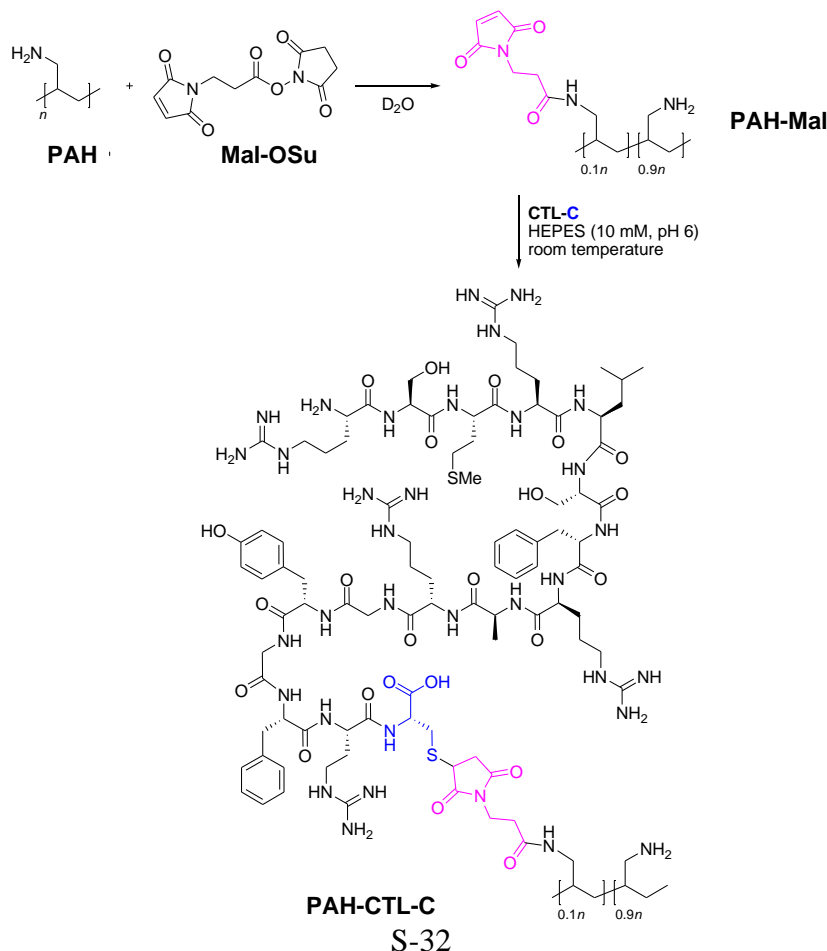
HA^{FITC} -CTL-C polymer has been prepared from HA-Mal. This polymer was labeled with 1%
FITC as described above to provide HA^{FITC} -Mal. Then, CTL-C peptide reacted with free
maleimide group to lead to HA^{FITC} -CTL-C as a yellow solid. The procedure used to get

HA^{FITC}-CTL-C was identical to the one described above to prepare HA-CTL-C. ¹H NMR (D₂O) spectra of HA^{FITC}-CTL-C was identical to the spectra of HA-CTL-C.

2.4. Preparation of PAH-CTL-C

810 All the following reactions were carried out in NMR tube and monitored by ¹H NMR (D₂O). To a solution of PAH (22.16 mg) in D₂O (0.6 mL) was added Mal-OSu (15 mg). The resulting mixture was stirred mechanically at room temperature until RMN monitoring indicated total consumption of the reagent (48 h). Evaporation of solvent provided the desired PAH-Mal (27 mg) as a white solid which was used without further purification. The DS of PAH-Mal was determined by ¹H NMR (400MHz, D₂O) and defined as the number of maleimide groups per 100 allylamine units of PAH. NMR Spectrum was realized in D₂O and calibrated at δ 4.79 ppm (residual water). The singlet at δ 6.9 ppm was assigned to the two symmetric protons on the double bond of the maleimide group. By comparing the integration of this signal with the singlet at δ 3.1 ppm, assigned to the CH₂-NH protons of the allylamine group, the DS was determined equivalent to 10%.

¹H NMR (D₂O, 400MHz, δ ppm): δ 6.9 (s, maleimide), 3.8 (t, Mal-CH₂-CH₂-CO), 3.1 (br s, CH₂ of PAH), 2.7 (t, Mal-CH₂-CH₂-CO), 2.1 (br s, CH of PAH), 1.6 (br s, CH₂ of PAH).



The coupling reaction between PAH-Mal 10% (1.5 mg) and CTL-C (15.6 mg) was performed overnight at room temperature in 0.6 mL of 10 mM HEPES buffer solution in D₂O (pH 6). Then, the mixture was dialyzed (cut-off: 12 000 – 14 000 Da) against MilliQ water for 6 days (water was changed every day). Peptide-conjugated PAH, named PAH-CTL-C, was freeze dried to provide a white solid (17 mg). This quantitative yield and the ¹H NMR described below allowed us to define a 10% DS (number of CTL-C peptide per 100 allylamine units of PAH).

¹H NMR (D₂O, 400MHz, δ ppm): 7.35 (br s, Ar aminoacid of CTL-C), 7.25 (br s, Ar aminoacid of CTL-C), 7.13 (br s, Ar aminoacid of CTL-C), 6.86 (br s, Ar aminoacid of CTL-C), 3.67-4.68 (m, CTL-C), 3.10 (br m, PAH and CTL-C), 2.50-2.68 (br m, CTL-C), 1.34-2.18 (br m, PAH and CTL-C), 0.96 (br s, CTL-C).

3. References

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- [4] S. T. Wall, K. Saha, R. S. Ashton, K. R. Kam, D. V. Schaffer, K. E. Healy, *Bioconjug. Chem.* **2008**, 19, 806.

4. Supplementary Figures

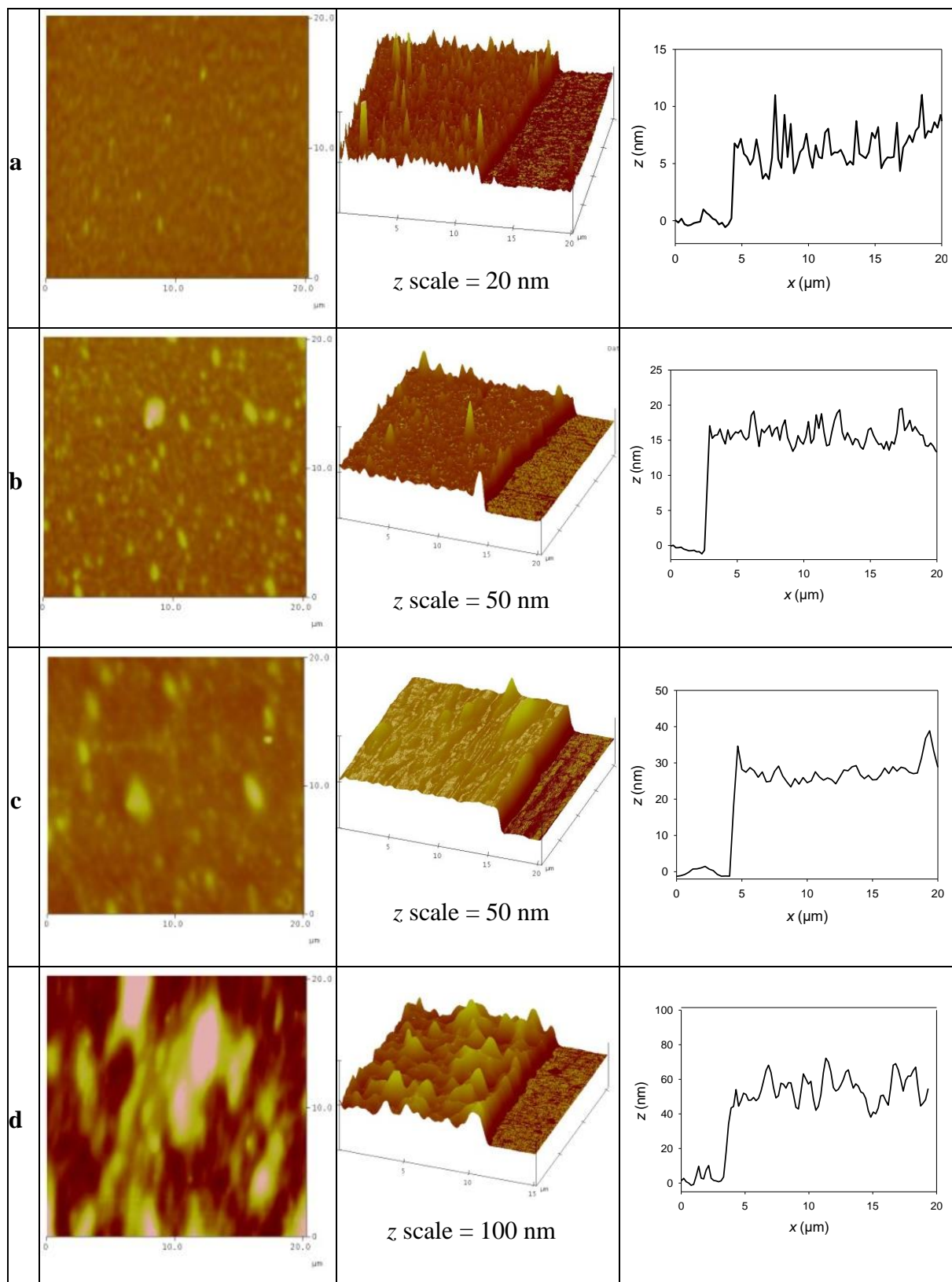


Figure S-1: AFM images of non-scratched and 3D images and their profilometric sections of scratched PEI-(HA-CTL-C/CHI)_n films built at (a) 5, (b) 10, (c) 15 and (d) 30 bilayers obtained in height mode in dry state.

860

Table S-1. Thicknesses and roughnesses of PEI-(HA-CTL-C/CHI)_n films built at different number of deposited pair of layers, *n*. The errors on the thickness values measured by AFM were obtained from the measurement of three different areas.

<i>n</i>	<i>d</i> _{AFM} (nm)	RMS (nm)
5	5 ± 1	1.6 ± 0.1
10	17 ± 2	4.2 ± 0.8
15	28 ± 3	4.6 ± 0.9
30	52 ± 9	16.5 ± 2.8

865

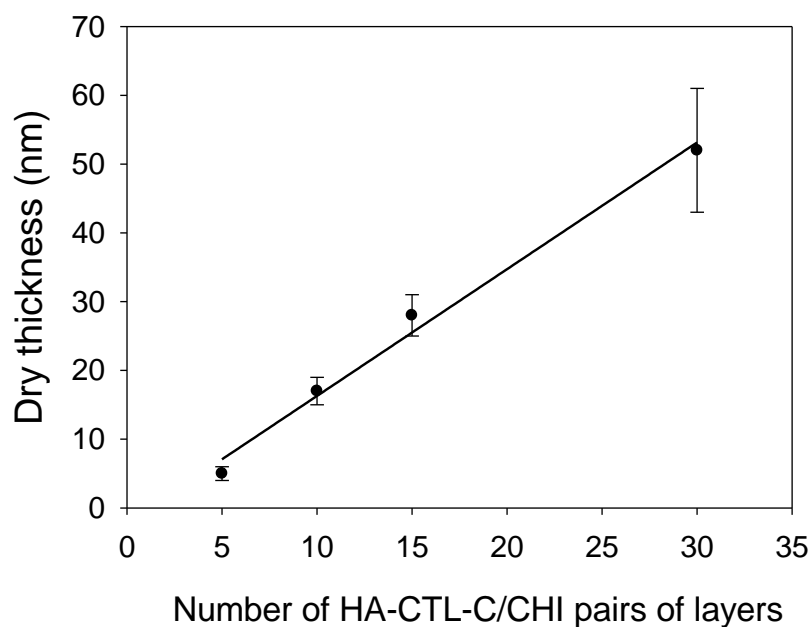


Figure S-2: Evolution of the thickness of (HA-CTL-C/CHI) films, measured by AFM in dry state after scratching, at different number *n* of pairs of layers.

870

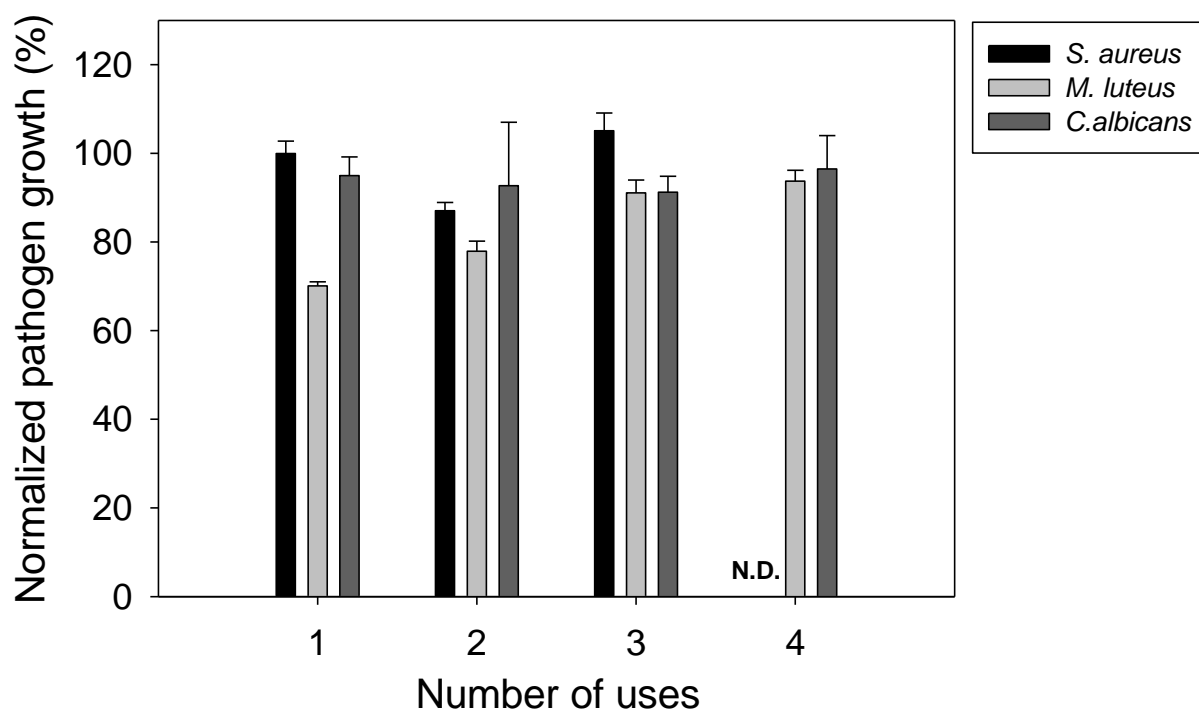


Figure S-3: Normalized growth of *S. aureus*, *M. luteus* and *C. albicans*, incubated for 24 h in contact with the supernatant withdrawn from HA-CTL-C/CHI films used in Figure 2d. Every 24 h, the film was put in contact with a fresh pathogen suspension. After 24 h of incubation with the film, the supernatant was withdrawn and incubated for 24 h with a fresh pathogen suspension. The normalization was performed with respect to OD₆₂₀ measured in the absence of film and antibiotics taken as 100% growth and in the presence of antibiotic taken as 0% growth. ND means not determined.

875

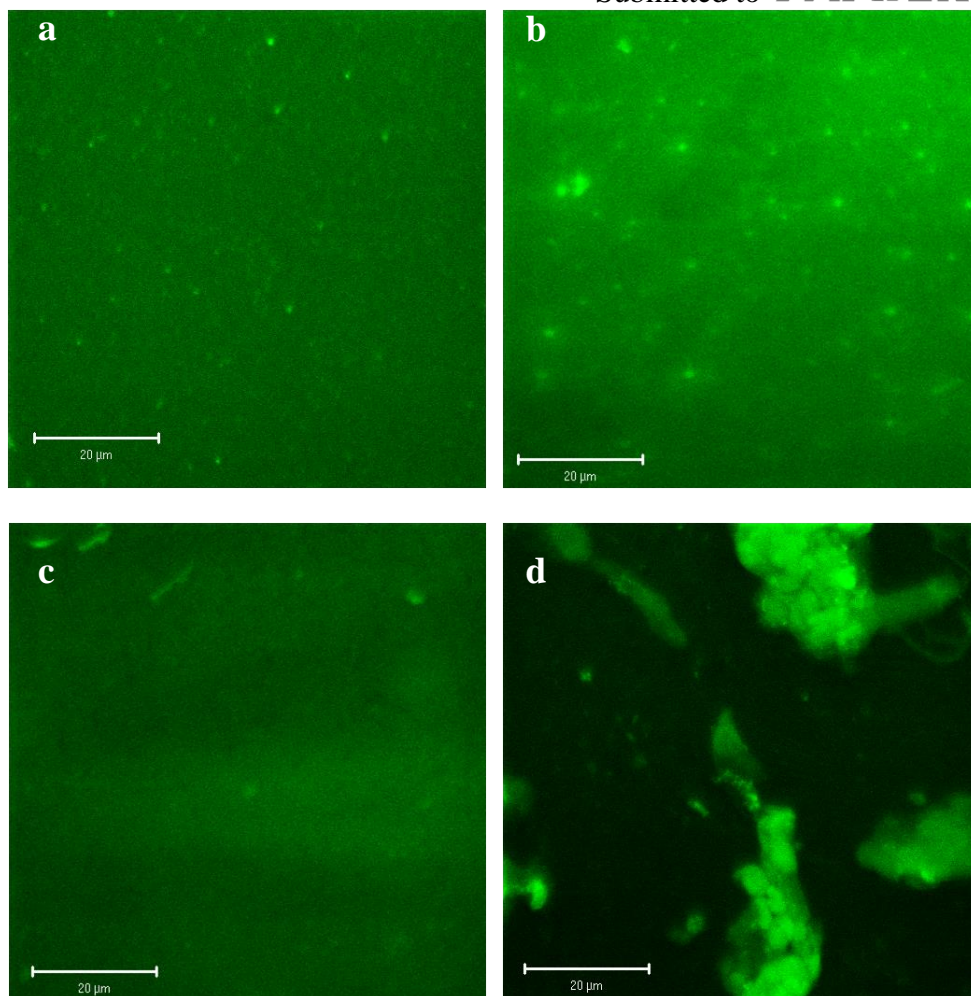


Figure S-4: CLSM images of PEI-(HA^{FITC}/CHI)₁₅ (a) before and (b) after paraformaldehyde (PFA) treatment and of PEI-(HA^{FITC}-CTL-C/CHI)₁₅ (c) before and (d) after PFA treatment. PFA treatment was performed to allow the observation of polysaccharide multilayer films after contact with pathogens. The films are put into contact for 30 min with 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.3 and, after several rinsing with PBS, were covered with Moviol 4-88 (Aldrich, Steinheim, Germany). The scale bars represent 20 μm.

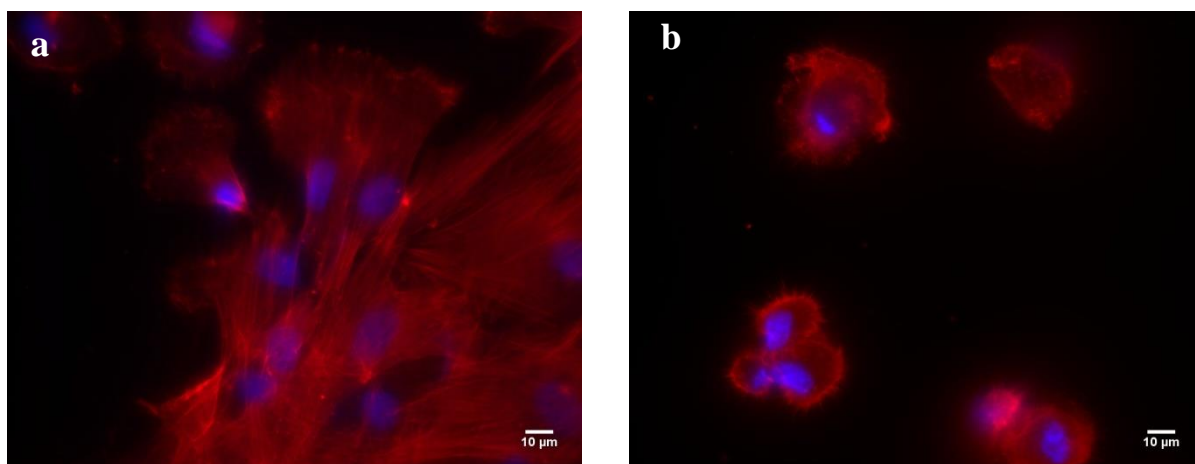


Figure S-5: Immunolabeling of HGFs after 24 h of culture on (a) a glass substrate and (b) on PEI-(HA-CTL-C/CHI)₁₅ film. The cytoskeleton was visualized by actin filament immunocytochemistry staining with phalloidin® (red labeling) and the nuclei with DAPI counterstaining (blue labeling) of HGFs. The scale bars represent 10 μm.