Encapsulation of Commensal Skin Bacteria within Membrane-in-Gel Patches

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The commensal skin bacterium *Staphylococcus epidermidis* provides a range of benefits to human hosts, contributing to skin equilibrium and good health, even though specific strains also act as pathogens if entering the host body. Therefore, skin-applicable devices allowing to benefit from some factors secreted by *S. epidermidis* while keeping the bacteria at a desired location are important for the development of bacterio-therapeutical applications. Here, a membrane-based gel patch is fabricated to trap *S. epidermidis* while keeping its metabolic activity. The pores of a track-etched membrane are modified with layer-by-layer (LbL) multilayers to anchor bacteria, followed by coating the membrane with a thick layer of agarose gel, leading to a membrane-in-gel soft patch. LbL multilayers comprising antibacterial polycations are then deposited over the patch. By varying the multilayer composition, thickness and nature of the last layer, both probability of escape and metabolic activity of entrapped bacteria can be tuned. Poly(ethyleneimine)-comprising multilayers prove to be particularly well-suited for the fine control of bacterial escape and activity. These bacterial patches are thus complex living materials in which the living component is controlled by a careful combination of soft macromolecular components.

1. Introduction

Skin together with its microbiota plays an essential role in human health, being a critical interface between human body and the environment.[1] In past years, culture-based and non-culture-based approaches, such as 16S rRNA gene sequencing and shotgun metagenomic sequencing, have been applied to identify and count bacterial communities on different skin sites.[2] The environmental, interpersonal and temporal diversity of skin microbiota has been proved, with a composition rich in the bacterial genera *Staphylococcus*, *Propionibacterium*, *Micrococcus* and *Corynebacterium*.[1a, 3] These skin bacteria are in a dynamic equilibrium which is responsive to the external environment, with infections or other skin disorders such as atopic dermatitis, psoriasis, and acnes, considered to arise at least partly from an alteration of these bacterial communities.[2a, 4]

Staphylococcus epidermidis (*S. epidermidis*), a permanent member of the human skin microbiota, has been extensively studied because of its prevalence on skin sites and its numerous benefits to hosts. On the one hand, some strains of *S. epidermidis* have been shown to promote the production of antimicrobial peptides (AMPs) by keratinocytes,[5] and induce T-cell development,[6] resulting in the enhancement of the innate immune protection. The extent and duration of the immune response has also been reported to be controlled by *S. epidermidis* which diminishes excessive inflammation after injury.[7] On the other hand, diverse molecules, such as phenol-soluble modulins, [8] serine proteases,[9] and nucleobase analogs,[10] can be produced by specific strains of *S. epidermidis*, leading to the suppression of adjacent microorganism growth. *S. epidermidis* itself can also secrete AMPs, which have been shown to synergize with the innate immune response to protect the host against pathogens.[11] Therefore, instead of using antibiotics, which have the potential to cause bacterial resistance, bacteriotherapy has been advocated as a promising alternative to prevent skin colonization by pathogenic bacteria.[12] The introduction of *S. epidermidis* on patients' skin has been shown to

significant reduce the colonization by *S. aureus* and group A *Streptococcus*.[8-9, 11b, 13] The short-chain fatty acids produced by *S. epidermidis* fermentation have been demonstrated to control the growth of *Cutibacterium acnes*,[11a, 14] and of *Propionibacterium acnes*,[15] which are both important acne triggers.

Human beings are colonized by a variety of *S. epidermidis* strains showing a diversity in pathogenicity.[16] For example, the non biofilm-forming strain ATCC 12228, which lacks the *ica* operon,[17] is a non-pathogenic commensal. However, some *S. epidermidis* strains are invasive and are major pathogens in nosocomial infections when they pass through the skin surface and enter the bloodstream via medical devices, for instance. For instance, 47% of coagulase-negative *Staphylococci* infections are due to *S. epidermidis* strains which often are methicillin- and multidrug-resistant.[18] Genomic analysis of both commensal and nosocomial strains demonstrate that they share approximately 80% of their genomes, with the 20% remaining portion being variable. Nevertheless, strains from both healthy individuals and hospital-associated infections may have nearly identical genomes, indicating that opportunity and environment can also contribute to clinical infections.[16b] Accordingly, *S. epidermidis* has been claimed to "live at the edge between commensalism and pathogenicity", [19] and is considered as an opportunistic pathogen.[20] Therefore, it is important to develop methods allowing us to reap the benefits of living *S. epidermidis* while keeping in check bacteria proliferation and migration.[11a, 21] Additionally, even if non-pathogenic strains are carefully selected for therapeutic applications, it remains important to confine them to the location where their effect is most desired.

Encapsulation has emerged as an attractive method to incorporate bacteria into matrices that not only preserve the metabolic activity of bacteria[22] while preventing their proliferation but also offer a way for the modification of the surface of bacteria cells.[23] Methods of cell encapsulation have been reviewed;[24] encapsulation was proposed within a wide variety of shells and capsules, based on a materials as diverse as silica,[25] gold, [26] calcium carbonate,[27]

metal-organic frameworks,[28] layer-by-layer (LbL) assemblies,[29] calcium phosphate,[30] polydopamine,[31] tannic acid,[32] or (interpenetrated) networks of hydrogels.[33] Since it was shown that stiffer materials generally prevent microbial growth,[24k] we restrict our focus to studies in which bacteria are coated by soft (bio)organic shells, which leads to an already large and non-exhaustive list of bacteria which can be encapsulated while preserving their metabolic activity such as *Alcaligenes faecalis*,[30b] *Allochromatium vinosum*,[34] *Bacillus (coagulans*,[29a] *subtilis*[29c, 29d, 35]), *Bifidobacterium (adolescentis*,[36] *breve*,[23c, 35a] *longum*[37]), *Enterococcus mundtii*,[38] *Escherichia coli*,[29e, 29f, 33d, 35a, 39] *Gluconacetobacter xylinus*,[39b] *Lactobacillus (acidophilus*,[29b, 40] *bulgaricus*,[41] *casei*,[42] *paracasei*,[41b] *plantarum*,[43] *reuteri*,[44] *rhamnosus*,[45] *zeae*[46]), *Micrococcus luteus*,[47] *Staphylococcus aureus*,[35a] *S. epidermidis*,[21, 48] a variety of probiotics,[33b, 49] and cyanobacteria.[50] Encapsulation of bacteria was shown to be able to prolong their storage time for periods as long as 1.5 year.[51] More relevant to our aim are studies showing that bacteria can be kept growing and metabolically-active while being prevented from escaping; for instance, confinement without escaping during 72 h, > 3 days, 7 days, > 20 days and 5 weeks were reported for, respectively, *E. coli* in an alginate core surrounded by a tough shell made of chemically-crosslinked polyacrylamide and physically-crosslinked alginate,[33d] *S. epidermidis* in polysulfone microtubes,[48b] *B. subtilis* in porous micro-needles of poly(ethylene glycol) diacrylate,[35d] and *M. luteus* in a poly(vinyl alcohol) (PVA) core surrounded by a shell of pH-responsive poly(*N*,*N*-diethylamino ethyl methacrylate)[47] or poly(methyl methacrylate).[51]

In contrast to the extensively studied probiotics encapsulation for food industry and gastrointestinal therapy,[22b, 45, 52] the encapsulation of commensal skin bacteria was only considered recently due to a growing interest for bacteriotherapy. Surface modification of *S. epidermidis* with biocompatible multilayers has been demonstrated to control bacteria growth depending on multilayer thickness.[21a] In another study, the thermo-responsive triblock polymer Pluronic F127, which hardens upon administration on skin, was selected to encapsulate

Bacillus subtilis. A patch for skin treatment was thereby obtained and enabled the continuous secretion of metabolic proteins and enzymes for the *in vivo* inhibition of *Candida* growth.[35c] A *B. subtilis*-encapsulating wound healing agarose hydrogel was built by 3D printing, offering on-demand production of patches for individual patients.[53] Electrospinning was also tested for the encapsulation of bacteria in the fibers of patches, which can be directly applied on skin.[12c, 38, 48b, 49b] A nanofibrillar hybrid biopatch composed of a mat of organic nanotubes entrapping *S. epidermidis* was also demonstrated by our group to be able to moderately control bacteria proliferation and activity.[21b] Although these preliminary studies showed promising results, the encapsulation of commensal skin bacteria remains in infancy. The main challenge remains to control bacterial proliferation while keeping them strongly metabolically-active over periods of time compatible with a topical application, *i.e.*, typically 24 h, which is a prerequisite for efficient bacteriotherapy. Additionally, the patch should confine bacteria at a specific location of the skin without risk of spreading, and be able to adapt conformally to skin.

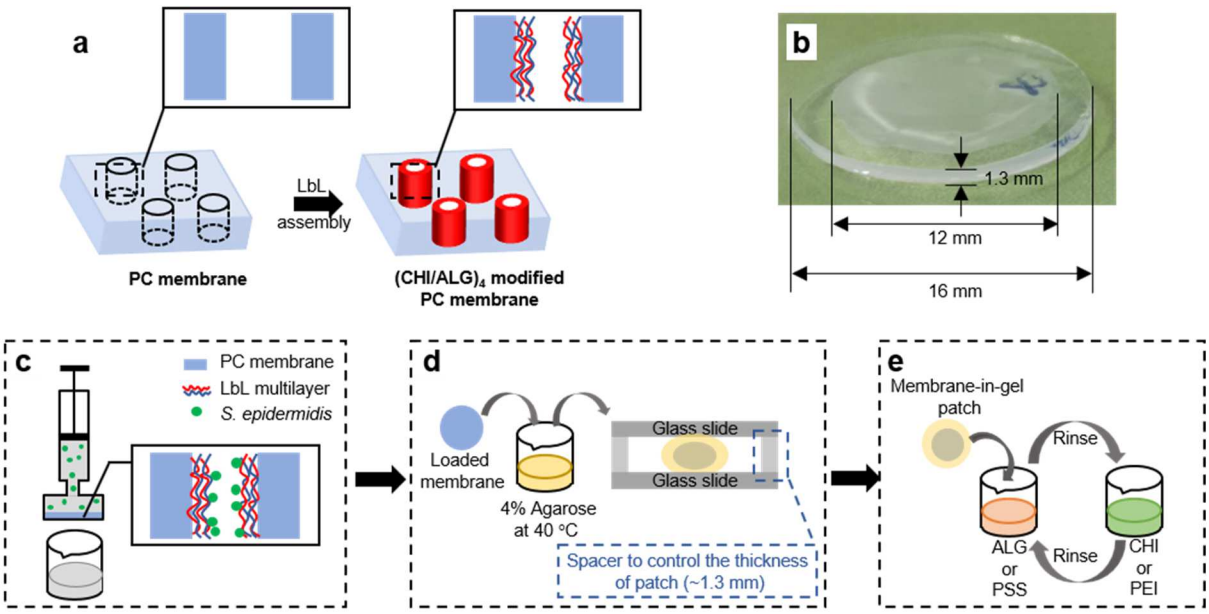
In this context, we develop a new bacteria encapsulation method for the fabrication of an *S. epidermidis*-containing patch for topical applications. The inner surface of the pores of a track-etched polycarbonate (PC) membrane is modified with layer-by-layer (LbL) assembled multilayers, providing anchoring for *S. epidermidis*; the membrane is then embedded in a thick layer of agarose gel, leading to a membrane-in-gel patch which can be applied on skin. To prevent the release of viable bacteria, LbL multilayers composed of bactericidal components are coated on the membrane-in-gel patch. We study the impact of multilayer composition, thickness, and type of last layer on bacterial escape and activity, and demonstrate that encapsulating *S. epidermidis* in such a membrane-based patch can maintain a significant bacterial activity. In addition, the inclusion of bactericidal components in the multilayer coating of the membrane-in-gel patch is demonstrated to not only tune the metabolic activity of bacteria but also to play a key role in regulating bacterial release, which can be prevented over a time compatible with topical applications (*ca.* 1 day) with a proper design. Our work thus provides

a practical route for the fabrication of compliant patches for future skin applications, but also shows how the inclusion of bactericidal components on a gel is an efficient route for the prevention of bacterial release.

2. Results

Different questions were considered in turn, aiming at obtaining a patch confining living bacteria over a reasonable period of time for a topical application. These questions are addressed below and comprise the methodology that can be used to encapsulate a significant number of metabolically-active bacteria in a soft patch that can be sticked on skin, and ways to limit the outside proliferation of bacteria.

2.1 Encapsulation of bacteria in membrane-in-gel patch



Scheme 1. a) Modification of the pores of a PC membrane with LbL assembled $(CHI/ALG)_4$ multilayers. b) A digital image of the membrane-in-gel patch. c-d) Fabrication of a membrane-in-gel patch: c) encapsulation by filtration of *S. epidermidis* in the $(CHI/ALG)_4$-modified membrane; d) coating of the membrane with agarose gel. The bacteria-loaded membrane was

dipped in the agarose solution then placed between two glass slides to form the patch. e) Surface modification of the membrane-in-gel patch with (ALG/CHI) or (PSS/PEI) multilayers at pH 6.0.

We selected track-etched PC membranes as frameworks for trapping the bacteria; such membranes are interesting because they exhibit a well-defined pore diameter, are transparent, and can be modified by LbL assembly. LbL is one of the most studied approaches for creating functional surfaces, allowing to cover a wide range of substrates of variable shapes, size, and chemical composition.[54] To anchor bacteria in the pores of PC membranes, the inner surface of the pores was first modified with LbL-assembled multilayers based on alginate (ALG) and chitosan (CHI) (**Scheme 1a**). Subsequently, bacteria were filtered through these surface-modified pores to trap them in the membrane (**Scheme 1c**). Several parameters, including the pore diameter of the membrane, the chemical nature of the last layer of multilayers, and the concentration of *S. epidermidis* were varied to optimize the trapping efficiency. Due to the complexity of encapsulating living bacteria, fluorescent latex microbeads (Figure S1a, supporting information) with a negatively charged surface and a similar size as *S. epidermidis* were initially used as model particles to rapidly screen trapping conditions. Although such beads are very poor models of bacteria, they nevertheless allowed us to eliminate conditions that were unlikely to a successful bacteria encapsulation for purely physical reasons. A PC membrane of 1.6 µm pore size and 10^7 pores cm^{-2} (Figure S1b) showed the best trapping efficiency for a suspension concentration of 1.5×10^8 microbeads mL^{-1}.

Four bilayers of CHI/ALG were selected for deposition in the pores of a PC membrane at pH 4.0. Since each CHI/ALG bilayer contributes only 2.7 nm to the dry thickness on flat surfaces,[55] the reduction of pore size due to the surface modification is negligeable. However, during LbL deposition, the membrane is dipped in polyelectrolyte solutions, resulting in the deposition of multilayers not only inside the pores, but also on the top and bottom surfaces of the membrane. To avoid this, the top surface of the membrane was quickly decrusted with a cotton pad in a

3 M NaCl solution at the end of the deposition process, fully removing the polyelectrolyte crust and leading to completely open pores on the top surface (Figure S1c), which is favorable for bacteria loading while limiting adsorption of the bacteria on the top surface. The bottom surface of the membrane was decrusted every second bilayer during LbL deposition in a gentle saline solution of 150 mM NaCl; this gentler condition effectively eliminates the crust adsorbed on the surface of the membrane, but tends to smear and spread the polyelectrolyte layer, leading to partial closure of the pores on the bottom surface (Figure S1d), which may improve the encapsulation efficiency.

To visualize the modification of the pores, poly(allylamine) tagged with rhodamine (PAH-Rh) was used as polycation instead of CHI in the last two bilayers of the LbL deposition, and the membrane was observed with a confocal laser scanning microscope (CLSM). As shown in **Figure 1a-b**, the pores of the membrane are fluorescently-labeled along their length, demonstrating the successful modification of pores with the LbL multilayers. The orthogonal images of a LbL-modified membrane in which a suspension of stained *S. epidermidis* was filtered are shown in **Figure 1c-d**. By checking 1800 pores on a membrane, we established that 40% of the pores contain from 1 to *ca.* 5 microorganisms, which are well-distributed along the whole length of the pores.

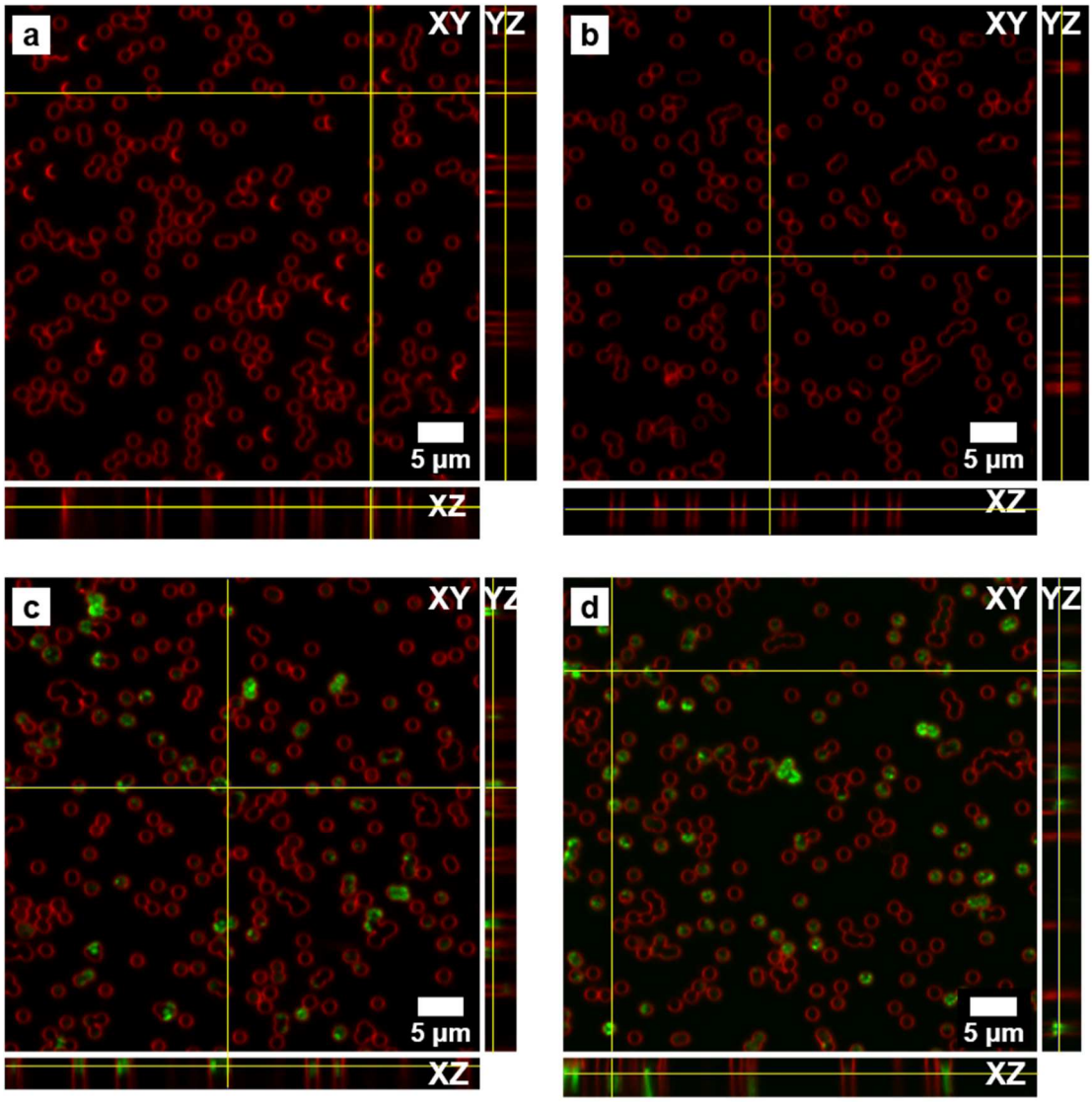


Figure 1. Orthogonal images of a,b) a fluorescently-tagged (red) $(CHI/ALG)_2$(PAH-Rh/$ALG)_2$-modified membrane and c,d) a membrane trapping SYTO 9-stained (green) *S. epidermidis*. The membranes were observed by CLSM with Z-stack scanning from a,c) the top surface and b,d) the bottom surface. In a) and c), the top edge of the XZ view corresponds to the top surface of the membrane; in b) and d), the top edge of the XZ view corresponds to the bottom surface of the membrane.

Agarose gel was cast around the bacteria-containing membrane to create a flexible and permeable layer, leading to a membrane-in-gel patch (**Scheme 1d**). The agarose gel acts as a physical barrier to prevent bacteria to freely disperse, which could easily happen since the

membrane pores are at least partially open on both sides. The movement of bacteria in a gel was investigated by other researchers, who proved that bacteria without flagella like *S. epidermidis* have no significant motion in such gels.[56]

Bulk hydrogels have been widely applied for cell encapsulation because they permit an easy exchange of nutrients and water with the environment.[57] Rhodamine B was used as a model molecule to test the outward diffusion of small molecules such as nutrients through the agarose gel. A $(CHI/ALG)_4$-modified membrane was dipped in a rhodamine B solution for loading, then the membrane was coated with the agarose gel. The diffusion of rhodamine B toward the broth was then measured. As shown in Figure S3, the fluorescence intensity of the broth quickly increased, due to the fast diffusion of rhodamine B from the membrane pores to the broth, confirming that small molecules can diffuse easily in the gel. The inward penetration of larger molecules in the agarose gel was also studied, by dipping a gel patch (devoid of membrane) into solutions of FITC-dextran of different molar masses. The diffusion of FITC-dextran in the gel layer was determined with CLSM (Figure S4 and S5). After a dipping time of 2 h, the 3-5 kg mol^{-1} FITC-dextran already penetrated by at least 480 μm into the agarose gel, which is almost half the thickness of the patch. Since the antibacterial compounds produced by *S. epidermidis* typically have a molar mass lower than 5 kDa,[58] our membrane-in-gel patch should thus be permeable to them. By contrast, the diffusion thickness of 40 kg mol^{-1} FITC-dextran macromolecules was small. After a dipping time of 4 h, patches dipped in 3-5 kg mol^{-1} and 10 kg mol^{-1} FITC-dextran showed a similar profile. However, no change was observed for the patch dipped in 40 kg mol^{-1} FITC-dextran, demonstrating that the diffusion of high molar mass molecules is limited by the pore size of the agarose gel.

The metabolic activity of bacteria-containing membrane-in-gel patches was tested by culturing these patches in broth. Free suspended bacteria and a bacteria-containing $(CHI/ALG)_4$-modified membrane (without agarose gel coating) were also tested. AlamarBlue™ reagent was added to the broth and the emission fluorescence intensity (at 585 nm) was used to evaluate the metabolic

activity of bacteria: indeed this indicator switches from a non-fluorescent to a fluorescent form upon reduction by the bacteria. Given that alamarBlue™ is introduced in the broth at the start of the culture, the measured fluorescence intensity provides the metabolic activity of bacteria integrated up to corresponding time point. This integrated activity reaches a plateau when the conversion of the dye is complete. The activity of the free bacteria displays a typical exponential increase due to bacterial growth after a lag time of a few hours (**Figure 2a**). Bacteria encapsulated in the membrane without agarose layer and bacteria encapsulated in the patch show a similar trend, with the fluorescence intensity of the supernatant increasing to the expected plateau after 12 h culture.

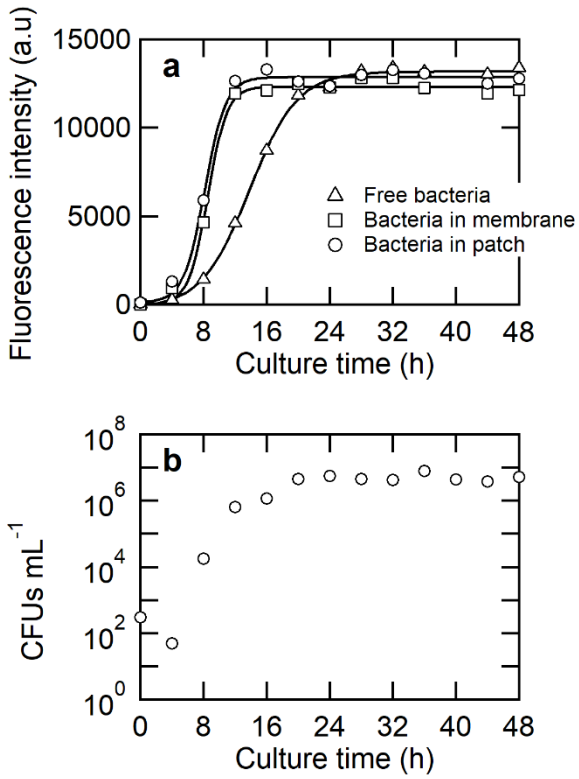


Figure 2. a) Integrated metabolic activity of free bacteria, bacteria trapped in a $(CHI/ALG)_4$-modified membrane and bacteria encapsulated in a membrane-in-gel patch. The samples were placed in 2 mL broth and 200 µL alamarBlue™, then cultured at 37 °C in the incubator. As a positive control, free bacteria were cultured in identical conditions by adding ~5×10^6 bacterial cells in a broth/alamarBlue™ mixture. The fluorescence intensity of the supernatant at 585 nm (470 nm excitation wavelength) is reported for different time points, with curves drawn to guide the eye. b) Bacteria released in the broth from a membrane-in-gel patch tested using the single plate serial dilution spotting technique. After successive 4 h culture times, the supernatant was taken from the culture, serially diluted and spotted on an agar plate. After two days culture of the agar plate, the number of bacterial colonies was counted and colony forming units per mL (CFUs mL^{-1}) were calculated and reported *vs* the culture time of the patch. The results of this figure were reproduced on biological duplicates (Figure S6).

The release of bacteria from the membrane-in-gel patch was measured using a single plate serial dilution spotting technique.[59] As shown in **Figure 2b**, bacterial colonies were observed in the supernatant from the very beginning of the culture, showing the escape of living bacteria to the broth as soon as the patch was immersed in the broth. With time passing, these escaped bacteria proliferated, as shown by the continuous increase of CFUs in the supernatant. Consequently, the strong increase of integrated metabolic activity essentially arises from the growth of escaped bacteria in the broth. Although encapsulation of bacteria in a membrane-in-gel patch can maintain bacterial viability, the agarose gel is not efficient enough to prevent bacterial release, and another way should be used to control bacterial spreading.

2.2 Control of bacterial escape with LbL coatings

Therefore, the patch was coated by LbL multilayers comprising bactericidal components (**Scheme 1e**). LbL assembly has been used early on to prepare antimicrobial coatings,[24c, 24j, 54b, 60] for applications in biomedicine, healthcare, and marine engineering.[61] Antimicrobial LbL coatings generally act via three main mechanisms: adhesion-resistance,[62] release-killing,[63] and contact-killing.[64] These mechanisms can work synergistically to protect a surface against infections.[65] In antibacterial contact-killing LbL coatings, polycations such as poly(ethyleneimine) (PEI) are often used since they can kill bacteria by disrupting cell membranes.[61b, 65b] Chitosan is also a frequently-used antibacterial polysaccharide, which can be assembled with ALG. The antibacterial effects of CHI/ALG multilayers have been studied before, showing a higher bacterial inhibition when increasing the number of bilayers.[66]

Here, ALG/CHI and poly(styrene sulfonate) (PSS)/PEI multilayers were thus explored as a supplementary way to control the release of bacteria from the membrane-in-gel patches. We first demonstrated that LbL multilayers can be successfully deposited on an agarose gel, with a penetration limited to the external skin of the gel (Supplementary Information, Figures S7, S8 and Table S1). Subsequently, $(ALG/CHI)_{5.5}$ multilayers were deposited on the membrane-in-

gel patch, and the resulting patches were cultured in broth implemented with alamarBlue™. The escape of encapsulated bacteria from the LbL coated membrane-in-gel patch was tested by the single plate serial dilution spotting technique.

No bacterial colony was observed in the supernatant for the first 4 h of culture (**Figure 3b**), showing that the $(ALG/CHI)_{5.5}$ coating slightly delays the escape of living bacteria from the membrane-in-gel patch, or prevents their growth in the broth. During these first 4 h of culture, the fluorescence intensity of the broth nevertheless moderately increased (**Figure 3a**), showing that the encapsulated bacteria are metabolically-active. After 4 h of culture, living bacteria start to escape and grow in the broth (**Figure 3b**), which explains the strong increase of integrated metabolic activity seen in **Figure 3a**.

Compared to the porous agarose gel, LbL multilayers have a less open and permeable structure,[67] and therefore may play the role of slight physical barrier to delay bacteria release. However, a bactericidal effect of $(ALG/CHI)_{5.5}$ cannot be ruled out either at this stage. Nevertheless, with culture time increasing, bacteria quickly proliferate in the membrane-in-gel patch and the $(ALG/CHI)_{5.5}$ coating loses its capability to contain bacterial migration. Therefore, a more bactericidal polycation PEI was included in the coatings. The minimum inhibitory concentration (MIC) of PEI towards *S. epidermidis* was measured to be 2 μg mL^{-1}, much lower than the MIC of CHI which was found to be 8 μg mL^{-1} (results not shown). To prevent killing the bacteria in the patch by direct contact, $(ALG/CHI)_{1.5}$ multilayers were first deposited on the patch, acting as a primer film for subsequent PEI/PSS deposition. The effects of PEI/PSS bilayers number and nature of last layer on bacterial activity and release were then studied.

The release of living bacteria from the $(ALG/CHI)_{1.5}(PEI/PSS)_n$-coated membrane-in-gel patch, with *n* varying from 1 to 4, are shown in **Figure 3c-f**. For the membrane-in-gel patch coated with $(ALG/CHI)_{1.5}(PEI/PSS)_1$, no bacterial colony was observed in the broth at 0 and 4 h culture times (**Figure 3c**). Starting from 8 h culture, the CFUs increased and eventually reached a maximum value after 28 h culture. Impressively, no CFUs could be detected for the membrane-

in-gel patch coated with $(ALG/CHI)_{1.5}(PEI/PSS)_2$ until 28 h culture (**Figure 3d**), demonstrating that the presence of $(ALG/CHI)_{1.5}(PEI/PSS)_2$ either prevents the release of living bacteria for at least 28 h, or prevents the growth of any escaped living bacteria. From 32 h culture on, living bacteria started to escape the patch and grow in the broth. However, only one or two bacterial colonies were observed for each 20 µL droplet; therefore, the CFUs mL^{-1} remained below 10^2. For the membrane-in-gel patch coated with $(ALG/CHI)_{1.5}(PEI/PSS)_{3 \text{ or } 4}$, no bacteria colony was observed during culture (**Figure 3e-f**), demonstrating the absence of release of living bacteria from the patch or, if released and living, the inhibition of their growth by the PEI-containing coating. The release and growth of living bacteria is thus significantly delayed by coating the membrane-in-gel patch with PEI/PSS, with a strong dependence on the number of bilayers.

The integrated metabolic activities of the membrane-in-gel patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_{1\text{-}4}$ are shown in **Figure 3a**. These curves are best explained by comparing them with the data on bacterial escape and growth (**Figure 3c-f**). For one PEI/PSS bilayer, the fluorescence intensity is slowly increasing in a first phase (0-4 h), when the bacteria do not escape or at least do not grow in the broth; this corresponds to the metabolic activity (including growth) of bacteria trapped in the patch. After 8 h culture, bacteria escape from the patch, and the fluorescence intensity rapidly increases mainly due to the growth of the escaped bacteria in the broth. For patches coated with three and four PEI/PSS bilayers, for which living bacteria do not escape in the broth or at least do not grow in the broth, the fluorescence intensity moderately increases and a plateau is reached after a few hours of culture. This again corresponds to the integrated activity of trapped bacteria, which raises the question as to why their metabolic activity stops after some time. For the intermediate case of two PEI/PSS bilayers, the fluorescence intensity vigorously increases for times at which bacteria do not grow in the broth (first ~28 h). It then increases further with living bacteria detected in the broth. Compared with CHI/ALG multilayers, which show no significant impact on encapsulated bacteria,

PEI/PSS proves thus to be an efficient coating to prevent the escape and/or the growth of living bacteria in the broth and modulate the metabolic activity of bacteria. With an increase of the number of PEI/PSS bilayers, outside growth of the bacteria can be increasingly delayed; this bacterial trapping also results in a decrease of bacterial metabolic activity, and eventually its arrest.

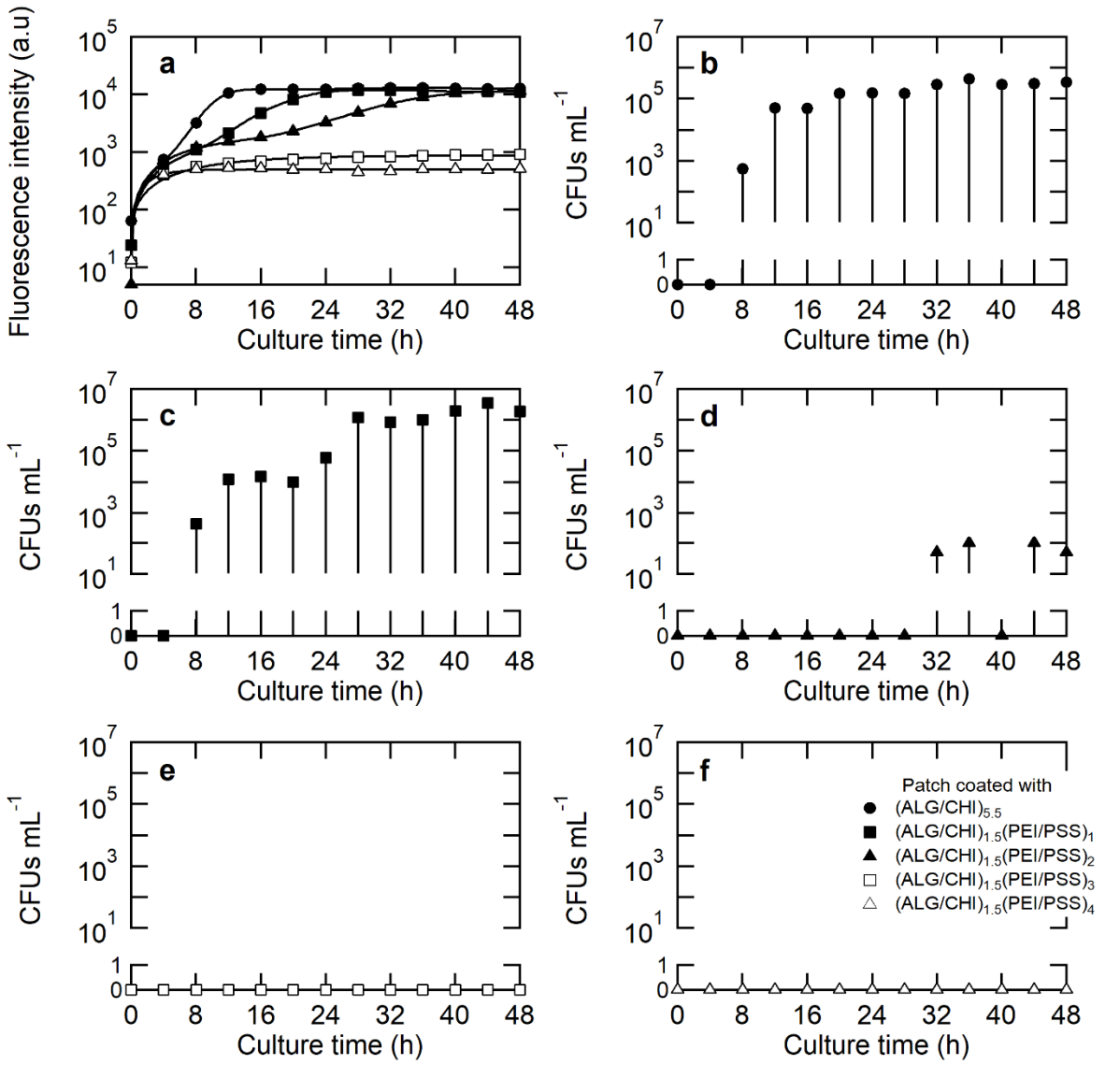


Figure 3. a) Integrated metabolic activity of *S. epidermidis* encapsulated in membrane-in-gel patches with different LbL coatings *vs* culture time. Bacteria were loaded in a $(CHI/ALG)_4$-modified PC membrane followed by agarose gel coating. Then, LbL multilayers incorporating antibacterial polycations were deposited on the membrane-in-gel patch. The LbL modified membrane-in-gel patch was cultured at 37 °C in 2 mL broth with 200 µL alamarBlue™ cell viability reagent. The fluorescence intensity of the supernatant at 585 nm (excitation wavelength 470 nm) was measured every 4 h during incubation; curves are drawn to guide the eye. b-f) Release of living bacteria from membrane-in-gel patches coated with different LbL films, *vs* culture time. Every 4 h culture, a sample of supernatant was pipetted, serially diluted

and spotted on an agar plate. After two days culture of the agar plate, the number of bacterial colonies was counted and the number of CFUs mL^{-1} was calculated. The results of this figure were reproduced on biological duplicates (Figure S9).

The PEI/PSS LbL multilayers deposited on the patch thus either act as mechanical barriers restricting bacterial migration or act as contact killing layers against escaping bacteria. Both effects are thickness-dependent, leading to controlling the time-dependence of the metabolic activity by the number of PEI/PSS bilayers. Another factor that may affect the bacteria is the chemical nature of the outer layer of multilayers. Due to charge overcompensation, the polyelectrolyte deposited as the outer layer consists of looser segments dangling in solution, which can exchange ions with the solution until reaching an equilibrium.[68] Therefore, membrane-in-gel patches coated with multilayers ended with a PEI layer, namely $(ALG/CHI)_{1.5}(PEI/PSS)_{n.5}$, were also prepared to study the metabolic activity of encapsulated bacteria as well as their release (**Figure 4**).

No release and growth of bacteria in the broth were detected for patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_{1.5,\ 2.5\ and\ 3.5}$ (**Figure 4c-e**). The fluorescence intensity measurements performed in presence of these patches clearly indicate a behavior similar to what was observed for patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_{3\ and\ 4}$: the integrated metabolic activity first increases due to growth of trapped bacteria, then saturates (plateaus of 1100 a.u, 900 a.u and 400 a.u, for 1.5, 2.5 and 3.5 (PEI/PSS) bilayers, respectively). However, for the membrane-in-gel patch coated with $(ALG/CHI)_{1.5}PEI$ (**Figure 4b**), bacterial colonies were observed in the broth only at 24 h and 40 h culture, at which times the CFUs remained small. For this patch, a significant fluorescence intensity growth was observed before viable bacteria release, indicating that trapped bacteria are responsible for the increase of integrated metabolic activity, similarly to patch coated with $(ALG/CHI)_{1.5}(PEI/PSS)_2$ (**Figure 3**).

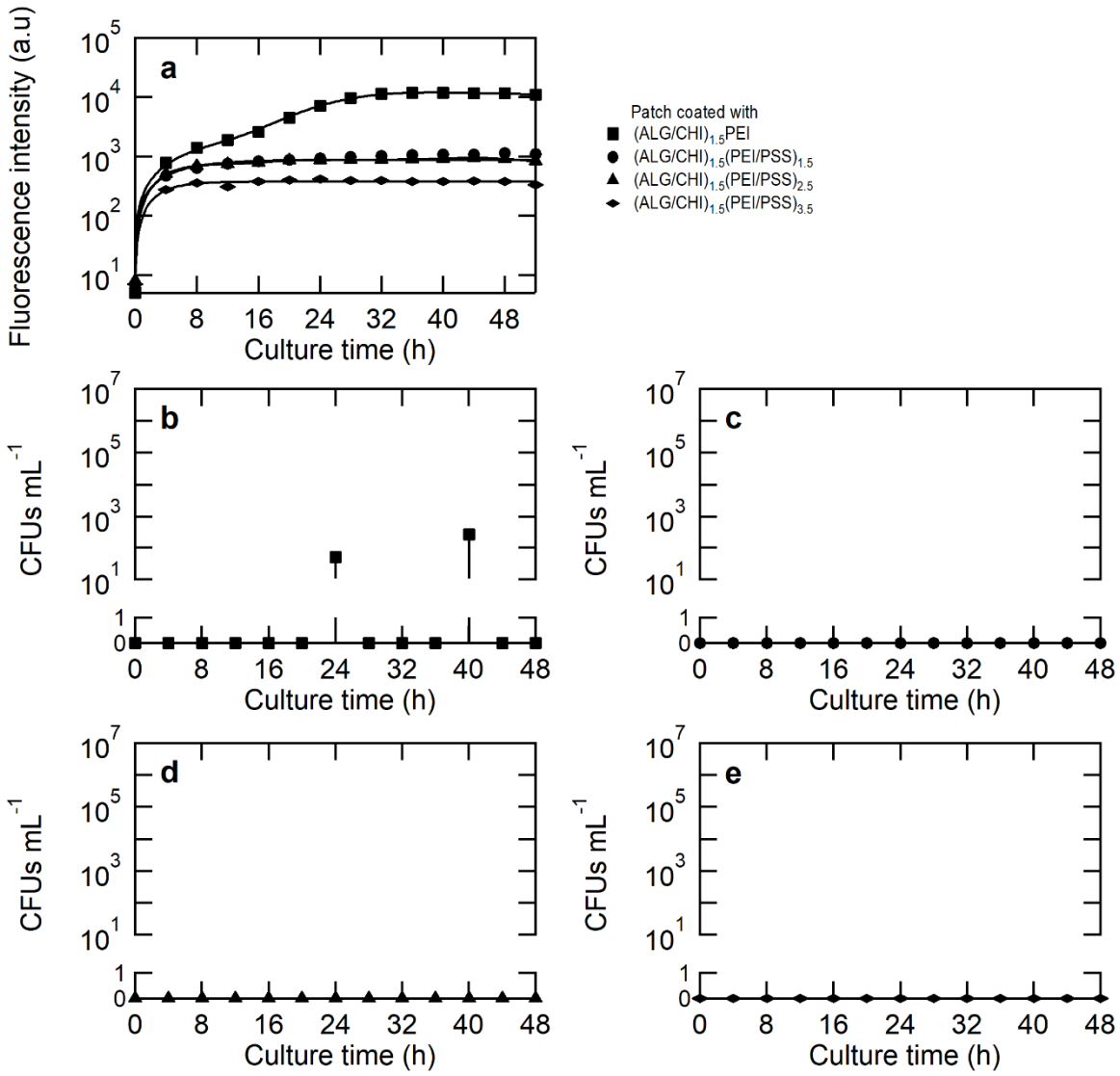


Figure 4. a) Integrated metabolic activity of *S. epidermidis* encapsulated in membrane-in-gel patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_{n.5}$ (n = 0-3). Bacteria were encapsulated in a $(CHI/ALG)_4$-modified membrane, followed by agarose gel coating. The LbL multilayers were then deposited on the membrane-in-gel patch, which was cultured at 37 °C in 2 mL broth with 200 µL alamarBlue™ cell viability reagent added. The fluorescence intensity of the supernatant at 585 nm (excitation wavelength 470 nm) was tested every 4 h during incubation; curves are drawn to guide the eye. b-e) Release of *S. epidermidis* from membrane-in-gel patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_{n.5}$ *vs* the culture time. Every 4 h culture, the supernatant was pipetted and serially diluted and spotted on an agar plate. After two days culture, the number of

bacterial colonies was counted and CFUs mL^{-1} was calculated. The results of this figure were reproduced on biological duplicates (Figure S10).

The comparison between patches coated either by $(ALG/CHI)_{1.5}(PEI/PSS)_n/PEI$ or $(ALG/CHI)_{1.5}(PEI/PSS)_{n+1}$ reveals that the presence of PEI as the outer layer systematically decreases bacterial leaching and growth in the broth compared to PSS-ended coatings with the same number of PEI layers. Clearly, PEI-ended coatings are more efficient to inhibit viable bacteria release and their proliferation in supernatant.

2.3 The impact of the LbL coating on free bacteria

When bacteria are released from a membrane-in-gel patch, two bacterial communities exist in the culture system. The first one is composed of bacteria trapped in the patch. The second comprises escaped free bacteria in the supernatant. The LbL coating may contact-kill bacteria trying to escape through the coating, and/or restrict the growth in the supernatant of escaped free bacteria. To confirm this second possibility, complete membrane-in-gel patches without encapsulated bacteria were placed in a free bacterial suspension, which was subsequently cultured.

The integrated metabolic activity of these free bacteria (**Figure 5**) and their CFUs (Figure S11) were measured as a function of culture time: The integrated metabolic activity indicates that the free bacteria cultured in the presence of a patch without LbL coating behave similarly to free bacteria; the same hold true for free bacteria added with patches coated by $(ALG/CHI)_{5.5}$ and $(ALG/CHI)_{1.5}(PEI/PSS)_1$. For these samples, the CFUs in broth either increased with time or saturated at a high level, depending on the coating (Figure S11a-c and e). However, when patches were coated with either $(ALG/CHI)_{1.5}PEI$ or $(ALG/CHI)_{1.5}(PEI/PSS)_4$ multilayers, the increase of fluorescence intensity was delayed by *ca.* 24 h, showing the inhibitory effect of these coatings. The CFUs mL^{-1} first decreased over the first 20 h of culture, before catching up

again. The decrease of the initial number of surviving bacteria indicates that these two types of coating exhibit bactericidal effects, which result in a delay of the exponential growth phase. Therefore, LbL coatings in the complete patches do not only prevent bacteria from escaping through them; they also kill the living bacteria that would have succeeded to escape.

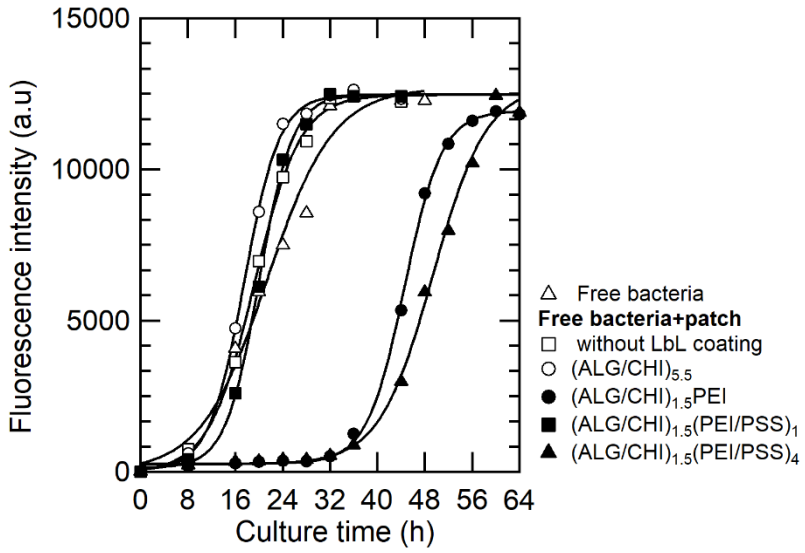


Figure 5. Integrated metabolic activity of free bacteria cultured in the broth in the presence of patches. Membrane-in-gel patches coated with/without LbL multilayers were added in free bacterial suspensions, with added alamarBlue™. The fluorescence intensity of the supernatant at 585 nm (excitation wavelength 470 nm) was recorded *vs* the culture time; lines are drawn to guide the eye.

2.4 The state of bacteria in membrane-in-gel patches

It was shown before that, for patches coated with more than two PEI/PSS bilayers (**Figure 3a**), or patches coated with $(PEI/PSS)_{n.5}$ (n>0) (**Figure 4a**), the integrated metabolic activity first increases then reaches a plateau before a complete conversion of alamarBlue™; this plateau illustrates the absence of metabolic activity of trapped bacteria after some culture time. To identify whether this is due to the death of the trapped bacteria, two membrane-in-gel patches

coated with $(ALG/CHI)_{1.5}(PEI/PSS)_4$ were prepared and placed in broth to start culture (hereafter called patch 1 and patch 2). After 8 h culture, when the integrated metabolic activity became constant, the two patches were removed from the broth. **Figure 6a** shows the variation of the fluorescence intensity of the supernatant *vs* time; after removal of the patches, the fluorescence intensity remains constant since no living bacteria escaped from the patches. Just after its removal from the first culture, the agarose gel layer of patch 2 was removed and both samples were then transferred to new culture tubes. The metabolic activity of bacteria in the new culture tubes was also measured and is shown in **Figure 6b**: no significant increase of fluorescence intensity was observed for the complete patch 1. In contrast, patch 2 exhibited a significant metabolic activity after a lag time of *ca.* 40 h, showing that the trapped bacteria could grow again, therefore testifying for the existence of live bacteria in the patch.

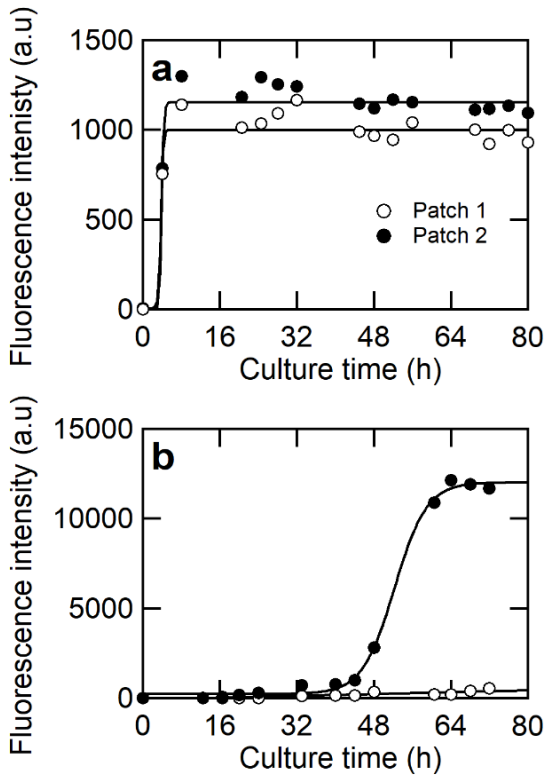


Figure 6. The state of bacteria in membrane-in-gel patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_4$. Two membrane-in-gel patches were prepared and coated with

$(ALG/CHI)_{1.5}(PEI/PSS)_4$, named patch 1 and patch 2. The patches were placed in a broth with alamarBlue™ to start incubation. After 8 h culture, the patches were removed (panel a). The agarose gel coating of membrane-in-gel patch 2 was removed, and both samples were transferred to a new broth to continue culture (panel b). The fluorescence intensity was measured as indicated before.

3. Conclusions

A bacteria-containing membrane-in-gel patch, built by embedding bacteria in the modified pores of a track-etched membrane, followed by embedding the membrane in a thick layer of agarose gel, was shown to be an efficient way to encapsulate living bacteria. The addition of a LbL multilayer shell on the agarose gel provides an anti-escape barrier, whose properties can be modulated by playing with bilayer composition and the number of bilayers. Whereas ALG/CHI bilayers only delay very moderately bacterial escape and subsequent growth, the inclusion of PEI in the multilayers provides antibacterial activity to the coating, which therefore prevents the escape of live bacteria and furthermore is efficient to kill possibly alive escaped bacteria. This is especially true when PEI is used as outer layer for the coating, most probably due to looser segments of the chains dangling at the solid/water interface. Interestingly, by changing the number of PEI/PSS bilayers, the metabolic activity and escape probability of trapped bacteria can be finely controlled. When the bacteria remain trapped, their integrated metabolic activity continuously increases for some time, then saturates at a plateau value at which the bacteria are still alive but in a dormant stage.

Comparing all samples with a topical application in mind, the patches coated with $(ALG/CHI)_{1.5}(PEI/PSS)_2$ show the most interesting prospects for the treatment of skin dysbiosis. Some strains of *S. epidermidis* have been proved to benefit skin by secreting AMPs and phenol soluble modulins, or by regulating the innate immune response of the host, both of which are highly related to the production of beneficial factors. Therefore, maintaining a high metabolic

activity of *S. epidermidis* is needed, which is the case for this specific sample for which bacterial release is suppressed for at least 28 h, when the integrated metabolic activity of bacteria has already reached a high value of 5000 a.u. This time is compatible with topical applications, for which a daily replacement of the patch can be considered; however, longer times without bacterial escape are also possible by increasing the number of LbL bilayers, at the expense of metabolic activity. Hence, our system allows us to generate a set of samples differing by the time before bacterial escape and by the metabolic activity. It is thus difficult to compare our system to previous work on encapsulated bacteria.[33d, 35d, 47, 48b, 51] This is compounded by the fact that different reports use different bacteria genera, strains, culture conditions, and methods of characterization of the metabolic activity. Further work is thus needed to benchmark these different systems using a standard methodology. Complementary studies are also needed in the presence of media such as artificial skin sweat to confirm that these results are fully relevant for topical applications. Since the composition of human sweat differs from the broth used in the present study,[69] it is likely that growth rates will be different; likewise, the culture conditions on skin are very different from culture in a broth.

Our methodology of encapsulation of bacteria in membrane-in-gel patches provides a soft system that can be easily and conformally applied to skin, and prevents adventitious release of the bacteria while allowing to deliver secreted beneficial factors. It should also be compatible with other bacteria genera, although this still remains to be demonstrated experimentally. Compared to generally studied methods of bacterial encapsulation, such as chemical/UV initiated bulk gel encapsulation, electrospinning, or 3D printing, our membrane-based patch allows us to encapsulate bacteria in biocompatible and gentle aqueous conditions without using sophisticated instruments or chemically aggressive reagents. The characteristics of the patch, including pore diameter, patch diameter, and thickness of agarose gel can all be tuned very easily according to the type of cell. The bactericidal LbL multilayers deposited on the patch can be easily modulated in both composition and thickness, bringing diverse possibilities for the

control of cell release and surface modification. This external multilayer may also protect the patch from other, external bacteria. Therefore, our versatile technology should be of interest for the development of a range of applications, beyond the specific case of commensal skin bacteria encapsulation.

4. Experimental Section

Materials: NaCl, poly(ethylenimine) (PEI, M_w 750 kg mol^{-1}), fluorescent latex microbeads (carboxylate-modified polystyrene, 1 µm particle size), sodium alginate (ALG), rhodamine B, FITC-dextran (M_w 3-5 kg mol^{-1}, 10 kg mol^{-1} and 40 kg mol^{-1}) and agarose were purchased from Sigma-Aldrich. Poly(styrene sulfonate) (PSS, M_w 70 kg mol^{-1}) was obtained from Acros. Rhodamine-labeled poly(allylamine hydrochloride) (PAH-Rh, M_w 15 kg mol^{-1}, 1:392 mol:mol monomer ratio in dye) was purchased from Surflay Nanotec GmbH. Chitosan (CHI, M_w 50-150 kg mol^{-1}, deacetylation degree > 90%) was provided by NovaMatrix. Glycerol (bioreagent), Live/Dead^{TM} bacteria viability kit and alamarBlue^{TM} Cell Viability Reagent were purchased form ThermoFisher Scientific. Freeze-dried *S. epidermidis* was purchased from ATCC (ref. ATCC 12228). Nutrient broth (BD 234000) and nutrient agar (BD 213000) were purchased from BD Bioscience.

Polymer solutions were prepared at 1 mg mL^{-1} in 150 mM NaCl except for PAH-Rh (0.5 mg mL^{-1}). Track-etched PC membrane (10^7 pores cm^{-2}, pore diameter 1.6 µm, thickness 24 µm) was provided by it4ip S.A., Belgium and was used as received. Milli-Q water with a resistivity of 18.2 MΩ.cm was used in all experiments. All solutions except for ALG and CHI were autoclaved then cooled to ambient temperature before use. ALG and CHI solution were sterilized by filtering through a 0.45 µm filter. All containers were sterilized or autoclaved before use.

(CHI/ALG)$_4$ modification of PC membrane: (CHI/ALG)$_4$ multilayers were deposited on the inner surface of the pores of PC membranes at pH 4.0 (**Scheme 1a**). A 6×6 cm^2 PC membrane was alternately dipped in polyelectrolyte solutions for 20 min in the first CHI/ALG adsorption cycle, then for 10 min from the second adsorption cycle on. Each adsorption was followed by two rinsing steps (2 min each) in a 150 mM NaCl solution. The PC membrane was decrusted with a cotton pad every second bilayer on its bottom surface. When finishing the LbL deposition, the top surface of the PC membrane was decrusted in a 3M NaCl solution and this process was performed for 2 min to prevent damaging the (CHI/ALG)$_4$ multilayers deposited in the pores. The PC membrane was then triply rinsed with MilliQ water and dried for storage. To prepare the rhodamine-tagged PC membrane for fluorescence microscopy observation, PAH-Rh was used instead of CHI in the last two bilayers.

Revival of S. epidermidis: Freeze-dried *S. epidermidis* was revived overnight in 8 mL broth (8 g L^{-1}) at 37 °C, 180 rpm, in a vented tube. Then, the broth was transferred to a vented culture flask containing 400 mL fresh broth to continue incubation. The optical density of the broth was measured at 540 nm (OD_{540}) until it reached ~0.5. The broth was centrifuged at 4 krpm for 5 min to remove the supernatant and *S. epidermidis* was resuspended in 100 mL fresh broth. Then 100 mL water/glycerol (50/50 v/v) mixture was added into the bacterial suspension and was gently shaken before cryo-preservation at -80 °C.

The cryo-preserved *S. epidermidis* was cultured to prepare fresh bacteria for encapsulation. Cryo-preserved *S. epidermidis* was added in 8 mL broth in a vented tube. After overnight incubation, the broth was transferred to a vented culture flask containing 200 mL fresh broth. Bacteria were cultured until the OD_{540} reached ~0.5. Then, the broth was centrifuged and *S. epidermidis* was triply rinsed with 150 mM NaCl (pH 6.0). Finally, fresh *S. epidermidis* was suspended in 10 mL of the same NaCl solution and stored in the fridge at 4 °C.

Fabrication of membrane-based patches (Scheme 1c-e): An *S. epidermidis* suspension of 0.042 OD_{540} was prepared, corresponding to 1.5×10^8 bacteria mL^{-1} (Figure S12). 1 mL of this suspension was diluted by a factor of 50 with 150 mM NaCl (pH 6.0). A circular sample of $(CHI/ALG)_4$-modified membrane with a diameter of 12 mm was placed in a Swinney filter unit. Then, 10 mL of the diluted *S. epidermidis* suspension was filtered through the PC membrane for 2 min (**Scheme 1c**). This step was repeated until having filtered 50 mL of the diluted bacterial suspension. After loading the bacteria in the pores of the modified-PC membrane, the top and the bottom surfaces of the membrane was triply decrusted with a cotton swab to remove deposited *S. epidermidis*. The obtained membrane was stored in 150 mM NaCl solution.

A 4% agarose solution was autoclaved and gradually cooled to 40 °C, at which temperature the agarose solution is viscous and semi-transparent. The bacteria-containing PC membrane was dipped in the agarose solution for 3 s. Then, the membrane was transferred between two glass slides separated by a gap of ~1.3 mm to obtain a well-defined membrane-in-gel patch as shown in **Scheme 1b**. The diameter of the membrane-in-gel patch was 16 mm. After gelation of the agarose, two types of polymer coatings composed of (ALG/CHI) or (PSS/PEI) bilayers were deposited on the surface of the membrane-in-gel patch at pH 6.0. For this, the membrane-in-gel patch was alternately dipped in ALG (or PSS) and CHI (or PEI) solutions for 1 min; between each polyelectrolyte adsorption, the patch was twice rinsed with 150 mM NaCl. **Scheme 1d** and 1e show the process to coat the membrane with agarose gel and the subsequent surface modification of the patch with LbL multilayers.

MIC measurement of CHI and PEI: A collection of aqueous PEI and CHI solutions (pH 6.0) was prepared by serial dilution by factors of two. *S. epidermidis* was diluted with broth to a concentration of 10^6 bacteria mL^{-1}. Then, 1 mL of a diluted polycation solution was mixed with 1 mL of bacterial suspension. A pure broth and a bacterial suspension without polycation were also prepared as negative and positive controls, respectively. All samples were cultured at 37 °C,

180 rpm and their turbidity was checked with the naked eyes after one day of culture. MIC was determined as the concentration from which no turbidity was observed in the culture tube.

Culture of bacteria in patch: A membrane-in-gel patch was placed in a sterilized well plate containing 2 mL broth, and cultured at 37 °C, 120 rpm. A positive control of free bacteria was prepared by adding 10 µL of bacterial suspension (OD_{540} = 0.042) in 2 mL broth, corresponding to 1% of the number of bacteria used for membrane loading. Pure broth was also cultured as a negative control. AlamarBlue™ cell viability reagent (200 µL) was added in each plate at the starting time. The fluorescence intensity of the supernatant was measured every four hours to evaluate the integrated metabolic activity of bacteria. For each test, 200 µL supernatant was pipetted in a 96-well plate and the fluorescence intensity at 585 nm (excitation wavelength 470 nm) was measured with a Tecan Infinite 200 pro microplate reader. After measurement, the supernatant was placed back in the initial well plate to maintain a constant volume of broth. In case of water evaporation, 10 µL water was added into the broth every 4 h culture.

A single plate serial dilution spotting technique was used to determine the number of viable bacteria escaped from the membrane-in-gel patch. For each time point (4 h), the supernatant was taken from the well plate then serially diluted by a NaCl solution (150 mM, pH 6.0). Thus, six suspensions with dilution factors of 1, 10^1, 10^2, 10^3, 10^4, and 10^5 were obtained from each supernatant. An agar plate was divided in six sectors onto which 20 µL of each suspension was micro-dropped. Then, the agar plate was left in the safety cabinet for 5-10 min to dry the micro-drops. After incubation at 37 °C for two days, the number of bacterial colonies on the agar plate was counted. Unless stated otherwise, sectors comprising from 5 to 100 bacterial colonies were used to calculate the number of colony-forming units (CFUs) in the supernatant.

$$\text{CFUs mL}^{-1} = \frac{\text{Number of bacteria colonies} \times \text{dilution factor}}{20 \times 10^{-3}\ \text{mL}}$$

The impact of alamarBlue™ reagent on the growth of bacterial colonies spotted on agar plate was also tested since 10 vol% alamarBlue™ was added in the broth during culture. A series of diluted bacterial suspensions with 10 vol% alamarBlue™ were prepared and 20 µL of each bacterial suspension was micro-dropped on an agar plate. After drying for 5-10 min to evaporate the liquid, the agar plate was incubated at 37 °C for two days. The number of bacterial colonies was counted; no difference was observed between bacterial suspensions with and without alamarBlue™.

Effect of patches coated with polyelectrolyte multilayers on free suspended bacteria: Membrane-in-gel patches without encapsulated bacteria were prepared to study the effect of ALG/CHI or PSS/PEI multilayers on bacteria free suspended in solution. $(CHI/ALG)_4$-modified membranes were directly dipped in 4% agarose solution to get membrane-in-gel patches devoid of bacteria. Then ALG/CHI or PEI/PSS multilayers were deposited on these patches, following the previously-described protocol. These patches were placed in 2 mL broth containing 10 µL of bacterial suspension (OD_{540} = 0.042) and 200 µL alamarBlue™. During incubation at 37 °C, 120 rpm, the fluorescence intensity of the supernatant at 585 nm (excitation wavelength 470 nm) was measured with a Tecan Infinite 200 pro microplate reader. The concentration of viable bacteria in the supernatant was tested using the previously-described single plate serial dilution spotting technique. In case of water evaporation, 10 µL water was added into the broth every 4 h culture.

Confocal laser scanning microscopy characterization: The observation of the loading of bacteria in PC membrane was performed by confocal laser scanning microscopy (CLSM, Zeiss LSM 710) using a fluorescently-tagged $(CHI/ALG)_2(PAH\text{-}Rh/ALG)_2$-modified membrane

loaded with stained *S. epidermidis*. For this, a *S. epidermidis* suspension (OD_{540} = 0.042) was stained with SYTO 9 (ThermoFisher) for 20 min in the dark. Then this bacterial suspension was diluted by a factor of 50 with 150 mM NaCl and filtered through a $(CHI/ALG)_4$-modified membrane tagged with PAH-Rh. After triply decrusting both surfaces of the membrane to remove the non-encapsulated *S. epidermidis*, the membrane was observed using Z-stack scanning from the top and the bottom surfaces, respectively.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author: morphology of fluorescent latex microbeads and LbL-modified membrane; diffusion measurements of microbeads, rhodamine B, FITC-dextran and PAH-Rh in agarose gels; biologically-duplicated experiments; effect of multilayers deposited on the patches on free bacteria proliferation; and optical density of *S.epidermidis* suspension at 540 nm (OD_{540}) *vs* bacteria concentration.

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Conflict of Interest

The authors declare no competing financial interest.

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Table of Content

Skin-applicable devices allowing to benefit from *Staphylococcus epidermidis* while preventing its uncontrolled proliferation are important for the development of bacterio-therapeutical applications. Here, bacteria are anchored within the pores of a membrane coated with a thick layer of agarose gel surrounded by an antibacterial shell, leading to a bacteria-containing membrane-in-gel soft patch in which bacteria display metabolic activity and limited proliferation.

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Encapsulation of Commensal Skin Bacteria within Membrane-in-Gel Patches