

**Encapsulation of Commensal Skin Bacteria in Soft Patches
Based on Layer-by-Layer Assembly**

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Abstract

The commensal skin bacterium *Staphylococcus epidermidis* provides a range of benefits to human hosts, contributing to skin equilibrium and good health. However, it can also act as a pathogen when entering the host body. Encapsulation is thus considered as a way to maintain the benefits of bacteria and control bacteria dispersion. Among various materials, porous polycarbonate (PC) membranes modified by layer-by-layer (LbL) assembled multilayers attract an increasing interest due to their biocompatible fabrication process and tunable properties. In this thesis, two membrane-based methods have been studied to entrap bacteria.

In the first method, a microtube mat is fabricated to encapsulate *S. epidermidis*. A bicompartimentalized LbL microtube, which contains an erasable external compartment, is designed to release the inner tube without having to dissolve the membrane template in non-biocompatible organic solvents. A hydrogen-bonded poly(*N*-vinyl caprolactam) (PVCL)/poly(methacrylic acid) (PMAA) system is chosen to build this erasable compartment. We investigate the growth and disaggregation of PVCL/PMAA LbL films, depending on pH, NaCl concentration, and PVCL molar mass. The use of PVCL/PMAA multilayers as a sacrificial compartment is proved to provide an efficient release of nano/micro-structures in biocompatible close-to-neutral aqueous conditions. *S. epidermidis* can be entrapped in LbL microtube under optimized loading conditions. After tube liberation and collection, the resulting microtube mat is shown to maintain the viability of *S. epidermidis*. However, the bacterial metabolic activity remains limited.

Therefore, a second method of entrapping *S. epidermidis* in a membrane-in-gel patch is studied. *S. epidermidis* is loaded in LbL-modified membrane, followed by coating the membrane with a thick layer of agarose gel. The membrane-in-gel maintains the metabolic activity of bacteria. LbL multilayers comprising antibacterial polycations are then deposited over the patch. By varying the multilayer composition, thickness, and nature of the last layer, both the probability of escape and the metabolic activity of entrapped bacteria can be tuned. This encapsulation methodology thus offers strong interest for the development of a range of applications in which bacteria need to be controlled, beyond the specific case of commensal skin bacteria encapsulation.

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List of abbreviations and acronyms

<i>A. muciniphila</i>	<i>Akkermansia muciniphila</i>
AAO	Anodic aluminum oxide
ALG	Alginate
AMPs	Antibacterial peptides
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
CHI	Chitosan
CLSM	Confocal Laser Scanning Microscope
<i>E. coli</i>	<i>Escherichia coli</i>
HA	Hyaluronic acid
H-bonded	Hydrogen-bonded
HSA	Human serum albumin
<i>L. acidophilus</i>	<i>Lactobacillus acidophilus</i>
<i>L. plantarum</i>	<i>Lactobacillus plantarum</i>
<i>L. rhamnosus</i>	<i>Lactobacillus rhamnosus</i>
LbL	Layer-by-layer
LCST	lower critical solution temperature
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MT	Microtube
NT	Nanotube
OD	Optical density
<i>P. acnes</i>	<i>Propionibacterium acnes</i>
PAA	Poly(acrylic acid)
PAAm	Poly(acrylamide)
PAH	Poly(allylamine hydrochloride)

PAH-Rh	Rhodamine-labeled PAH
PC	Polycarbonate
PDADMAC	Poly(diallyldimethylammonium chloride)
PEAA	Poly(ethacrylic acid)
PEG	Poly(ethylene glycol)
PEI	Polyethylenimine
PEO	Poly(ethylene oxide)
PET	Polyethylene terephthalate
PGA	Poly(glutamic acid)
PHEA	Poly(2-hydroxyethyl acrylate)
PLA	Poly-L-arginine
PLL	Poly-L-lysine
PLAA	Poly(L-aspartic acid)
PMAA	Poly(methacrylic acid)
PNIPAAM	Poly(<i>N</i> -isopropyl acrylamide)
PSS	Polystyrene sulfonate
PVA	Poly(vinyl alcohol)
PVCL	Poly(<i>N</i> -vinyl caprolactam)
PVME	Poly(vinyl methylether)
PVPON	Poly(vinyl pyrrolidone)
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
SEM	Scanning Electron Microscope
TA	Tannic acid
UCST	Upper critical solution temperature

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Chapter 1 Objectives and plan of the work

Among plenty of microorganisms living on the body, skin commensal bacteria are essential communities for human health. These bacterial communities are in a dynamic equilibrium and fluctuations in bacterial composition and number can promote common skin disorders, such as atopic dermatitis, psoriasis, and acne. Therefore, bacteriotherapy, which can either re-built the bacterial equilibrium or take advantage of the positive effect of bacteria, has gained increasing attention. *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. However, some *S. epidermidis* strains are known to be opportunistic pathogens, which can lead to nosocomial infections when they penetrate into the human body. Therefore, a good control of *S. epidermidis* dispersion and release is essential for skin bacteriotherapy. Bacterial encapsulation is one among possible methods to harvest the benefits of *S. epidermidis* by maintaining its biological activity, and to prevent its dispersal into the human body.

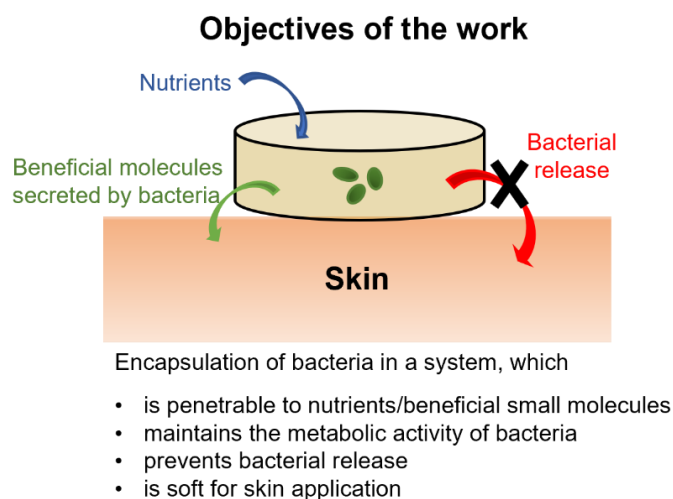
Encapsulation in bulk hydrogels is one of the most used methods, especially for skin bacteria encapsulation, because the resulting hydrated microenvironment contributes to maintain bacteria viability on skin. However, bacteria escaping from porous hydrogels is a limitation for clinical use. Moreover, gelation processes based on chemical reactions or UV irradiation are stressful for bacteria and can result in a lower activity. Fibrillar structures mimicking tissue have also been fabricated by electrospinning to entrap skin bacteria. However, use of high voltage can lead to a significant reduction of bacterial viability. Therefore, biocompatible encapsulation methods with better control of bacteria escape are proposed in this thesis.

Layer-by-layer (LbL) assembly is a widely used surface modification technique, especially for biological applications. Compared to other bacterial encapsulation methods such as microencapsulation in gel, extrusion, emulsion, electrospinning, and spray drying, LbL assembly is conducted in mild conditions without using organic solvents, high voltage, high temperature or chemical reagents for gel crosslinking. LbL assembly in the nanopores of

membranes results, after membrane dissolution, in nanotubes (NTs). Such NTs were developed prior to this work by researchers at UCLouvain, and used for the encapsulation and delivery of biomolecules. Instead of liberating LbL tubes, the LbL-modified PC membrane can also directly serve as a matrix to entrap and protect bacteria. In this thesis, we planned to build on this expertise regarding LbL in porous polycarbonate (PC) membranes in order to entrap *S. epidermidis* for topical applications. As shown in Scheme 1.1, the specifications of the final encapsulating system were the following:

- To allow the transfer of nutrients and maintain the metabolic activity of bacteria;
- To allow the diffusion of beneficial small molecules secreted by bacteria;
- To provide control over bacterial dispersion while preventing the penetration of *S. epidermidis* into the human body;
- To permit convenient skin administration.

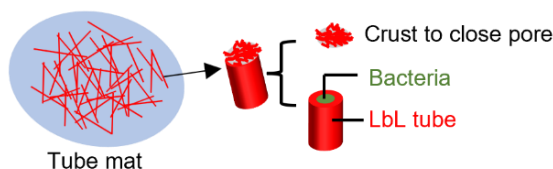
Two methods were thus developed and are detailed in this thesis, as summarized in Scheme 1.2.



Scheme 1.1 Our objectives of encapsulating bacteria for topical applications

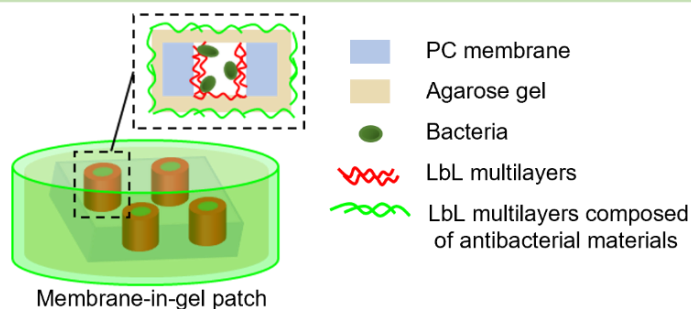
Plan of the work

Method 1: Encapsulation of bacteria in a tube mat



- **Limitation of tube mat for bacterial encapsulation** : Non-biocompatible organic solvents are used to liberate LbL tubes for the tube mat preparation.
- **Our strategy**: Fabrication of LbL tubes with an erasable external compartment to release LbL tubes in aqueous solution.
- **Advantages**:
 Tube mat fabrication and bacterial encapsulation conditions are biocompatible.
 The composition and compartment thickness are tunable by LbL assembly.
 The opening or closure of the tubes can be controlled.

Method 2: Encapsulation of bacteria in a membrane-in-gel patch



- **Our strategy**: Bacteria are encapsulated in a membrane-in-gel patch, which is composed of (1) a porous PC membrane modified by LbL multilayers to entrap bacteria; (2) an agarose gel coating; (3) LbL multilayers composed of antibacterial materials.
- **Advantages**:
 The patch is permeable to nutrients and small molecules secreted by bacteria.
 Bacterial activity and bacterial release can be controlled by the bactericidal coating.

Scheme 1.2 Illustration of two bacterial encapsulation methods studied in this thesis: (1) bacterial encapsulation in a tube mat and (2) bacterial encapsulation in a membrane-in-gel patch.

Chapter 1

The content of this thesis is the following. Chapter 2 provides an overview of commensal skin bacteria, materials and methods used to encapsulate bacteria. Then we specifically introduce the LbL assembly technique, which is essential in this thesis.

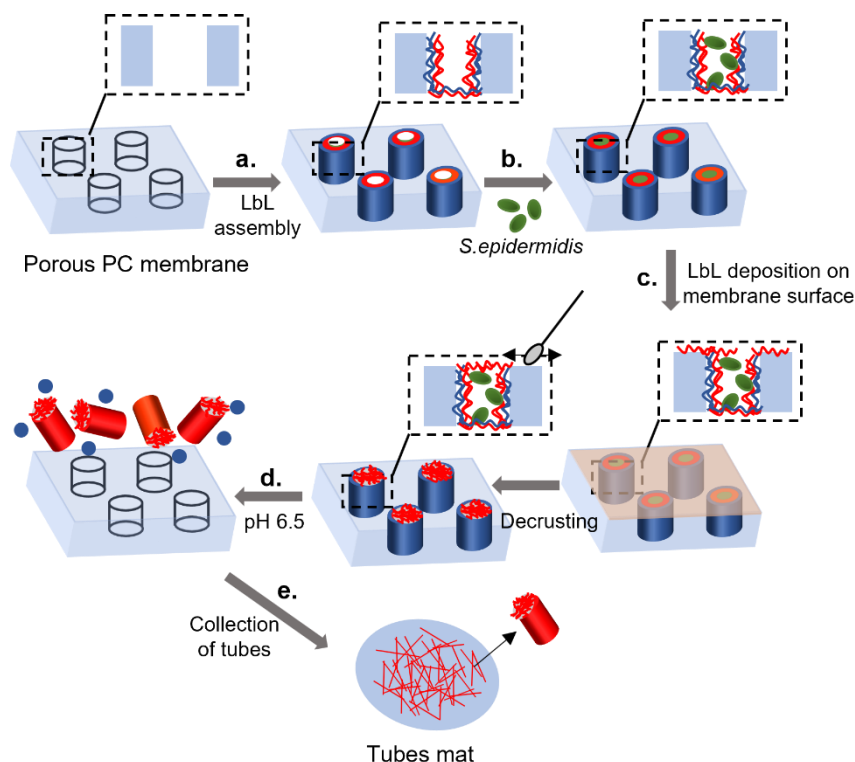
In chapters 3 to 5, two bacterial encapsulation methods based on LbL assembly are studied to encapsulate bacteria (Scheme 1.2). The first method which consists in entrapping bacteria in a mat of tubes, is based on the following steps:

- Preparation of LbL tubes in the PC membrane; for this, the pores of a PC membrane are modified by LbL multilayers to create bacteria-anchoring compartments;
- Loading of bacteria in tubes;
- Release and collection of tubes loaded with bacteria.

A major challenge faced in this method is that the tube release step, in which an organic solvent, such as dichloromethane, is generally used to dissolve the PC template. To avoid killing bacteria, a new tube release method using biocompatible aqueous solutions was studied. As shown in Scheme 1.3, two compartment-tubes were fabricated, composed of an external sacrificial compartment (dark blue) and an inner compartment (red). For the sacrificial compartment, hydrogen-bonded (H-bonded) LbL systems were selected owing to their tunable stability to solution parameters such as pH and salt concentration.

To encapsulate *S. epidermidis*, which is active at close to neutral pH (6-7), the sacrificial compartment should be stable in 150 mM NaCl solution at *ca.* pH 6 and should be erasable at a slightly higher pH. To this purpose, in chapter 3, H-bonded poly(*N*-vinyl caprolactam)/poly(methacrylic acid) (PVCL/PMAA) films are intensively studied. The growth of PVCL/PMAA films on a flat surface and their disaggregation are studied depending on several parameters, such as PVCL molar mass, salt concentration, and pH. Using PVCL/PMAA LbL multilayers as a sacrificial compartment, the liberation of LbL structures is then demonstrated as shown in Scheme 1.3.

In chapter 4, *S. epidermidis* is then loaded in bicompartimentalized PC membranes. A negatively charged microbead is used as a model particle to optimize the loading parameters, including membrane pore size, microbead concentration, and charge type of innermost layer. To maintain as many microbeads in LbL tube as possible during tube release, the pores are closed by decrusting. (Scheme 1.3c). The metabolic activity of bacteria in tube mats is then tested.

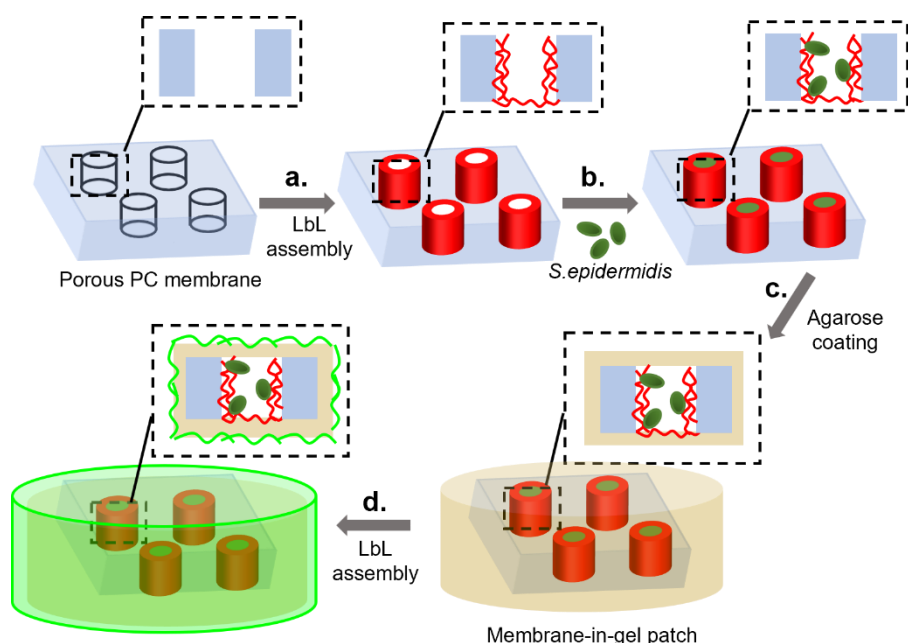


Scheme 1.3. Schematic illustration of bacteria entrapped in a tube mat. This method is comprised of (a) LbL modification of the pores of porous PC membrane, (b) loading of *S. epidermidis* in pores (LbL tubes), (c) closure of pore by decrusting the LbL-assembled multilayers on the PC membrane surface (d) release of tubes from the PC membrane, and (e) collection of tubes to obtain a tube mat. Two compartment-LbL tubes are prepared with an external PVCL/PMAA compartment (blue) that is erasable in pH 6.5 aqueous solution, and with an inner compartment (red) made up of chitosan (CHI) and alginate (ALG).

For the second method of encapsulation (chapter 5), *S. epidermidis* is entrapped in a membrane-in-gel patch (Scheme 1.4), according to the following steps:

- Surface modification of the pores of a PC membrane to anchor bacteria;
- Loading of bacteria in pores;

- Coating of PC membrane with agarose gel to get a membrane-in-gel patch;
- Coating of patch with LbL multilayers composed of antibacterial components.



Scheme 1.4. Schematic illustration of bacteria entrapped in a membrane-in-gel patch. (a) The pores of a porous PC membrane are modified by CHI/ALG multilayers; (b) *S. epidermidis* is loaded in the pores; (c) Agarose gel is coated around the membrane, getting a membrane-in-gel patch. (d) LbL multilayers composed of antibacterial materials are coated on patch.

Compared with the first method, the second one does not rely on the release of tubes, which can reduce the bacterial loss. Furthermore, the use of an agarose gel coating can delay the free diffusion of bacteria while allowing molecular diffusion to the environment. However, the escape of bacteria through the agarose layer remains possible. Therefore, we developed a strategy to prevent bacterial release from the agarose gel in order to avoid penetration into the human body. For this, we explored the deposition of LbL multilayers which have a denser structure compared to agarose gel, on agarose gel. Moreover, antibacterial polyelectrolytes are used to build these LbL multilayers, in order to attempt to kill bacteria when they are trying to escape

from patch. The effect of antibacterial multilayer composition and thickness are described, which supplies a new way to regulate bacterial activity and bacterial escape.

This thesis is ended by chapter 6, in which we summarize the two bacterial encapsulation methods based on LbL assembly and discuss the prospects of our patch for topical applications.

Chapter 2 State of the art

This chapter highlights the benefits and importance of commensal skin bacteria for skin health, for which bacterial encapsulation has emerged as an attractive method for maintaining metabolic activity. Various bacterial encapsulation methods and materials are reviewed including a general surface modification technique, layer-by-layer assembly, which is specifically introduced. The fundamentals of LbL assembly, and its wide application for the fabrication of functional multilayers, indeed make it an attractive technique for the encapsulation of bacteria.

2.1 Commensal skin bacteria

As the primary surface of human body, skin plays a role of “physical” and “chemical” barrier.^[1-3] Structurally, the skin consists of two layers: epidermis and dermis (Figure 2.1). Between the epidermis and dermis is a basement membrane, which is a narrow, multilayered structure that anchors the epidermis to the dermis. The epidermis can be divided into several layers, namely stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The keratinocytes, which constitute up to 90% of the epidermal cells, are formed by stem cells in the stratum basale, and the differentiation of keratinocytes leads to the formation of a layered epidermis.^[2]

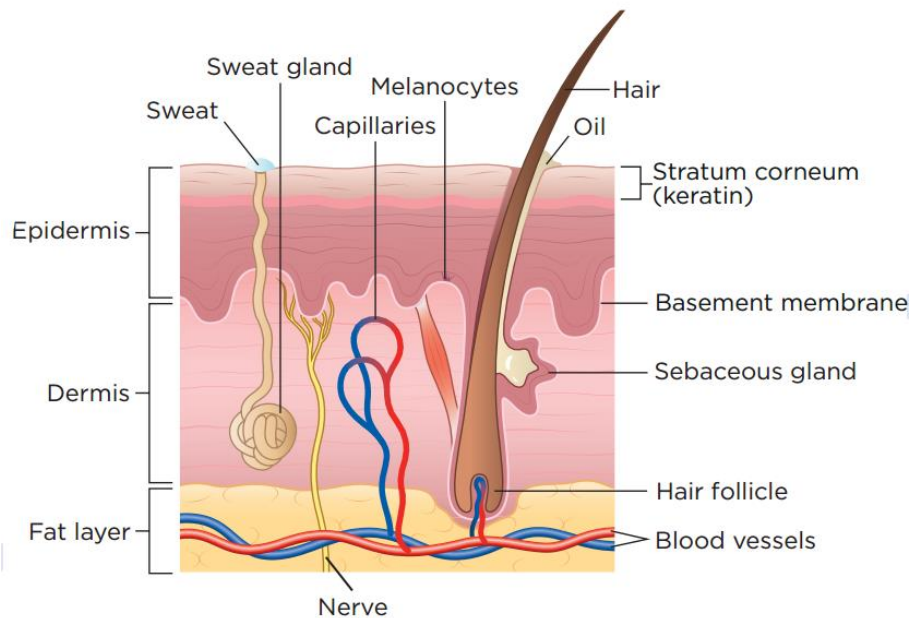


Figure 2.1. Cross-section through the skin.^[2]

The densely-packed keratinocytes in the stratum corneum form a waterproof layer that protects the host from attacks by pathogens. In contrast to moist mucosal surfaces, the skin has low moisture levels which prevents colonization by unfavorable bacteria, providing a “physical” protection. Several skin defense molecules produced by epidermal cells also act as a “chemical” barrier. These molecules, including defensins, proteases, and cytokines, can directly inhibit the growth of pathogens on the skin. On the other hand, these molecules serve as mediators that can regulate the innate

immune response. The synergy of beneficial effects underscores the importance of the skin in maintaining a healthy host.

At birth, human skin is colonized by millions of microorganisms, including bacteria, fungi, and viruses. Most microbes on the skin are harmless to humans and some microbes actually are beneficial to the human body by modulating the immune response or secreting antibacterial substances that form an essential part of the skin's "chemical" barrier. Bacteria can be commensal and symbiotic, or parasitic relative to the host, which depends on their inherent properties.^[3] The beneficial effect of commensal bacteria is best evidenced by studies on probiotics in the gastrointestinal tract. In addition, studies have demonstrated that two of the most frequently isolated and investigated commensal skin bacteria, *Propionibacterium acnes* (*P. acnes*) and *S. epidermidis*, can inhibit the growth of pathogens, thus acting as an additional "biological" barrier.

Compared to other mucosal surfaces, skin lacks nutrients for bacteria growth beyond basic proteins and lipids. To survive under these conditions, skin bacteria should obtain nutrients from the secretions of the stratum corneum, sebaceous glands, eccrine glands, and apocrine glands.^[4] Eccrine sweat is one of two types of sweat glands on the skin surface that play an essential role in thermoregulation by evaporating water while acidifying the skin, making the skin site unfavorable for pathogen colonization. Studies also show that the eccrine sweat glands can produce antibacterial peptides (AMPs) that show promise as a defense against infection.^[5] Therefore, the varying microenvironments of the skin, including pH, temperature, humidity, salinity, and sebum content, result in fluctuations in the bacterial communities on skin sites. Figure 2.2 shows the microbiomes and bacterial composition on normal-appearing human skin.

Skin sites are colonized by three main bacterial genera, including *Corynebacterium* spp, *Staphylococcus* spp. and *Propionibacterium* spp. They are generally lipophilic bacteria dominating on sebaceous sites such as face, chest and back.^[6] *P. acnes* can use lipases to degrade triglyceride lipids in sebum, allowing it to thrive in anoxic sebaceous glands. The amount of sebum on the cheek has been shown to correlate positively with *P. acnes*.^[7] *Corynebacterium* spp. and *Staphylococcus* spp. can tolerate a high concentration of salt in sweat, allowing them to thrive in moist areas such as the feet and crook of the elbow.

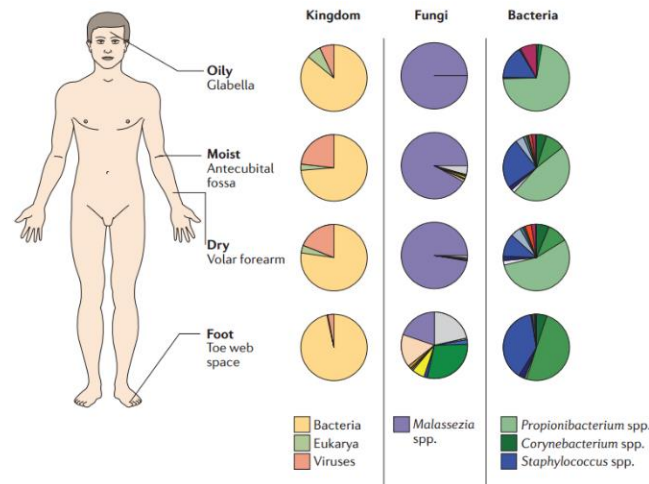


Figure 2.2. Skin microbial communities are shaped by physiological characteristics of skin sites^[1]

The identity and composition of skin bacteria fluctuate over time but are in a delicate balance with the environment and the host. The imbalance of bacterial communities is related to common skin diseases and inflammations. For example, the overgrowth of *Staphylococcus aureus* (*S. aureus*) is responsible for various infections such as impetigo, boils, and acne vulgaris. Interestingly, studies on bacteriotherapy demonstrate that *S. epidermidis* can inhibit the growth of *S. aureus*, providing a potential way to treat skin disorders.^[8-10] Lactic acid bacteria have been found to function as immune modulators to inhibit the release of pro-inflammatory cytokines, bringing beneficial effects in case of skin inflammation.^[11] Culture filtrates of *Lactobacillus plantarum* (*L. plantarum*) were shown to enhance the phagocytosis of *Pseudomonas aeruginosa*, indicating a potential for treatment of *Pseudomonas aeruginosa*-induced infections of burns.^[12] Administration of *L. plantarum* or *rhamnosus*-containing ointment on cutaneous wounds resulted in a significant decrease of inflammation by preventing the growth of Gram-positive bacteria.^[13, 14]

Bacteria can adhere to a variety of surfaces, including glass, metals, polymers, and even other bacteria or eukaryotic cells.^[15] The microenvironment on the surface differs from the bulk liquid; thus adhesion of bacteria on a surface can be promoted even when the nutrients are insufficient to sustain planktonic cells growth.^[16] For example, surface-attached *Caulobacter crescentus* is able to optimize nutrient uptake.^[17]

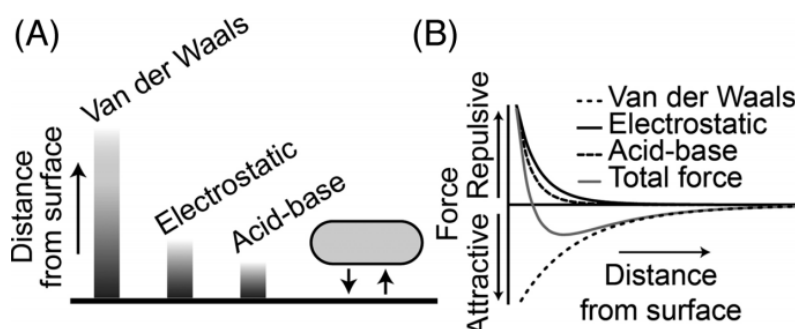


Figure 2.3. (A) Schematic representation of forces acting on a bacterium when it approaches a surface; (B) Adhesion forces as a function of distance from the surface.^[15]

The interactions between bacteria cells and solid surfaces have been extensively studied since they concern biofilm formation, bioenergy and biofouling. The attachment of bacteria cells on a solid surface is a complex process including three steps: (1) the approach of bacteria to the surface, (2) initial reversible adhesion and (3) irreversible adhesion, in regards to the liquid, bacteria and surface.^[18] The attachment of bacterial cells generally begins with the passive or active approach of planktonic bacterial cells to the solid surface. The initial attachment occurs rapidly. However, it is a reversible process mediated by a sum of van der Waals interactions, electrostatic forces, acid-base and hydrophobic interactions (Figure 2.3). Van der Waals forces are attractive, electrostatic forces are generally repulsive and acid-base forces might be either. The total force reaches an energy minimum, which determines the separation of the bacterium from the surface.^[15] Following reversible adhesion, the bond strengthening is generally facilitated over a time window of seconds to minutes by rearrangements of both bacteria and surface components or the production of adhesins, leading to irreversible cell attachment. For example, the displacement of interfacial water enhances the hydrophobic interactions between *S. epidermidis* and surface, promoting bacterial cell adhesion.^[19]

The properties of bulk liquid, the composition of bacterial cell surface and the nature of solid surface, altogether determine the attachment process. Flow and shear help to transport bacterial cells to the surface. For example, bacterial cells are transported or pulled away at a high flow velocity ($>30 \text{ s}^{-1}$). By contrast, a random swim of bacterial cells can be observed at a low flow condition (6.4 s^{-1}).^[20] Appendages such as flagella, pili, and curli were also demonstrated to promote the adhesion of bacterial cells on a surface. The physicochemical properties of the surface, such as the charge density,

potential and wettability have been shown to regulate the bacterial cell adhesion process. For example, many studies focused on the use of superhydrophobic surfaces in preventing bacterial adhesion and biofilm formation.^[21-23] Moreover, the adhesion of *S. epidermidis* and *Escherichia coli* (*E. coli*) has been demonstrated to be higher on a surface with a higher stiffness.^[24] It was also shown that bacterial cell attachment and detachment can be controlled by tuning the properties of LbL multilayers (*i.e.*, hydrophilicity, hydrophobicity, stability etc.).^[25-27]

2.1.1 *Staphylococcus epidermidis*

Gram-positive *S. epidermidis*, to distinguish from *S. aureus*, belongs to the group of coagulase-negative staphylococci. *S. epidermidis* is a facultative anaerobe with a large diversity of strains. *S. epidermidis* cells are spherical with a diameter of 0.5-1.5 μm at an optimum culture temperature of 30-37 $^{\circ}\text{C}$. Scanning electron microscopy (SEM) image (Figure 2.4) of *S. epidermidis* cells show that small clusters of 2 to 4 cells are frequent while occasionally a single cell is observed.^[28]

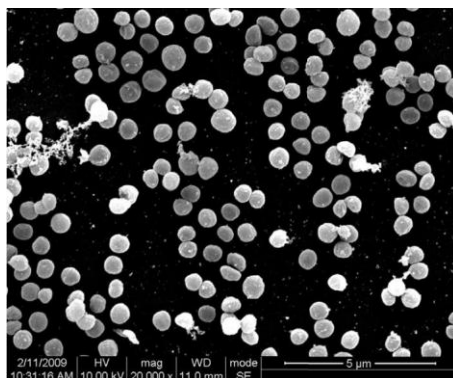


Figure 2.4. SEM image of *S. epidermidis* cells^[28]

As a commensal of the skin, *S. epidermidis* is considered to benefit hosts both directly and indirectly. Direct effects include the production of bacteriocins or metabolites (depending on strain) that play a role in preventing colonization or reducing biological activities of pathogens. Bacteriocins are antimicrobial peptides that have antibacterial activity against bacteria closely-related to the producer and are known as alternative antibiotics. Some strains of *S. epidermidis* can produce lantibiotics, belonging to class I bacteriocins of less than 5 kDa in size.^[29] Compared to antibiotics, bacteriocins have a narrow spectrum of bactericidal activity and are effective against antibiotic-resistant bacteria, such as methicillin-resistant bacteria. The bacteriocins produced by

S. epidermidis such as Pep5, epidermin, pilancin K7 and epicidin 280 have been shown to be effective against seven clinical isolates of common ear infections, including *S. aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*.^[30-33]

Phenol-soluble modulins (PSMs), a family of small peptides with an amphipathic α -helical structure, play an important role as virulence factors for staphylococcal pathogens. PSMs were first discovered in *S. epidermidis*, then were shown to be produced by all staphylococcal species, including PSM α - ϵ and PSM-*mec*, which vary in their amino acid sequences.^[34] The biological function of PSMs includes the induction of the synthesis of proinflammatory cytokines. In addition, the migration of neutrophils and monocytes to the inflammatory site can be induced by PSMs and plays a role in regulating the expression of cell adhesion molecules. To date, five α -type PSMs and two β -type PSMs have been derived from *S. epidermidis*. It was found that PSM δ and PSM ϵ produced by *S. epidermidis* destroy microbial membranes and selectively inhibit the growth of *S. aureus* and group A *Streptococcus*.^[35]

AMPs are immune defense molecules that can lead to cell lysis and death by interacting with the negatively charged membrane of microorganisms. Gallo and co-authors demonstrated that AMPs produced by *S. epidermidis* are strain-specific and highly effective in selectively killing *S. aureus*.^[36] These AMPs acting synergistically with PSMs have been shown to affect pathogen survival and to enhance the innate immune system of the host.^[29, 35, 37] In addition to preventing pathogen growth, other small molecules produced by *S. epidermidis* have also been studied and shown to inhibit the proliferation of tumor cells administrated intravenously or topically, as well as UV exposure-induced neoplasia.^[38, 39]

Indirect benefits of *S. epidermidis* result from the various immuno-modulatory properties of these bacteria.^[40] *S. epidermidis*-induced T-cell development on the epidermis was demonstrated to strengthen the immune system.^[41] Toll-like receptors (TLRs), a group of transmembrane receptors on the surface of sentinel cells, are important components of the innate immune system. It has been reported that a small molecule produced by *S. epidermidis* increases the expression of AMPs via TLR2, thereby enhancing the immune response to pathogens.^[41] The polysaccharide intercellular adhesin produced by *S. epidermidis* was demonstrated to induce interleukin-8 (IL-8) expression in human astrocytes partly via TLR2, showing that *S. epidermidis* can contribute to the regulation of pro-inflammatory cytokine production.^[42] Moreover, *P. acnes* can induce the expression of proinflammatory cytokines (IL-6 and tumor necrosis factor- α (TNF)) via the activation of TLR2 receptors in keratinocytes. As shown in Figure 2.5, lipoteichoic acid (LTA) produced by *S. epidermidis* can activate the TLR2 to induce the expression miR-143, which targets the 3'UTR of TLR2 mRNA and thereby induces a decrease of the

production of TLR2 protein. As a consequence, *P. acnes*-induced proinflammatory cytokines are suppressed by *S. epidermidis*.^[43] In addition to suppressing pathogen invasion, *S. epidermidis* has also been shown to diminish excessive inflammation after injury by limiting the production of undesirable inflammatory cytokines.^[44, 45]

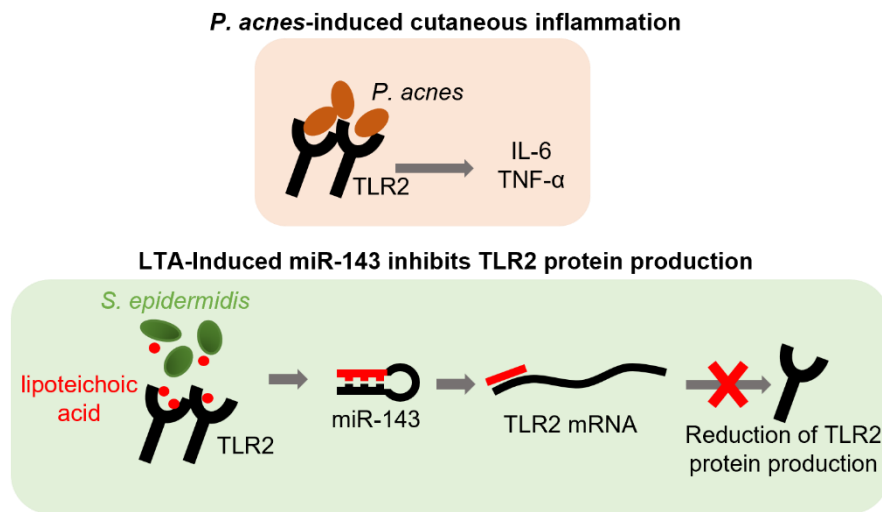


Figure 2.5. Mechanism developed by *S. epidermidis* to inhibit *P. acnes*-induced cutaneous inflammation. Staphylococcal lipoteichoic acid (LTA), a by-product of *S. epidermidis*, interacts with TLR2 receptors of keratinocyte to inhibit *P. acnes*-mediated inflammatory response (expression of IL-6 and tumor necrosis factor- α (TNF)) by induction of miR-143 in keratinocytes.^[43]

Though *S. epidermidis* has gained increasing interest in regulating skin homeostasis, it is also accepted as an opportunistic pathogen since it can cause sepsis when entering the bloodstream.^[46-51] *S. epidermidis* is one of the most commonly-isolated bacterial pathogens in nosocomial infections, including cardiovascular infections, nosocomial bloodstream infections, and organ infections.^[46, 47] These infections are usually associated with the invasion of *S. epidermidis* from the patient skin when inserting indwelling medical devices.^[52] The transformation of *S. epidermidis* from commensal to pathogen is mainly due to the formation of biofilms on medical devices. The presence of a biofilm protects bacteria against antibiotics and human immune reaction. In addition, the prevalence of multi-resistant *S. epidermidis* strains also becomes a challenge for the treatment of nosocomial infections. Accordingly, well controlling the dispersion of *S. epidermidis* is required for its application in bacteriotherapy.^[53-55]

2.2 Encapsulation of bacteria

Encapsulation techniques have been extensively investigated for particles or drugs, and rapidly extended for the immobilization of microorganisms with the aim to produce “living materials”. Both individual bacterium encapsulation or bacterial colony encapsulation are investigated. Encapsulation and culture of a group of bacteria provides the average information about hundreds of thousands of bacteria. By contrast, individual encapsulation allows in-depth profiling of single cell, which is increasingly investigated for diagnostics.^[56, 57] Encapsulation is an effective way to maintain the activity and function of bacteria and avoid cross-contamination during storage. With encapsulation, environmentally sensitive bacteria are incorporated into a matrix that shields them from the impact of external stresses, including variations of pH, temperature, ionic strength, and moisture. Encapsulation also allows to incorporate bacteria into materials such as bacteria-loaded particles, fibers, hydrogels, or patches, for applications in various fields. Additionally, encapsulation offers a way to combine bacteria with new functions, such as electronic, magnetic, and release properties, opening to the design of multifunctional living materials.

2.2.1 Encapsulation materials

Encapsulation of bacteria consists in the formation of a semi-permeable shell around the cell, aiming at shielding bacterial cells from environmental aggressions while allowing the diffusion of nutrients and oxygen to ensure their metabolic activity and functionality. Therefore, to be effective, the shell has to be composed of a non-toxic material to preserve bacterial viability. Moreover, it has to be porous enough to allow exchanging with the external medium but not too much to avoid the potential escape of cells. The mechanical resistance of the shell is also an essential parameter since growing bacteria inherently exert mechanical forces. In this section, mainly used organic, inorganic or hybrid encapsulation materials are discussed with a summary of their properties in Table 2.1. Depending on the applications, the shell could also need to be pH- or temperature-resisting or biodegradable in certain conditions.

Organic materials. Commercial polysaccharides are the most common agents used to encapsulate bacteria due to their chemical nature close to the natural bacteria environment. Polysaccharides can protect bacteria in two aspects: they can interact with lipids, protecting cell membranes under dehydrated conditions;^[58] on the other hand, they form hydrogen bonds with proteins present on cell membranes, thus preventing the denaturation of membranes during a drying step.^[59] Figure 2.6 shows the chemical structures of three most regularly studied polysaccharides.

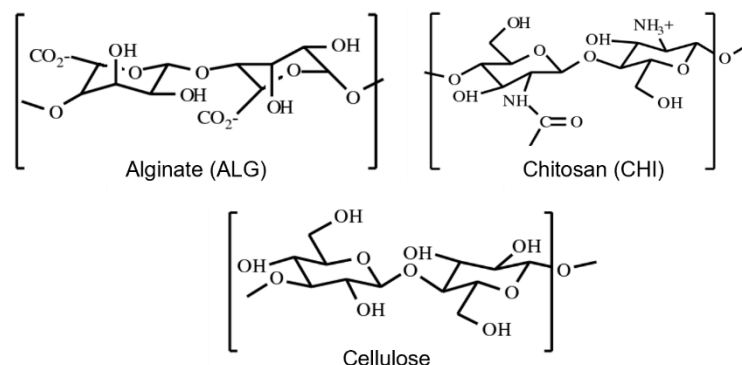


Figure 2.6. Chemical structure of ALG, CHI, and cellulose.

Among commercial polysaccharides studied to encapsulate bacteria, ALG is undoubtedly the most used. This natural polymer derived from brown algae or bacterial sources shows a sol-gel transition in the presence of divalent metal ions such as calcium cations. Therefore, the extrusion of a concentrated solution of sodium ALG into a solution of calcium ions results in rapid gelation. Due to this easy, mild and rapid gelation process, calcium ALG hydrogel is widely applied to encapsulate various bacterial strains.^[60-62] For example, gastrointestinal therapy requires preserving the survival of probiotics in acidic stomach fluid and ensuring their release in the neutral conditions found in the targeted small intestine.^[63-65] Therefore, different strategies have been explored over the years to prepare ALG-encapsulated probiotics. A pH-responsive shell based on a complex of ethylenediaminetetraacetic (EDTA)-calcium-ALG was developed to encapsulate *Lactobacillus rhamnosus* (*L. rhamnosus*). At lower pH, the in-situ release of calcium cations from EDTA-calcium compound resulted in reinforcing the ALG-calcium binding so that the hydrogel structure remained compact. However, at neutral pH, calcium ions were gradually released from the complex hydrogel, leading to its destruction and consequently the cell release.^[66] In another system, *L. plantarum* was loaded in cellulose-based microgels, then the particles were coated with calcium ALG hydrogel to form a core-shell structure.^[67] The porous structure of cellulose microgels allowed for the accommodation of a large number of bacteria (as high as 10 log CFU/g). It was evidenced that the particles remained intact in the simulated gastric fluids but cells were released due to the slow disintegration of ALG shell in simulated intestinal fluids.

The poor stability of ALG hydrogel in neutral/basic conditions strongly limits its use for gastrointestinal therapy based on oral administration of encapsulated probiotics. Thus multilayered ALG shells (Figure 2.7) were

produced by emulsion technique to encapsulate *Bifidobacterium breve*, *S. aureus*, *E. coli* and *Bacillus subtilis* (*B. subtilis*).^[68] These multilayered shells were shown to maintain the viability and the bioactivity of bacteria in a wide pH range corresponding to the gastrointestinal tract. Moreover, a continuous liberation of a metabolic enzyme produced by encapsulated *B. subtilis* was observed, demonstrating the potential interest of such capsules for controlled release. Biocomposite shells incorporating ALG with various biomaterials, such as milk soy and whey proteins, silica, cellulose, gelatin and xanthan gum, have also been developed to maintain bacterial viability. For instance, whey protein isolate with an excellent buffering capacity and gelation property was combined with ALG to form a complex hydrogel, leading to an enhanced tolerance of encapsulated bacteria towards low pH.^[69, 70] ALG hydrogel incorporating caseinate and soy protein isolate exhibited a positive effect on the gastric resistance and storage stability of encapsulated *Lactobacillus zeae*.^[71]

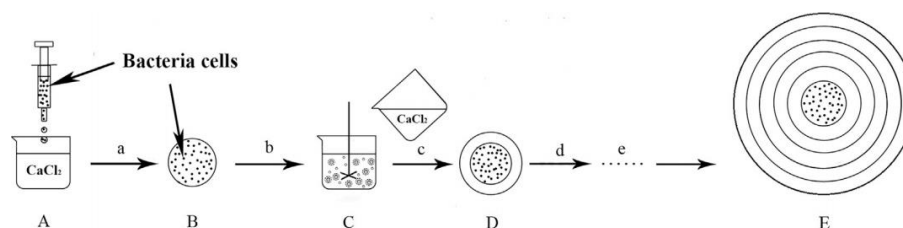


Figure 2.7. Preparation of bacteria cells encapsulated in multilayer ALG particles. (A) Injecting ALG solution containing bacteria cells into CaCl_2 solution; (B) bacteria cells encapsulated in core beads; (C) two different types of droplets in emulsion system; (D) the bead with a new layer; (E) bacteria cells encapsulated in five-layer ALG particles; (a) the beads were taken out and rinsed; (b) the beads were immersed in ALG solution and poured into oil phase with surfactant while stirring; (c) CaCl_2 solution was added into the emulsion system; (d) the beads were taken out and rinsed on a sieve; (e) repetition of processes (b)-(d).^[68]

Other polysaccharides known to form gels such as carrageenan gum, locust bean gum, xanthan gum, and guar gum were also tested alone or in combination with ALG to encapsulate bacteria. The acid and bile tolerance tests performed on ten different probiotics encapsulated with these biopolymers evidenced that carrageenan gum and xanthan gum showed equivalent cell viability protection as ALG.^[72] Moreover, *Lactobacillus acidophilus* (*L. acidophilus*) encapsulated in xanthan gum-ALG biocomposite capsules could survive in simulated gastric fluids at pH 1.2.^[73] It was also shown that the addition of xanthan gum in the ALG

solution during the encapsulation process led to a significant increase of the viability of probiotic bacteria during the storage since 82% of encapsulated bacteria were maintained bioactive after six months.^[74]

CHI, a cationic polysaccharide known to reduce the diffusion of H^+ , was tested in combination with ALG to form capsules around probiotics. More precisely, ALG/CHI multilayers were coated on commensal bacteria using the LbL deposition technique, demonstrating the control of bacteria proliferation.^[54] *Bacillus coagulans* coated with ALG/CHI shell (Figure 2.8) revealed a significant survival degree compared to non-coated bacterium when exposed to simulated gastric juice.^[75] The mucoadhesion property of coating ended by CHI was highlighted, showing to benefit the gastrointestinal delivery of probiotic bacteria.

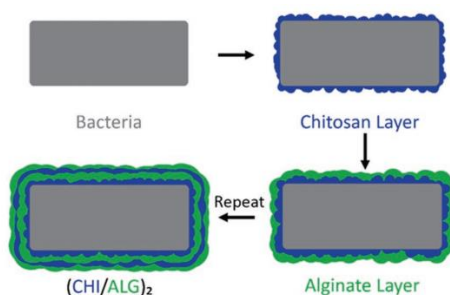


Figure 2.8. Schematic LbL encapsulation of *Bacillus coagulans* with CHI and ALG^[75]

The presence of CHI on the surface of ALG shell has been proved to strengthen the ALG particles at high pH thanks to ionic crosslinks.^[62, 76-78] For instance, more than 8 log CFU/mL *Bifidobacterium breve* were maintained in ALG-CHI coated ALG matrices after 4 hours exposure to simulated gastric fluid. Moreover, due to the pH-sensitive property of ALG, bacteria could be released in the small intestine at pH 7.2. Therefore, ALG-CHI shells not only enhance bacteria viability but also allow a controlled release of probiotic bacteria in the intestinal target as required for gastrointestinal therapy.^[79] However, the bactericidal properties of CHI limits its use as a protective agent for bacteria.

Starch is another attractive agent to protect probiotics since it is an easily digestible carbohydrate and is known for its prebiotic effect on the growth of intestinal microflora.^[59, 80] *L. plantarum* coated with soluble starch showed a higher degree of viability and a lower content of injured cells, compared to bacteria covered with various carbohydrates with lower glass transition

temperatures, such as glucose, sucrose, lactose and maltodextrin.^[59] Another approach consists of adsorbing probiotics on the surface of insoluble starch granules and then coating the resulting particles with gelatinized starch.^[81] To increase the loading content of probiotics, similar experiments were performed with partially hydrolyzed starch granules with a high porous structure, which facilitates the diffusion of bacteria. Encapsulated bacteria showed a higher degree of viability in acid bile salt and better resistance to heating treatment than free bacteria.^[80] Bacterial beads were successfully produced by extruding a mixture of *Lactobacillus casei*, ALG and starch granules in a calcium chloride gelling solution.^[82] It was shown that the presence of the solid starch filler in the beads significantly improved the cell viability during lyophilization and storage. Synergistic protection based on a complex of ALG and cationic starch derivative was also achieved on *L. acidophilus*, a milk bacteria.^[83] It was demonstrated that the viability and fermentation activity of the encapsulated bacteria were not impacted compared to free bacteria. Moreover, the coated bacteria can be re-used several times to perform pre-fermentation.

Cellulose is a natural dietary fiber that has been widely used in biomedical applications due to its beneficial effects on human health. However, native cellulose is not soluble in aqueous solution, which considerably limits its use for bacteria encapsulation. Therefore, a mild process based on the treatment in alkaline/urea solution at low temperature was developed to produce cellulose microgels with a porous fibrous structure. This cellulose hydrogel could host up to 9 log CFU/g of probiotics such as *L. plantarum*.^[84] The combination of cellulose microgels with ALG contributed to bacterial acid resistance and survival. Due to the fact that native cellulose is not soluble in aqueous solution, various soluble cellulose derivatives such as carboxymethyl cellulose and hydroxypropyl cellulose have been tested to fabricate microgels or edible films hosting probiotics.^[85-87] Experimentally, these gels are crosslinked in mild conditions by using, for instance, citric acid, and probiotics are incorporated during the gelling process or after its preparation by bacteria diffusion or adsorption.^[87] Interpenetrating polymer network hydrogels entrapping probiotics were also successfully produced by combining soluble cellulose derivatives with CHI^[86], gelatin^[88] or k-carrageenan.^[89] Multilayers of carboxymethyl cellulose and CHI were successfully deposited on *L. acidophilus*, resulting in a significant enhancement of bacterial survival rate in gastric pH as well as during freezing and freeze-drying.^[90] The higher survival of encapsulated bacteria in gastric conditions was attributed to the stability of the coating and its impermeability to enzymes responsible for proteolysis.

Interestingly bacteria such as *Acetobacter xylinum* can produce cellulose matrix as a natural protective barrier against external aggressions.^[91, 92] This

Chapter 2

bacterial cellulose has been used to prepare capsules or engineered hydrogels.^[93-95] Co-cultures involving strains producing cellulose were tested to perform bacterial encapsulation. For instance, recombinant *E. coli* and *Gluconacetobacter xylinus* were co-cultured, leading to the formation of “living membranes” for potential cell-based sensor applications (Figure 2.9).^[96] More robust composite scaffolds were produced by incorporating silk fibroin in the bacterial cellulose matrix. It was shown that the viability and the recombinant function of *E. coli* entrapped in these membranes were preserved under UV light irradiation, offering an efficient strategy for encapsulation of functionalized cells.^[97]

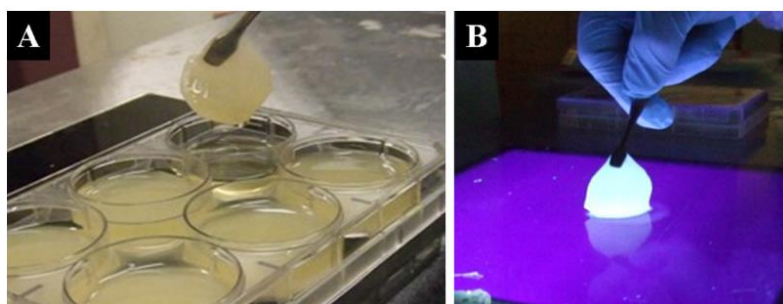


Figure 2.9. Encapsulation of *E. coli* in bacterial cellulose membrane. (A) The cellulose membrane produced by *Gluconacetobacter xylinus*; (B) GFP fluorescent signal expressed by *E. coli* entrapped in the cellulose membrane observed under UV light.^[96]

Proteins are other important agents used to encapsulate bacteria due to their high nutritional value and structural/physicochemical properties, such as thermal/ionic/chemical gelation ability, biocompatibility, and biodegradability. Moreover, proteins strongly interact with bacterial surface molecules, leading to strong adhesion between bacteria and encapsulating materials.^[98] The encapsulation of bacteria in protein matrices was notably developed to deliver probiotics to human gut via fermented dairy products. However, the use of non-dairy agents as encapsulating materials may affect the flavor and sensory evaluation of dairy food. Therefore, proteins extracted from milk such as casein, whey proteins and milk fat globule are the most popular materials for embedding probiotic bacteria.^[99] Sodium caseinate and soy protein isolate were notably used in combination with ALG to produce microparticles entrapping a model probiotic bacterium.^[71] It was evidenced that ALG/caseinate matrix provided a higher protection to probiotic cells during storage, whereas ALG/soy protein isolate blends enhanced the gastric resistance of cells. Whey protein was also used in combination with prebiotic

carbohydrates to encapsulate *L. plantarum* and it was evidenced that these blends provided a higher survival rate to probiotics under simulated gastric fluid and over the storage period of 60 days.^[100]

The use of transglutaminase to crosslink the whey proteins during the encapsulation process and the deposition of an ALG or xanthan gum coating on the obtained microparticles contribute to reinforce the barrier and structural properties of the envelop, leading to a better viability of probiotic cells in simulated gastric juice and bile.^[98, 101] However, protein matrices are sensitive to proteolytic enzymes present in gastric juice. So to prevent the degradation of gelatin microspheres by pepsin in the gastrointestinal tract, a calcium ALG layer was deposited on the surface of the particles. The buffering effect of the robust wall surrounding bacteria contributed to maintain significantly the survival of *Bifidobacterium adolescentis*.^[102]

Various charged biopolymers such as poly(glutamic acid) (PGA), poly-L-lysine (PLL), albumin, lysozyme, gelatin A, protamine sulfate, and chondroitin sulfate were successfully LbL assembled on bacteria surface via electrostatic interactions in order to form protecting complex polyelectrolyte shells.^[103]

Synthetic polymers were also studied to encapsulate bacteria, notably when using electrospinning and electrospraying encapsulation techniques which require a fine control of the rheology of the feed-in solutions and consequently of the characteristics of the polymers. Water-soluble polymers such as polyvinyl alcohol (PVA), poly(ethylene oxide) (PEO) and polyvinylpyrrolidone (PVPON) showing a good ability to form fibers were frequently used to prepare electrospun fibers entrapping bacteria.^[104-107] Synthetic polyelectrolytes such as cationic poly(allylamine hydrochloride) (PAH), polyethylenimine (PEI), poly(diallyldimethylammonium chloride) (PDADMAC) and polyanions such as polystyrene (PSS) and poly(acrylic acid) (PAA) are attractive materials for bottom-up LbL encapsulation of bacteria through electrostatic interactions.^[103, 108, 109]

Synthetic polymers or their complex showing stimuli-responsive properties were used to prepare smart coatings onto bacterial cells in order to control their release and/or their handling under variations of pH, ionic strength or temperature.^[110, 111] Such an approach is of a great interest for gastrointestinal therapy and skin care applications. Pluronic F127, a triblock copolymer composed of a poly(ethylene glycol)-b-poly(propylene glycol)-b-poly(ethylene glycol) and exhibiting a reversible gelation temperature at about 17 °C, was frequently tested to fabricate temperature-responsive matrices entrapping bacteria.^[112, 113] At low temperature, the polymer solution is indeed poorly viscous but hardens after administration at a higher temperature. For instance, a composite Pluronic F127 hydrogel embedded with *B. subtilis* was

successfully administrated on human skin to inhibit fungal infections thanks to the antifungal agents secreted by *B. subtilis*.^[112] Cell-laden hydrogels incorporating various bacterial strains were also prepared according to this strategy then processed by 3D printing to fabricate structured multi-materials where cells communicated in a programmable manner, as shown in Figure 2.10.^[113, 114] Such customized living materials showing predictable dynamic functionalities are of interest for biosensing applications and disease treatment.

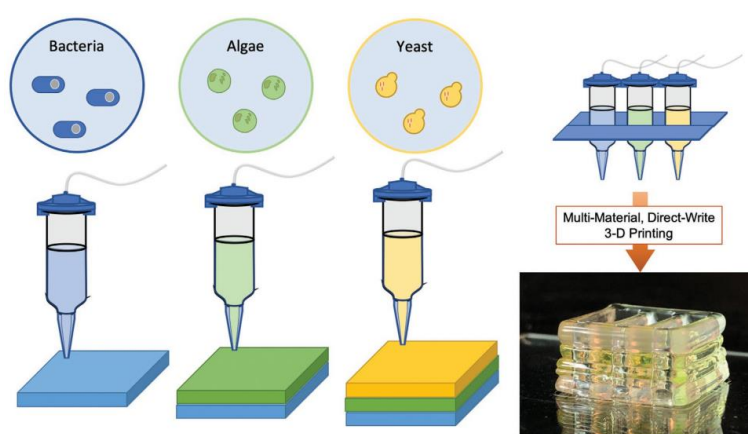


Figure 2.10. 3D printing cell-laden multilayered hydrogel prepared from Pluronic 127.^[113]

Inorganic materials. The deposition of metal nanoparticles on bacteria surfaces was developed to produce bioelectronic devices combining the biological response with electronic or magnetic properties. Such systems have a potential interest in biosensing or medicine. For instance, surface-modified gold nanoparticles were successfully adsorbed on Gram-positive *Bacillus cereus* via electrostatic interactions. A conductive layer was formed on bacteria and bacterial viability was maintained.^[115] It was shown that these coated bacteria could be used as a humidity sensor since the conductivity of the monolayer varied with the swelling degree of bacterial cells (Figure 2.11).^[116] Gold-coated bacteria were also used as weapons to destroy cancer cells. Indeed, the gold nanoshell deposited on cell surface provides photothermal properties that can be used wisely to destroy malignant cells. In this case, the gold nanoshell was in-situ prepared by self-nucleation of gold nanoparticles on bacteria surface. An antibody targeting malignant cancer cells was then adsorbed on the coated bacteria for an enhanced selectivity.^[117]

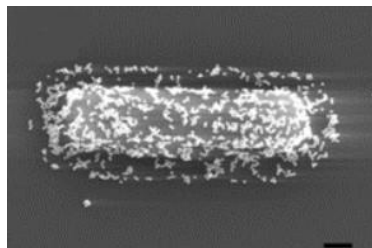


Figure 2.11. SEM image reveals the deposition of surface-modified gold nanoparticles (30 nm) on *Bacillus cereus*. Scale bar: 300 nm.^[116]

Porous silica gel matrices deposited on bacteria surface have been proved to be efficient in providing robust mechanical and chemical protection.^[118] These matrices are typically synthesized by a sol-gel process based on the hydrolysis and condensation of alkoxysilanes or silanols. However, the accumulation of alcohol produced along the reaction may cause a sharp loss of cell viability. Therefore, an alcohol-free route based on the controlled vacuum evaporation of the alcohol by-product during the process was tested.^[119] It was shown that the integrity of *E. coli* was essentially preserved using this encapsulation protocol. Other routes based on the acidification of sodium silicate and using of silica nanoparticles solutions were also developed to avoid producing alcohol in the reactive mixture.^[120, 121] Although the growth and viability of *E. coli* encapsulated according to this protocol were much higher compared to the conventional sol-gel process based on alkoxysilanes, it is worth noting that sodium ions tend to accumulate in the matrix, which are harmful for encapsulated cells.^[122]

Hybrid materials. The incorporation of inorganic particles in organic hydrogels allows the preparation of protective matrices with enhanced mechanical, thermal and chemical resistance. Moreover, the inorganic nanoparticles can fill the pores of the hydrogels, thus limiting the diffusion of salts, hydrogen ions and enzymes which are harmful to cells. Thereby, ALG-gelatin microgels doped with magnesium oxide (MgO) nanoparticles were used to encapsulate *Pediococcus pentosaceus*, a probiotic that has the potential to reduce inflammation, encephalopathy, obesity, and fatty liver in animal models.^[123] The comparison of probiotics encapsulated in microgels doped with and without MgO nanoparticles revealed that the incorporation of MgO enhanced significantly the cell viability after a long-term storage in an aerobic environment or a heat treatment, but also after exposure to the gastrointestinal transit. This feature is attributed to the ability of MgO to inhibit hydrogen protons and consequently to avoid acidic degradation of cells. The presence of MgO nanoparticles also reduced the diffusion of oxygen in matrix, which is detrimental for long time storage of probiotic.

Hybrid shells were fabricated on bacteria surface by combining the LbL assembly technique and sol-gel route. Thereby, cyanobacteria known for their ability to treat wastewater were coated with a hybrid multilayer composed of an external silica shell prepared by sol-gel, a cationic PDADMAC layer acting as a glue between the negatively charged cell wall and silica shell, and a PSS layer acting as a protective agent against ultraviolet light irradiation.^[124] It was shown that this hybrid coating offered a strong protection against harmful ultraviolet light irradiation while maintaining the viability and bioadsorption capacity of bacteria for heavy-metal ions in polluted solutions.

The addition of inorganic particles in the encapsulating matrix was also developed to provide novel physical properties such as magnetic, fluorescent, bioelectric, and photo-thermal functions. For instance, magnetite (Fe_3O_4) particles were incorporated in polymer microtubes (MTs) entrapping *E. coli* in order to manipulate them under a magnetic field.^[125] Polymer-based shells loaded with noble metal particles (Figure 2.12) were explored to characterize bacteria with surface-enhanced Raman scattering. It was demonstrated that this approach not only allowed to get important information about the bacterial cell wall biochemistry but also enabled the identification of single bacteria cell.^[126]

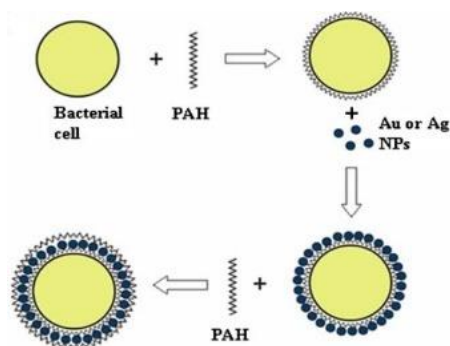


Figure 2.12. Layer-by-layer coating of bacteria with Au/Ag-nanoparticles for surface-enhanced Raman scattering.^[126]

Hybrid shells were also prepared by sol-gel route. For instance, hybrid shells were obtained by adding an organosilane and poly(ethylene glycol) (PEG) in the silica stock solution to prepare an encapsulating matrix.^[119] *E. coli* strain encapsulated in such organically modified silica showed longer viability compared with cells encapsulated in pure silica (18 versus 6 days, respectively). Following a similar approach, *Oenococcus oeni*, a bacterium used to make wine, was entrapped into an inter-penetrated network of silica

and ALG. The presence of ALG in the shell provides a hydrophilic microenvironment, while silica reduces the shrinking of capsules. As a result, bacteria entrapped in such hybrid capsules were maintained viable under a low pH of 3.3 or a high alcohol degree of 15%. Moreover, these bioactive capsules could be re-used for at least five times under winemaking conditions, whereas unencapsulated bacteria could not be re-used.^[127] An electrochemically assisted process was also developed to encapsulate bacteria in thin hybrid sol-gel films, as shown in Figure 2.13. For this, a tetramethyl orthosilicate solution used as a silica precursor was implemented with-PEG, CHI and trehalose additives. The presence of these organic materials contributed to increase the stability of the coatings. It was shown that *E. coli* entrapped in such hybrid capsules remained active for one month. In addition, the thickness of this hybrid coating could be tuned from less than 100 nm to more than 2 μm by varying the deposition time.^[128]

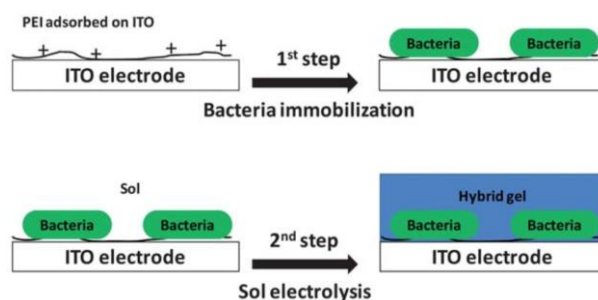


Figure 2.13. Mechanism of electrochemically assisted bacteria encapsulation in a thin sol-gel film. ITO: indium tin oxide.^[128]

Biofilm. In most natural ecosystems, bacteria grow in biofilms on a surface to enhance bacterial survival under extreme conditions (Figure 2.14A). Biofilm is a complex structure composed of a community of cells encapsulated within self-produced extracellular polymeric substances. The extracellular matrix composition varies with the bacteria type and the environment, including polysaccharides, proteins, and DNAs.^[129, 130] This extracellular matrix not only acts as an adhesin for bacteria to attach on a surface, but also protects embedded bacteria as a physical and chemical barrier by preventing molecule penetration.^[131, 132] Bacterial cells in the extracellular matrix exhibit differential gene expression because of heterogeneity in nutrients and oxygen supply. Therefore, bacteria with phenotypic diversities in antibiotic resistance, DNA repair, lipid biosynthesis and host immune modulation are obtained.^[129, 133, 134]

Inspired by this natural bacteria protection method, encapsulation of bacteria in biofilms was tested. Different from other encapsulation methods that provide a temporary protection before bacterial division, a self-produced biofilm provides a long-lasting protection to bacteria during growth. As shown in Figure 2.14B, self-coated *B. subtilis* showed a 125-fold greater oral bioavailability and a 17 times higher intestinal colonization than uncoated bacteria, demonstrating biofilm as a robust platform for a broad set of gastrointestinal biomedical applications.^[131] Interestingly, biofilm also shows prospects for delivery of probiotic cells to humans, evidenced by co-culture of *B. subtilis* with probiotic lactic acid bacteria.^[135] The extracellular matrix produced by *B. subtilis* could be shared with *L. plantarum* probiotic, contributing to maintain the survivability of *L. plantarum* cells during food preparation and gastric conditions.

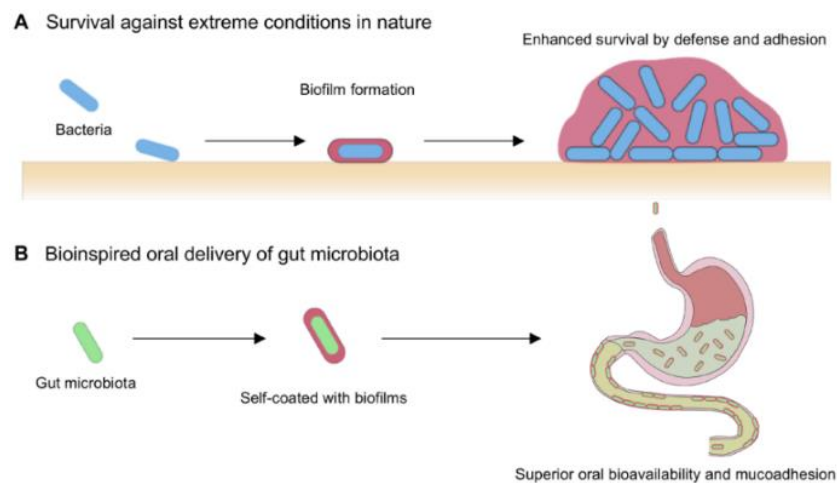


Figure 2.14. Schematic illustration of (A) biofilm formation of bacteria in nature to enhance survival by defense and adhesion under extreme conditions and (B) bioinspired oral delivery of gut microbiota with superior oral bioavailability and mucoadhesion by self-coating with biofilms.^[131]

Table 2.1 Summary of main materials used to encapsulate bacteria

Materials	Properties	Advantages	Drawbacks
ALG	-A sol-gel transition in the presence of Ca^{2+} (ionic crosslinking) -Ionically complexed with CHI	- A biocompatible material - Easy gelation - Widely applied in various encapsulation techniques	ALG gel can be disaggregated at high pH or after long storage
CHI	A polycation derived from macromolecules of natural origin that can form polyelectrolyte complexes with ALG	- A biocompatible material - Reduces the diffusion of H^+ - Strengthens the stability of ALG-based matrix	Bactericidal compound
Cellulose	A fibrous material with a high glass transition temperature	- High mechanical strength and high thermo-stability - Digestible	Cellulose is not soluble, thus cellulose derivatives are used
Proteins	Proteins (caseinate, whey protein, etc.) can complex with bacteria membrane to strengthen bacterial viability	- Biocompatible and digestible - Easy gelation - Popular for food industry applications	Can not be used in dairy free products.

Table 2.1 Summary of main materials used to encapsulate bacteria

Materials	Properties	Advantages	Drawbacks
Synthetic polymers	Molar mass (M_w) and properties of polymers can be controlled by chemical synthesis	- Both neutral polymers and polyelectrolytes can be used - Polymers have tunable M_w, mechanical properties, and functions	- Synthesis can be complex - Limited biocompatibility
Metal particles	Metal (Au, Ag, MgO, Fe_3O_4 etc.) nanoparticles have high physical, chemical and thermal stabilities	Attractive for preparing bioelectronic devices, such as sensors, and detectors	Can impact the bacterial viability
Silica gel	A sol-gel transition to encapsulate bacteria	- Tunable porosity and high mechanical strength - Chemically and thermally stable	Alcohol by-product can be accumulated during sol-gel process, which impacts bacterial viability
Biofilm	Self-produced extracellular matrix to embed bacterial community.	- Promote bacterial adhesion on surface - Long term protection of bacteria	Leads to antibacterial resistance of bacteria

2.2.2 Encapsulation methods

A series of bacterial encapsulation methods are reviewed in this section with a summary of these methods in Table 2.2.

Emulsion. Emulsion is a classic method to fabricate capsules. Experimentally, encapsulation of bacteria according to this method is performed by suspending cells in a small volume of an aqueous solution which is then dispersed in an oil phase in the presence of the polymer selected to form capsules. The resulting mixture is emulsified under vigorous stirring in order to form microdroplets of polymer solution around bacterial cells.^[136] Capsules are then obtained by gelling or solidifying the polymer around the cells.

ALG capsules were extensively produced by this technique: after dispersing ALG in water-in-oil emulsion, calcium chloride solution is added to the mixture to obtain ALG gel.^[72] After removal of the organic phase, for instance by evaporation, the harvested capsules can be freeze-dried. Their size ranges from tens of microns to several millimeters, depending on the agitation speed used to prepare the emulsion.^[72, 137] However, such an approach can be detrimental for bacteria since bacterial cells are exposed to organic solvent. For this reason, double water (water in oil in water (W/O/W)) emulsions were tested. The fabrication process consists of primary then secondary emulsification. In primary emulsification, water phase containing bacteria is dispersed into oil phase to produce water-in-oil emulsion. This primary emulsion is then emulsified in a second aqueous solution, followed by removal of the oil phase.^[138, 139] Indeed, double water emulsions offer the advantage of using less oil phase to limit cell exposure. Moreover, this technique allows for a better control of the capsule size.

Pseudomonas putida, a biotechnological model strain, was successful encapsulated in polyhydroxyalkanoate capsules using the double water emulsion approach. It was shown that the average diameter of the obtained particles was about 10 μm . Moreover, encapsulated bacteria exhibited excellent tolerance to alkaline and oxidative conditions with cells remaining viable after 2 h in alkaline solution and 24 h in 20 mM H_2O_2 .^[140] Acid sensitive *Akkermansia muciniphila* (*A. muciniphila*), an abundant bacterium in human gut identified as a potential new therapeutic microbe to prevent diet-induced obesity and type 2 diabetes, was embedded in capsules via the mild homogenization process of double water emulsion.^[141] The objective was to protect *A. muciniphila* during gastric passage when used in oral formulations. After 2 h in vitro culture in gastric acid, *A. muciniphila* remained entrapped in capsules and showed higher cell viability. In addition, the degradation of oil phase in bile extract allowed for the targeted delivery of *A. muciniphila* to the small intestine. The stability and integrity of microcapsules prepared by double emulsion can be enhanced by multiplying the number of polymer

layers deposited around the bacteria.^[67] For instance, a repeated ALG emulsion process was developed to get multi-layered capsules entrapping *B. subtilis*, used as a model of oral delivery system. It was shown that the multilayered ALG capsules not only protected bacteria from harsh conditions, but also allowed the long-term sustained release of bacterial metabolite. The amount of α -amylase produced by *B. subtilis* in multilayered capsules continuously increased for ten days, which contrasted with the burst release observed for free bacteria and bacteria embedded in single-layer ALG capsules.^[68]

However, the extraction of the organic solvent after the capsule formation may cause particle aggregation and cell damage. To overcome this problem, supercritical carbon dioxide fluid with a low viscosity and high diffusivity was tested to remove oil phase from emulsion in mild conditions.^[142-144] Due to the fast solvent extraction, a better control over particle size and distribution was observed compared with freeze-drying and solvent evaporation process.^[145] *L. acidophilus*, a bacterium known to reinforce the immune system, was entrapped in FDA-approved poly(lactic-co-glycolic acid) capsules with a diameter of 20 μm via a double water emulsion-supercritical emulsion extraction process. Although a high encapsulation efficiency of 80% was obtained, cell viability was strongly impacted with only 5% of viable bacteria maintained in the capsules.^[146] This suggested that double water emulsion-supercritical emulsion extraction technology is more appropriate for the encapsulation of dead bacteria used as therapeutic agents while avoiding their potential adverse effects.

As an alternative, pickering emulsion using solid nanoparticles adsorbed at the oil-water interface was developed to prevent capsule aggregation and coalescing. By reducing the energy difference between oil and water phases, the nanoparticles contribute indeed to obtain more stable emulsions, compared to those prepared with usual surfactants.^[147, 148] Moreover, nanoparticles such as modified silica, are more biocompatible than surfactants. The encapsulation of viable *L. plantarum* cells was successfully achieved by pickering emulsion. As shown in Figure 2.15, a pickering emulsion of water droplets containing bacteria and stabilized by hydrophobized silica particles, was templated with poly(organosiloxane) thanks to the interfacial reaction between alkylchlorosilane molecules. The viability of bacterial cells entrapped in the resulting hollow capsules could be fully preserved within the first three hours after encapsulation.^[149] A similar approach based on pickering emulsion stabilized with β -cyclodextrin (β -CD) inclusion complexes was successfully developed to entrap *Lactobacillus dellbrueckii*. It was shown that the survival of the entrapped bacteria was much higher when β -CD was used compared to Tween-80 surfactant.^[150]

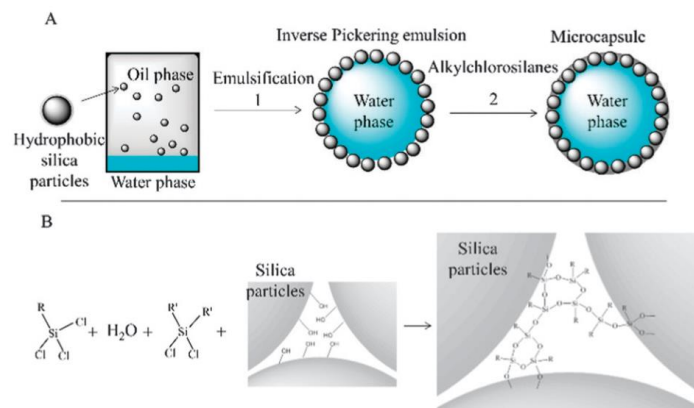


Figure 2.15. Methodology for the synthesis of microcapsules encapsulated with bacteria. (A) An inverse Pickering emulsion is produced by the emulsification of water in a dispersion of hydrophobic silica particles in n-heptane (1). The addition of alkylchlorosilanes to the inverse Pickering emulsion results in an interfacial reaction with water to produce polymer, thus forming microcapsules (2). (B) The reactive silanol groups on the primary stabilizing silica particles will also react with the poly(alkyl siloxane).^[149]

Extrusion. The extrusion-based method consists in forcing a polymer precursor solution through a syringe needle or a nozzle to form droplets which are subsequently solidified or gelled.^[151-153] To perform bacteria encapsulation, cells are simply dispersed in the polymer precursor solution. Solidification or gelation of the droplets can be achieved by ionic, chemical, photo or thermal crosslinking. Compared to emulsion technique, the extrusion is easier to perform, less costly and requires milder conditions since it does not use organic solvents. However, the size of the produced spheres needs to be optimized to limit the impact on cell viability, depending on a series of parameters such as the properties of the polymer precursor solution (*e.g.* surface tension, viscosity, concentration) and processing conditions (*e.g.* needle diameter, liquid flow rate, distance between the dripping system and the gelling solution).

ALG is the most used polymer precursor to produce beads by extrusion. The gelling of the extruded ALG droplets is achieved by dripping in a divalent cation solution, for instance a calcium chloride solution.^[154-158] ALG beads incorporating *Bifidobacterium bifidum* and *L. acidophilus* were successfully prepared according to this technique then incorporated in white-brined cheese. It was demonstrated that the count of unencapsulated bacteria decreased by approximately 3 log CFU/g for 90 days storage in the control cheese. However, the count decrease was limited to 1 log CFU/g when encapsulated bacteria

were used.^[99] Other biopolymers such as carboxymethyl cellulose, κ -carrageenan and pectin that can form gel in presence of divalent cations, were also used to encapsulate probiotics.^[89, 159] For instance, *L. plantarum* was entrapped in microspheres based on pectin/starch blend.^[159] Thanks to the resistance of pectin to both gastric and intestinal enzymes, encapsulated bacteria survived the sequential exposure to simulated gastric and intestinal fluid while free bacteria died. Moreover, the digestion of pectin in the colon by pectinases allowed for the colon-specific delivery of bacteria.^[159, 160]

Various strategies have been developed to optimize the size of the spheres produced by extrusion in order to improve the viability of encapsulated bacteria as well as the sensorial impact of the capsules when incorporated in food products. Using pressure at a high flow rate or droplet breaking systems such as co-axial air flow, vibrational encapsulator or jet-cutter can decrease the size of the droplets and consequently the diameter of the produced beads.^[155, 157] The variation of the vibration frequency of nozzle was also tested to produce particles with uniform size distribution.^[161] For instance, by using a 80 μm -diameter nozzle and applying a vibration frequency of 1740 Hz, ALG capsules entrapping *Lactobacillus reuteri* were produced with a perfect spherical shape and an uniform size of 100 μm . It was shown that such a diameter contributes to limiting the sensorial impact of capsules when incorporated in food products.^[157] However, the effect of the processing parameters decreases when the viscosity of the precursor polymer solution increases.

Co-extrusion has been developed to create core-shell capsules, offering better bacterial protection against the external environment. For this, external and internal nozzles are used simultaneously to extrude the core and the wall materials of the capsule, respectively.^[158] Figure 2.16 shows the differences between extrusion and co-extrusion techniques. Reservoir-type particles composed of ALG or ALG-shellac blend as the wall material and sunflower oil loaded with *L. acidophilus* probiotics as the core were successfully fabricated by co-extrusion. Bacteria encapsulated in such particles showed higher survival rates after storage and in simulated gastrointestinal fluids than the ones entrapped in ALG beads produced by simple extrusion.^[155] This can be attributed to the lower porosity of the wall inhibiting the diffusion of gastrointestinal fluids to trapped bacteria. However, the use of oil as a core medium may present limitations, notably for food products where lipids are undesired. For this reason, a co-extrusion approach based only on aqueous polymer solutions was developed: two ALG solutions with different concentrations (*i.e.* 0.5 and 1%) were co-extruded with the 0.5% ALG solution containing probiotics forming the core and the 1% solution forming the shell.^[156] The encapsulated *L. acidophilus* showed a much higher viability after 50 days storage in an acidic milk medium than free bacteria.

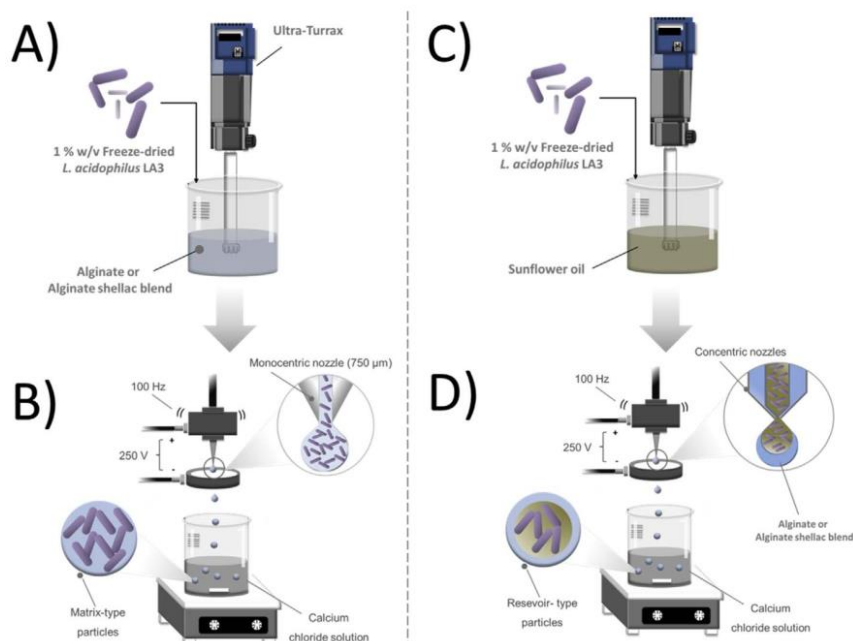


Figure 2.16. Differences between extrusion (A and B) and co-extrusion (C and D) techniques using a vibrational system for bacteria encapsulation.^[155]

As a fundamental and easy-operating technique, extrusion has been studied to optimize the protecting capsules surrounding cells in order to improve their storage in harsh conditions. However, other innovative encapsulation techniques have been developed for commercial developments, such as microfluidics, electrospinning, spray-drying, and spray freeze-drying, which will subsequently be discussed.

Microfluidics. Microfluidics has been used as a droplet generating system to encapsulate bacteria in spherical or cylindrical particles.^[162-164] Microfluidic devices are composed of circuits of microchannels allowing two or more streams to flow in contact with one another without mixing. Three most commonly used microfluidic devices: T-junction, flow-focusing, and co-flow devices are shown in Figure 2.17.^[165] This technique is well-adapted for cell encapsulation since it allows to produce monodispersed particles or fibers, depending on the conditions used.^[165, 166] The particles produced by microfluidics have a smaller diameter compared to the other techniques such as emulsion or extrusion. The large surface-to-volume ratio of particles facilitates the diffusion of nutrients and consequently the survival of encapsulated cells. Moreover, microfluidics offers simplicity in design and operation, has the advantages of low reagent consumption, short reaction time

Chapter 2

and real-time monitoring of the fabricated particles.^[167-169] The material used for the microfluidic circuits depends on the process selected to fabricate the particles. Typically, glass-based circuits are used when organic solvent or chemicals are used during the fabrication process, while polydimethylsiloxane based circuits are preferred when using aqueous solutions.^[166] Another advantage of microfluidics is its large production rate (up to twenty thousand particles per second), allowing for industrial applications.^[170]

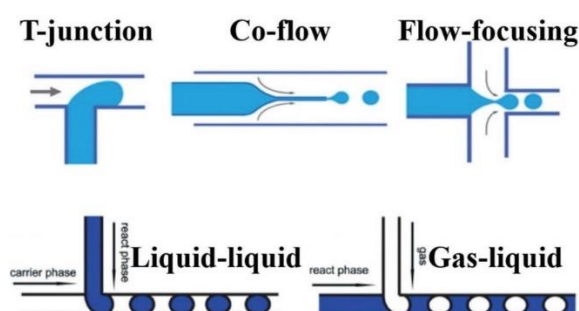


Figure 2.17. Schematic illustration of microfluidic flow relied on various channel geometries, including T-junction, co-flow, flow focusing; segmented flow relied on liquid-liquid interfacial and gas-liquid interfacial interaction.^[165]

The formation of particles in microfluidics systems is done sequentially by mixing components coming from different streams through a microchannel circuit with the nucleation being the first step, then the growth and the solidification.^[171] The characteristics of the produced particles depend on the geometry of the microcircuit and the materials used for their fabrication.^[165] *E. coli* was successfully entrapped in agar capsules by this technique.^[172] For this, an agar solution containing bacteria was injected in mineral oil to form an agar-in-oil emulsion. Then the agar droplets containing bacterial cells were allowed to cool down and solidify through the downstream channel. By combining simulation and experimental setup, it was shown that the size and the dispersity of the obtained particles depend on the agar solution concentration, the flow ratio between oil and agar phase and the capillary number. It was checked that encapsulated bacteria were able to grow in the agar particles over the time, which testified for their viability. Owing its capability to perform entrapment of individual cell, microfluidics was also used in combination with various sensitive detection techniques to produce platforms for ultrahigh-throughput screening, drug testing or to study cellular processes like cell-to-cell interactions.^[173-175]

Microfluidic devices can also be used to produce microfibers entrapping different bacteria. For this, a coaxial laminar flow is developed followed the solidification of the central flow containing bacteria by photo-crosslinking, ion crosslinking or chemical reaction.^[176-179] The stable laminar flow produced in the microchannel allows for the continuous embedding of bacteria in the produced fibers. The morphology, hydration, and thermal properties of fibers produced by microfluidics can be tuned by adjusting the composition of the precursor solutions.^[180]

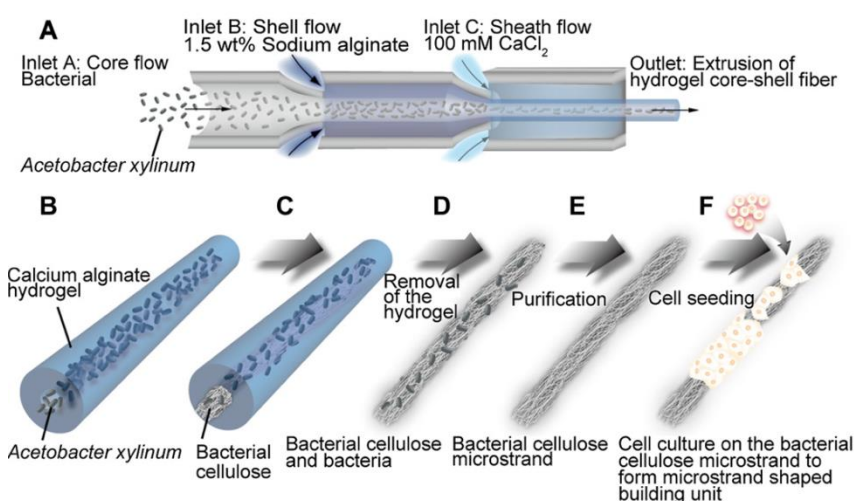


Figure 2.18. Schematic illustration of the process to fabricate bacterial cellulose microstrands. (A) Fabrication process of a bacterial/ALG fiber by a double co-axial laminar flow device. (B-F) Culture of the fiber to get bacterial cellulose microstrands.^[61]

By comparison with other fibers producing techniques like electrospinning, microfluidics does not use high voltage, organic solvent or high shear stress that can be detrimental for cells. For example, fibers encapsulating *Bacillus cereus* and *E. coli*. were successfully prepared using the co-axial approach. The core fluid was a photo-crosslinkable pre-gel of poly(ethylene glycol dimethacrylate) where bacteria were added and the sheath was an aqueous solution of PEG.^[180] More than 90% of bacteria was maintained in co-axial fibers, which was much higher than the viability rate obtained by electrospinning. Moreover, it was shown that the encapsulation process did not impact the growth and the metabolic activity of cells. *Acetobacter xylinum*/ALG core-shell fibers were also successfully produced by injecting in a double coaxial microfluidic device composed of a bacterial core flow, an

ALG shell flow and a calcium chloride sheath flow.^[61] Interestingly, the culture of fibers led to the formation of bacterial cellulose microstrands thanks to the ability of *Acetobacter xylinum* to form a cellulose network within the core of the hydrogel fiber (Figure 2.18).

Electrospinning and electrospraying. Electrospinning is an electrohydrodynamic process allowing to produce fibers in a broad size range. Practically, a jet of polymer solution extruded from a spinneret is charged by applying a high-voltage electric field. The electrostatic force resulting from the application of this voltage overcomes the surface tension of the polymer solution so that the natural hemispherical droplet pending at the tip of the spinneret is distorted into a conical shape. This leads to the formation of a continuous filament which solidifies during the journey to the collector by solvent evaporation. The principle of electrospraying differs from the electrospinning by the fact that the jet of polymer solution breaks up in droplets under the application of a high voltage.^[181-183] Indeed, when the solution concentration is low, the jet is destabilized due to various instability mechanisms and hence droplets are formed. These droplets are subsequently solidified to form small particles which are harvested on the collector. The typical laboratory-scale electrospinning and electrospray setups are shown in Figure 2.19 and 2.20, respectively.

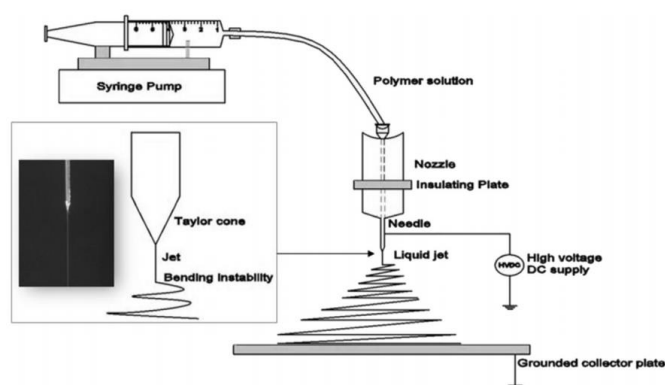


Figure 2.19. A typical laboratory-scale electrospinning setup. Inset shows the digital image and representation of electrospinning process.^[182]

Both electrospinning and electrospraying are attractive techniques because they are easy to operate and they allow to fabricate fibers and particles with narrow size distributions. Moreover, the size and the morphology of fibers or particles can be controlled by regulating different factors related to the polymer solution (*e.g.* concentration, viscosity, molar mass of polymer chains,

conductivity, etc.) and processing parameters (*e.g.* flow rate, voltage value, distance between the tip and the collector, etc.).

Water-soluble and synthetic polymers such as poly(ethylene oxide) (PEO) and PVA, and natural polymers such as ALG, CHI, gelatin, collagen, and hyaluronic acid (HA) have been intensively used to perform encapsulation by electrospinning and electrospraying.^[184] However, the processing conditions used for electrospinning and electrospraying can strongly impact bacterial viability. For instance, *Staphylococcus albus* and *E. coli* were successfully embedded in electrospun PVA nanofibers. However, viability assays showed a significant difference between bacteria. Indeed, *Staphylococcus albus* remained fully viable while only 19% of *E. coli* survived after encapsulation. The rapid dehydration of *E. coli* during drying was the main reason for the loss of viability.^[185] A similar study investigated the impact of electrospinning on the viability of lactic acid bacteria. Ten different species of bacteria were embedded in electrospun PEO nanofibers. It was found that the cellular viability was correlated with the hydrophobicity of bacteria. Indeed, the hydrophobic bacteria were more resistant than hydrophilic ones. This could be attributed to the presence of hydrophobic molecules at the surface of these strains supplying better protection during the encapsulation process.^[186] In addition, bacteria of extreme size like *Lactobacillus delbrueckii ssp. bulgaricus* showed a lower degree of viability compared to smaller species, proving that the morphology of cell can also be a parameter impacting viability during electrospinning.

It can be concluded from these observations that conventional electrospinning is poorly effective to protect bacteria against adverse conditions. This is notably due to the complexity of controlling the thickness of the polymer layer coated on bacteria. In regard to this limitation, co-axial electrospinning leading to double-layered fiber has been developed. Experimentally, bacteria are incorporated in the core fiber and the shell part provides additional protection. Moreover, the separation of two layers enables the specific functionalization of the external shell while reducing the negative impact on inner bacteria. Bifidobacteria encapsulated in PVA co-axial nanofibers remained viable for 40 days at room temperature and for 130 days at refrigeration conditions.^[187] The resistance of *L. plantarum* towards thermal treatment and gastric fluid exposure was also significantly improved when cells were encapsulated in core-shell fibers composed of a PVA core and PVA-ALG shell.^[188] Directly inspired by the fabrication of hollow capsules, hollow fibers entrapping bacteria were also produced by electrospinning. They were achieved by performing co-axial electrospinning with a sacrificial polymer used as a core. For instance, nanofibers based on poly-L-lactic acid shell and sacrificial PEG core were successfully fabricated according to this approach.^[189, 190] The incorporation of bacteria in the core solution and the

subsequent removal of the PEG component by aqueous washing resulted in the fabrication of poly-L-lactic acid tubules entrapping cells. Such a hollow structure was shown to be favorable for cell growth.

The proper deposition of electrospun fibers on a substrate leads to fibrillar structures mimicking extracellular matrix with mechanical properties, which can be used as artificial scaffolds or skin patches. Non-woven nanofibrous mats encapsulating bacteria beneficial for health were notably generated for wound healing,^[105, 191] or treating periodontal disease^[106] and skin disorders.^[107, 192] Due to its high surface area and porous structure, nanofibrous materials encapsulating bacteria were also tested for bioremediation of heavy metals and reactive dyes from wastewater. Moreover, it was shown that polysaccharides used for producing electrospun fibers could serve as an additional carbon source, contributing to promoting bacterial growth and consequently the depolluting performance of the mat.^[193]

However, a major drawback of electrospinning is the application of a high voltage, which can strongly impact bacterial viability. To overcome this problem, an ultralow voltage continuous electrospinning patterning technique (LEP) using a reduced voltage was developed.^[104] This technique is based on a lateral mechanical stretching force effect and an electrostatic field focusing effect to initiate polymer jetting. These combined effects could be obtained by using “initiators” localized on either side of the patterned route. When the jet moves away from the initiator, mechanical lateral force contributes to stretch fibers. And depending on the polymer solution conductivity, the initiator can be conducting or insulating materials. LEP is a versatile method allows to use a wide range of polymer and solvent systems and to perform direct printing of living bacteria on diverse substrates, offering a potential route for lab-on-chip applications.

Electrospray particles are advanced carriers for encapsulating bacteria in dry state while maintaining their viability for food industry applications. The application of high voltage is favorable to generate particles of narrow distribution because the electrostatic repulsion of high charged particles prevents agglomeration and coagulation. The size and integrity of particles not only depend on polymer solution but also on ambient and instrumental factors. Thus the electrospray conditions need to be optimized for each selected system to get the highest encapsulation efficiency and to minimize the loss of bacteria viability.^[194, 195] For instance, the concentration, the thermal treatment and the composition of the acacia gum solution were optimized to prepare highly uniform acacia gum microparticles entrapping *L. plantarum*.^[196] Viability assays of these particles showed that 96% of probiotic cells were still alive after electrospraying encapsulation. Moreover, it was shown that electrosprayed microcapsules conferred enhanced protection, leading to a reduced cell loss during storage at high relative humidity.^[195]

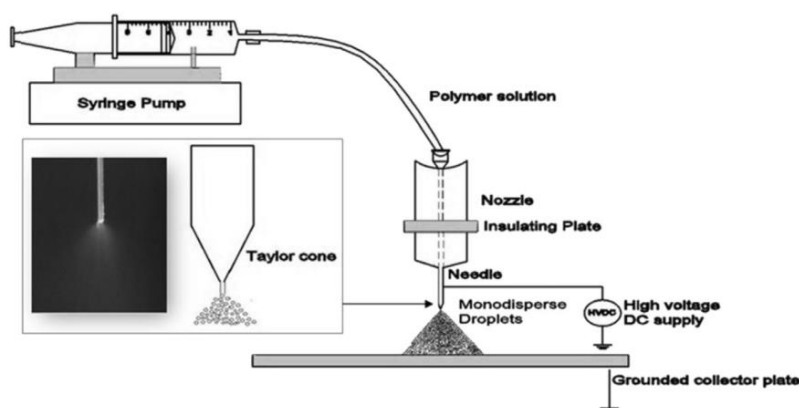


Figure 2.20. A typical laboratory-scale electrospray setup. Inset shows the digital image and representation of electrospray process.^[182]

Spray drying. Spray drying consists of the atomization of a fluid and the drying of the resulting droplets in a heated gaseous medium. A typical spray drying setup contains the following elements: a feed-in reservoir and a pump, an atomizer, a heater, an air supplier, a drying chamber, a cyclone, and a collector. As shown in Figure 2.21, the polymer solution is pumped into one or two nozzles to break the feed-in liquid into droplets. A heated gas is forced into the drying chamber to facilitate the evaporation of solvents. Then the dried solid particles obtained are sucked into a cyclone and collected by the container located in the bottom part of the instrument.^[197]

Because of the rapid drying process, spray drying is a popular technique for bacteria encapsulation, especially for lactic acid bacteria used for food applications.^[198, 199] However, the drying process can strongly impact the bacteria viability so that a proper selection of the encapsulating agent is necessary to limit the drying stress effects. Typically, encapsulation or co-encapsulation with carbohydrate polymers and proteins can improve the survival of bacteria during the process and can offer a robust protection during storage.^[71, 200] It was shown that blends of ALG and soy protein isolate provided the best protection to a *Lactobacillus* probiotic during simulated gastric digestion, whereas the best storage property was obtained using blends of ALG and caseinate.^[71] These results proved that the formulations offering the best protection during simulated gastrointestinal digestion did not necessarily supply the best protection for storage.

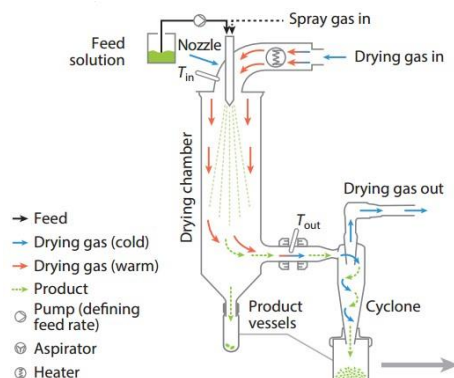


Figure 2.21. A schematic representation of the spray-drying process^[197]

Different carbohydrate molecules and carbohydrate polymers were tested as protecting agents to encapsulate *L. plantarum* by spray drying.^[59] It was shown that soluble starch used at a proper concentration provided the highest cell viability. This was attributed to the high glass transition of starch compared to the other molecules. The resulting encapsulated probiotics were tested as co-starters to perform fermentation of a fish product. It was proved that encapsulated probiotics could inhibit the growth of inoculated pathogens thanks to their ability to produce antagonistic substances and lactic acid. Moreover, the antibacterial activity of encapsulated bacteria was increased by using the starch shell as carbon source. The addition of some protectants such as fats, glycerol, lactose, maltodextrin and skim milk in the formulation could also contribute to preserving cell viability during the encapsulation.^[70, 201, 202] The presence of calcium cations could increase the intracellular calcium level, which might stimulate the activity of heat shock proteins such as Dnak.^[203] Therefore, certain calcium concentrations were shown to enhance the thermal resistance of lactic acid bacteria during the spray drying.^[204-206]

Spray drying, freeze drying and extrusion techniques were compared for the encapsulation of bacterial spores in ALG shells. It was shown that freeze drying was the most appropriate technique since it provided the highest cell survival rate.^[154] Spray drying, freeze drying and electrospray were also compared for the encapsulation of a probiotic in various protein capsules. Electrospray caused the highest reduction in bacteria survival after encapsulation and during storage, whereas freeze drying seemed to be the more appropriate technique to prolong cell viability and activity.^[207] Though freeze drying is less stressful to encapsulate living bacteria, it is energy-consuming and time-consuming, which greatly limits its scaling-up for applications.^[208] In contrast, spray drying is a more relevant technology for commercial applications since it provides high productivity and

reproducibility. However, the process involves the use of high temperature air to rapidly dry the droplets. It was reported that this step leads to a significant loss in probiotic cell viability. Therefore, spray-freeze-drying technique, a combination of spray drying and freeze drying was developed. It involves spraying of feed solution into a liquid nitrogen medium. The frozen droplets are subsequently dried under vacuum in a freeze dryer. The size of the droplets produced by this technique is much smaller than the ones fabricated by spray drying. Heat and mass transfer rates are higher in spray-freeze-drying than in freeze-drying process, which contributes to a significant reduction in the drying time. *L. plantarum* encapsulated in fructo-oligosaccharide-whey protein-maltodextrin formulations by this technique showed nearly 1.3 fold increased cell viability than spray drying bacteria under simulated oral-gastrointestinal conditions.^[100]

Table 2.2 Summary of encapsulation methods

Encapsulation methods	Encapsulating system	Control of size	Advantages	Drawbacks
Emulsion	Particle	Medium	Easy operation	Use of oil
Extrusion	Particle Fiber	Poor	Easy operation	- Wide size dispersion - Low repeatability
Microfluidics	Particle Fiber	Very good	- Good control of particle/fiber size and dispersion - Repeatability - In-situ monitoring/detection	- Complex chip design, costing equipment - Difficult to scale-up
Electrospinning	Particle Fiber	Medium	- Easily scalable	Use of high voltage
Spray draying	Particle	Medium	- Easy scalable - Dry solid particles for food industry	Stressful for thermally sensitive bacteria
LbL assembly	Depends on the template	Good	- Can be applied to encapsulate materials with any shape - Biocompatible encapsulation conditions, tunable coating properties	Difficult to scale-up

2.2.3 Skin bacteria encapsulation

Instead of using antibiotics, which can cause bacterial resistance, commensal skin bacteria have gained increased interest for skin treatment. Lactic acid bacteria have been revealed to play the role of immune modulators in the inhibition of pro-inflammatory cytokine release.^[11] An electrospun bio-scaffold comprising *Enterococcus mundtii* accelerated epithelialization, collagen deposition, and hair follicle formation. Harmful bacteria growth was inhibited by the encapsulation process; therefore, the electrospun patch was proposed as a potential patch to speed up burn wound healing.^[105] Autochthonous bacteria have also been incorporated in nanofibers. The nanofiber patch was demonstrated to inhibit periodontal pathogen growth, showing to improve current periodontal disease treatments.^[106]

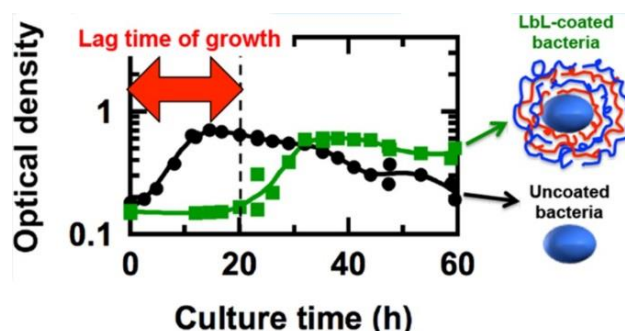


Figure 2.22. Evolution of the OD at 540 nm during the culturing of *S. epidermidis* without coating and coated by (CHI/ALG)₂ bilayers. The lag times are indicated by the thin vertical lines.^[54]

S. epidermidis is also known to inhibit the growth of pathogenic *S. aureus*.^[53, 209] However, direct administration of bacteria on human skin is unsafe due to their capability to turn into opportunistic pathogens when entering the human body. For this reason, its encapsulation was investigated by our group. The proliferation of *S. epidermidis* could be tuned by its encapsulation in a shell of LbL multilayers, such as ALG/CHI and PSS/PAH (Figure 2.22) with longer lag times before growth for thicker multilayers;^[54] however, the bacteria are not significantly metabolically-active during the lag time period, and can thus not be used for therapeutic purpose. Therefore, a mixture of templated LbL NTs and *S. epidermidis* was filtered on a porous membrane to fabricate a nanofibrillar biopatch, with a preserved metabolic activity of the bacteria and limited bacterial leakage over a few hours.^[55] Electrospinning, a more convenient technique to produce fibrous scaffold, was also used to encapsulate *S. epidermidis*. The scaffold was proposed as treatment for diabetic foot.^[107] To facilitate the release of bacterial metabolites, a MT array membrane was

prepared with encapsulated *S. epidermidis*. The MT array membrane was demonstrated to inhibit the *in vivo* growth of *Cutibacterium acnes* without the leaking of *S. epidermidis*, suppling a possible way to treat acne vulgaris.^[192]

Hydrogels are excellent encapsulation materials because of their high water content, which benefits bacteria growth and skin application. The triblock copolymer Pluronic F127 was selected to encapsulate *B. subtilis* (Figure 2.23). Pluronic F127 is thermally-responsive in water with a lower critical solution temperature (LCST) between room temperature and body temperature, and therefore hardens upon application of a solution on skin at 37 °C, forming a sticky hydrogel patch. The mechanical properties of the hydrogel are compatible with *B. subtilis* growth and allow the continuous secretion of metabolic proteins/enzymes which inhibit *in vivo* candida growth.^[112] A wound healing hydrogel containing *B. subtilis* has also been built by 3D printing, offering an alternative approach to produce custom-shaped patches.^[210]

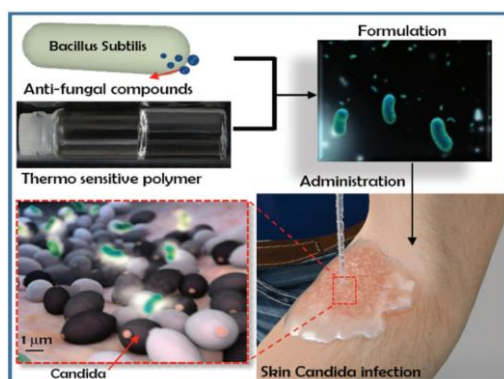


Figure 2.23. Encapsulation of *B. subtilis*, a food-grade bacterium, in a thermoresponsive formula that allows germination and proliferation. After administration, the transparent formula hardens and functions as a unique “factory” that continuously produces and releases natural antifungal agents locally.^[112]

2.3 Layer-by-layer assembly

LbL assembly is a versatile method to create functional coatings on multiple substrates.^[211] Since the first report of LbL polyelectrolyte multilayers, this technique has been strongly developed to produce nanomaterials of well-defined components and properties. According to the type of driving force for the assembly, this method can be classified into electrostatic, hydrogen-

bonding, hydrophobic, host-guest, and bio-specific LbL assembly. The working principle and the advantage of LbL assembly will be discussed in this section. Specifically, H-bonded LbL assembly and LbL assembly in porous membranes will be introduced since they are directly related to this work.

2.3.1 Basic notions

Electrostatic-driven LbL was first reported in the late twentieth century, opening a new age of surface modification.^[212, 213] Figure 2.24 shows a conventional electrostatically-driven LbL assembly. A substrate is sequentially dipped in polyelectrolyte solutions (steps 1 and 3) with the electrostatic interaction between polyanions and polycations leading to the alternated adsorption of polymeric chains on the surface. After each adsorption, a rinsing step (steps 2 and 4) is mandatory to remove the loosely-adsorbed polymers and avoid precipitation. Steps 1-4 are repeated until reaching the designed bilayer number. Substrates of any shape (planar or non-planar, porous, colloidal, cylindrical, etc.), size (nano- to millimeters), and chemical composition can be used for LbL assembly, showing the versatility of LbL assembly.^[214-216] Except for the early-studied electrostatic interaction, non-electrostatic interactions including hydrogen-bonding, hydrophobic, host-guest, and bio-specific interactions were also developed with time. Therefore, various materials have been incorporated as LbL building blocks, such as polyelectrolytes, neutral polymers, biological entities (enzyme, proteins, nucleic acids, peptides, drugs), and inorganic structures (carbon NTs, Au/Ag nanostructures, clays, metal oxides).^[216-218]

Apart from the conventional immersion technique, other LbL assembly methods have been developed, including spraying-, spinning-, electromagnetic-, and fluidic-based LbL assembly (Figure 2.25).^[218] Immersion is convenient to deposit a film on a substrate with any shape and size. Spin-coating followed by a drying step is used to fabricate more rapidly thin LbL films on flat surfaces.^[219] For LbL assembly on extremely large substrates, spray assembly is generally selected. The film composition, morphology, and property are then controlled by polymer type and concentration, spray duration, flow rate, washing duration, etc.^[220] Flow-based fluidic LbL assembly relies on polymer and washing solutions being pushed in a channel through a pump or capillary forces. Fluidic LbL assembly was used for the surface modification of particulate substrates, and claimed to minimize particle aggregation and to simplify particle collection.^[221] Electromagnetically-driven deposition is useful to fabricate coatings on metals.^[218]

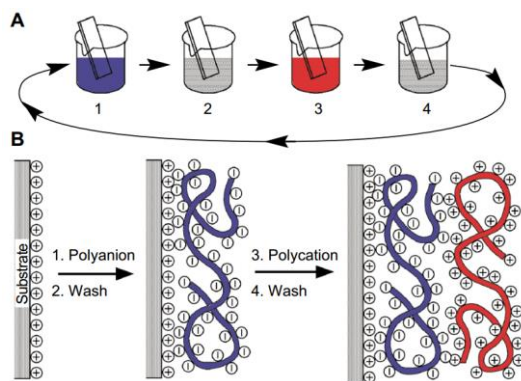


Figure 2.24. (A) Schematic representation of the LbL deposition process on a substrate. Steps 1 and 3 represent the adsorption of polyanion and polycation, respectively; steps 2 and 4 are rinsing steps. Repeating the 1-4 steps leads to LbL multilayers. (B) Simplified molecular picture of the first two adsorption steps, depicting film deposition starting with a positively charged substrate.^[212]

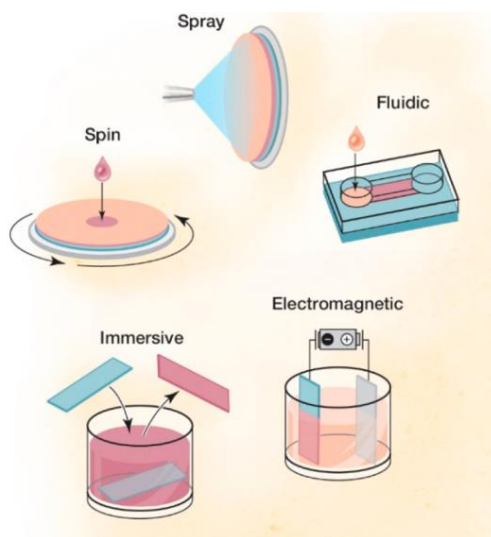


Figure 2.25. Conventional LbL assembly techniques, including immersive, spin, spray, fluidic and electromagnetic assembly.^[218]

LbL-assembled multilayers are promising for applications of storage, encapsulation, and controllable release because they can be readily engineered and functionalized with desired properties. Regarding applications in drug delivery, material protection or filtration, the permeability of LbL assembly multilayers is intensively studied. Antipov et al. demonstrated the sustained

release of fluorescein, a model substance for a large class of drugs, from PSS/PAH-based capsules.^[222] The permeability of LbL films is controllable by cross-linking and hydrolysis.^[223, 224]

External stimuli can strongly impact the permeability of LbL multilayers. For example, the permeability coefficient through LbL film was found to have an extreme nonlinear dependence on salt concentration. The salt-induced permeability changes can be explained by a partial screening of electrostatic interactions between oppositely charged polyelectrolytes. This results in the formation of defects or nano-cavities in the multilayers.^[225] For this reason, the diffusion of small molecules through LbL films can be increased by adding NaCl.^[226] The addition of salt in the medium can also induce a shrinking of LbL films due to the decrease of electrostatic repulsions between chains. For instance, dextran of 2000 KDa was successfully loaded into the PSS/PDADMAC capsules according to this salt-induced shrinking approach, demonstrating the controllable loading and release of material in LbL structures (as shown in Figure 2.26).^[227] The effect of salt on LbL films was also used to fuse polyelectrolyte capsules to form larger capsules.^[228] Indeed, the presence of high salt concentration in the medium led to the formation of defects and pores in the polyelectrolyte shell structure as well as a decrease of electrostatic repulsions and conformational changes of polyelectrolyte chains which facilitates the fusion of LbL shells.

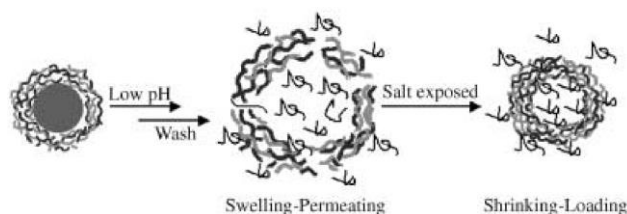


Figure 2.26 Schematic presentation showing the generation of a swollen capsule and the following loading of dextran (2000 kDa) by electrolyte-induced capsule shrinking.^[227]

LbL multilayers based on weak polyelectrolytes are pH-responsive due to protonation/deprotonation of charged groups. Therefore, the charges of weak polyelectrolytes and the permeability of LbL multilayers can be controlled by adjusting the pH of the solution. Reversible permeability change of LbL multilayers under pH has been investigated, showing a way to control the release of both small and large molecules over a wide pH range.^[229] For example, PVA/PAA-coated microcapsules demonstrated a pH-dependent release of vitamin B₁₂: a faster and larger release of B₁₂ was observed when

the pH was changed from 2 to 10, due to the high swelling of the microcapsule shell.^[230] The interactions between the diffusing molecule and the multilayer also contributes to the diffusion behavior: a small charged molecule of sodium dithionite ($S_2O_4^{2-}$) showed a non-Fickian diffusion in PSS/PAH because of the trapping of dithionites in positively charged layers of the multilayer, illustrating the subdiffusion of charged molecules in LbL multilayers.^[231-233]

2.3.2 LbL modification of bacteria

The possibility of preparing LbL multilayers in non-toxic aqueous conditions is attractive for the surface modification of bacteria cells. In addition, the step-by-step deposition of polymer layers allows a very specific control over the resulting functional properties of the assembly.^[234] Bacterial modification by LbL can be sorted into two categories: direct and indirect coating.^[235] The direct coating involves the deposition of LbL multilayers on single bacteria cells or cell aggregates. Linear growth of ALG/CHI multilayers was confirmed on *Bacillus coagulans*.^[75] A high bacteria survival rate was obtained after freeze-drying and long-time storage, showing the protective effect of polyelectrolyte multilayers.^[90, 111, 236]

The state of bacteria after encapsulation is tunable by tailoring the bilayer composition. Silk fibroin modified by PLL, poly-L-glutamic acid, and PEG side chains were applied to encapsulate *B. subtilis* and *E. coli*. As shown in Figure 2.27, H-bonded silk ionomer-co-PEG shells with a high PEG grafting density promoted individual bacterium encapsulation and showed the highest cytocompatibility. By contrast, electrostatically-assembly silk ionomeric shells promoted the formation of bacteria clusters.^[237] Our group reported an agarose gel-assisted LbL process to solve the problem of bacteria aggregation. The nanoporous agarose gel could entrap bacteria while allowing free diffusion of polyelectrolyte chains, thus eliminating bacterial aggregation during LbL coating. The LbL coating deposited on bacteria delayed their growth and the lag time highly depended on multilayer thickness and composition.^[54]

Bacteria can be modified with metal particles, which expands the application of living bacteria beyond food engineering. Both Gram-positive and Gram-negative bacteria were coated with Ag/Au nanoparticles by LbL assembly.^[126] A digital humidity sensor was prepared by depositing Au particles on bacteria, showing potential for fabricating electronic systems. By varying the deposition time, the number of particles on bacteria is tunable.^[116] PSS/PAH multilayers on *Synechococcus sp.* not only offered protection against UV but also provided a positive surface for silica coating. The multi-coating proved to improve the heavy metal bio-absorption capacity of *Synechococcus sp.*^[124] pH-sensitive materials have also been coated on bacteria, leading to site-specific release encapsulation systems.^[111, 238]

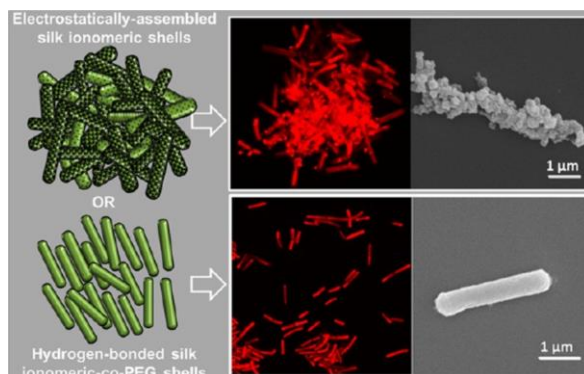


Figure 2.27. Scheme representing the formation of cell clusters coated by strong electrostatically-assembled silk LbL shells, and single cells coated by H-bonded silk shells.^[237]

Indirect encapsulation involves coating bulk cell capsules with LbL films. LbL multilayers have been demonstrated to stabilize the structure of bulk hydrogels and maintain their integrity, providing a second protection shell to cells.^[239] ALG/CHI multilayers were coated on ALG hydrogels containing bacteria, showing an improvement of bacterial survival under stimulated gastric treatment.^[76] In addition, LbL multilayers retard molecule transfer because of the smaller porosity of multilayers compared to hydrogel matrices. The release of bacteria was better controlled in the presence of LbL coatings, enabling the targeted delivery of probiotics to the intestine.^[78, 79] For bulk encapsulation of bacterium or bacteria aggregates, the thickness of capsule walls reduces the exchange of oxygen and material, which may cause core hypoxia and nutrient shortage. A hollow capsule entrapping *E. coli* was prepared by depositing multilayers on a sacrificial calcium carbonate microsphere. This approach showed that thickness-tunable LbL multilayers are an excellent alternative to improve the biocompatibility and permeability of bacteria-containing capsules.^[240]

2.3.3 Hydrogen-bonded LbL assembly

Stockton and Rubner first demonstrated hydrogen bonding-assisted LbL assembly. They showed that non-ionic polymers, such as poly(vinylpyrrolidone) (PVPON), PVA, poly(acrylamide) (PAAm), and poly(ethylene oxide) (PEO), could be assembled with polyaniline to form multilayers.^[241] In addition to aqueous solutions, organic or organic/water mixtures have been explored to fabricate H-bonded multilayers. Thereby, water-insoluble materials can also be used.^[242, 243] However, H-bonded LbL assembly from aqueous solutions is more attractive for applications in biological and medical fields. Generally, water-soluble polymers act as either

hydrogen-donor or acceptor in H-bonded LbL assembly. As shown in Figure 2.28, being weak polyelectrolytes, polycarboxylic acids are widely applied as hydrogen donors. However, polycarboxylic acids can be ionized or protonated, depending on the pH value of the solution. An acidic pH is necessary to keep the polyacid protonated and to form hydrogen bonds with hydrogen acceptor polymers. In contrast, ionization of polyacids at high pH leads to the disruption of hydrogen bonds and thus film delamination. This characteristic of H-bonded LbL multilayers has been widely used to control the release of materials.

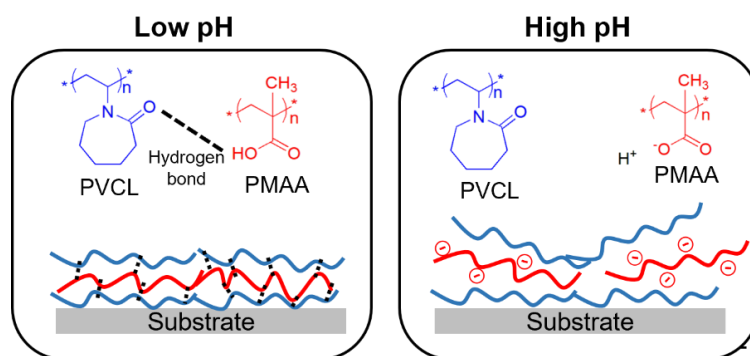


Figure 2.28. Schematic representation of the assembly of a H-bonded PVCL/PMAA film at low pH and film disaggregation at high pH because of the de-protonation of polyacid.

The use of weak polyelectrolytes allows also to control the composition and properties of H-bonded LbL multilayers by changing deposition conditions. The thickness increment of multilayers per bilayer depends on the conformation of polymer chains, with more loopy conformations contributing to thicker bilayers. The effect of polymer molar mass on bilayer thickness shows different behavior depending on the strength of hydrogen bonds. For weakly-bonded LbL systems, for example PEO/PAA, a significant increase in bilayer thickness was observed when the molar mass of PEO increased from 1.5 kDa to 20 kDa.^[244] However, the polymer molar mass has less influence on bilayer thickness for strong H-bonded systems: a similar bilayer thickness growth was found for the PVPON/PMAA system when the molar mass of PVPON increased from 55 kDa to 1200 kDa.^[245]

Ionic strength, pH, and temperature are the three main parameters determining film growth. H-bonded LbL bilayer growth dramatically decreases as the pH deposition approaches a pH_{crit} , above which LbL assembly is stopped because of the ionization of some H-bonding groups. Strong H-bonded LbL systems

have a higher pH_{crit} : For example, PVCL/PMAA and PVPON/PMAA have a pH_{crit} of 6.5^[246] and 4.5,^[245] respectively, compared to a pH_{crit} of 3.5^[244] for PEO/PAA.

The salt concentration affects the growth of H-bonded films in a complex way. Ions screen electrostatic charges and result in more coiled conformations of chains, leading to a thicker bilayer thickness. The addition of NaCl proved to increase the bilayer thickness of PMAA/PVPON films by 10% when 0.5 M NaCl was used.^[245] However, the excessive addition of salt may affect the solubility of polymers. For example, PAA precipitation was observed at a NaCl concentration of 0.8 M.^[247] The growth of PEO/PAA was inhibited at a lithium triflate concentration of 0.5 M.^[244]

For thermally-responsive polymers, the temperature of the polymer solution can affect bilayer thickness. Poly(*N*-isopropyl acrylamide) (PNIPAAm), PVCL, and poly(vinyl methylether) (PVME) have LCST of 32, 36, and 35 °C, respectively. A higher bilayer thickness and a much smoother film can be obtained when performing the assembly at higher temperatures; however, LbL assembly is stopped above the LCST. By contrast, polymers with an upper critical solution temperature (UCST) show an opposite behavior, with thinner bilayers obtained at a higher temperature.^[248]

As illustrated in Figure 2.28, hydrogen bonds are sensitive to environmental conditions, and LbL films stability can thus also be controlled by pH, salt concentration, and temperature. Increasing the degree of ionization of a polyacid can lead to the deconstruction of a LbL film. Table 2.3 shows the critical disaggregation pH of PMAA-based LbL films. Strongly H-bonded LbL films show higher disaggregation pH. The hydrophobicity of LbL system also impacts film stability. For example, PVCL has two extra methyl groups compared to PVPON, leading to a more hydrophobic contribution. Therefore, PMAA/PVCL films have a higher critical pH of 6.95.^[249] The use of polyacids with a higher pK_a value can also increase the stability of LbL films. Tannic acid (TA), a polyphenol with a pK_a of 8.5, was assembled with PVCL. The TA/PVCL film showed a high critical dissolution pH of 9.5. By contrast, PVCL/poly(L-aspartic acid) (PLAA) films have a critical dissolution pH of 3.3. TA/PVCL bilayers and PVCL/PLAA bilayers were either alternately or stacked deposited on a substrate (Figure 2.29). The hybrid film has a wide dissolution pH range of 3.0 to 9.5, showing an innovative approach to tune the dissolution pH of LbL systems.^[250]

Table 2.3. Critical dissolution pH for H-bonded polymer systems. The critical pH value is defined as the pH at which more than 90% of film does not dissolve in one hour.^[249]

Polymer System	Critical dissolution pH
PMAA/PHEA	4.0
PMAA/PEO	4.6
PMAA/PAAm	5.0
PMAA/PVME	6.0
PMAA/PNIPAAM	6.2
PMAA/PVPON	6.4
PMAA/PVCL	6.95

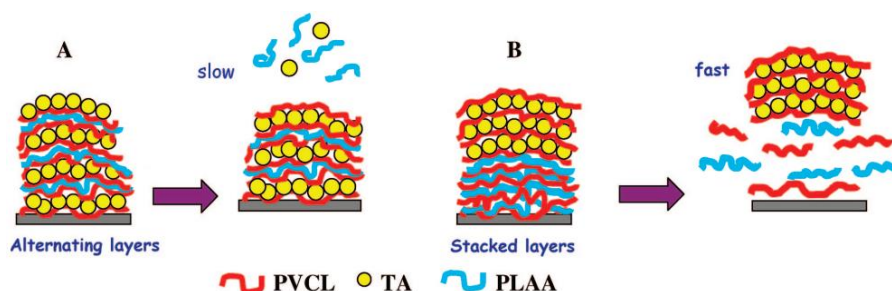


Figure 2.29. Schematic representation of film destruction for (A) alternating and (B) stacked hybrid multilayers.^[250]

H-bonded LbL films composed of thermally-responsive polyelectrolytes show thermally-dependent stability. As shown in Figure 2.30, LbL films built from PNIPAM, PVME, and PVCL have a higher critical pH at high temperature. By contrast, PAAM/PMAA films display a reduced critical pH at high temperature because PAAm exhibits a UCST.^[251]

Due to their stimuli-sensitive properties, H-bonded LbL multilayers have attracted significant interest to encapsulate and release materials. By using photo-, thermal-, or ionic-sensitive films as sacrificial compartments, free-standing films or cargos can be controllably released.^[250-253] As shown in Figure 2.31, a hybrid film structure composed of two compartments was fabricated to produce self-standing membranes: (1) a pH-responsive PAA/PEG LbL film formed via hydrogen bonds and (2) a stable PAH/PSS LbL film on top of 1. The disaggregation of PAA/PEG films under pH

increase, led to the release of self-standing PAH/PSS films. In addition, the controllable release feature of H-bonded LbL films enables their application for drug/cell delivery.^[254, 255] Here, inspired by the fabrication of free-standing LbL films, we propose the release of LbL tubes from PC membrane by using H-bonded multilayers as a sacrificial compartment.

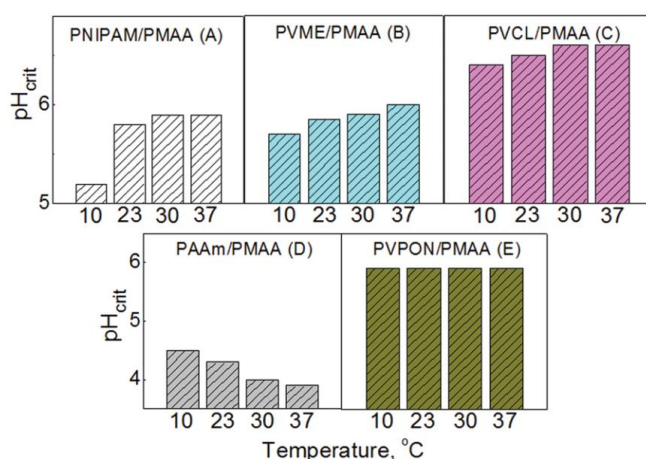


Figure 2.30. Effect of temperature on the pH-triggered disintegration of multilayers of PMAA with a series of neutral hydrogen-bonding polymers: (A) PNIPAM/PMAA, (B) PVME/PMAA, (C) PVCL/PMAA, (D) PAAm/PMAA, and (E) PVPON/PMAA. The five-bilayer films were deposited at pH 2.0 and 23°C.^[251]

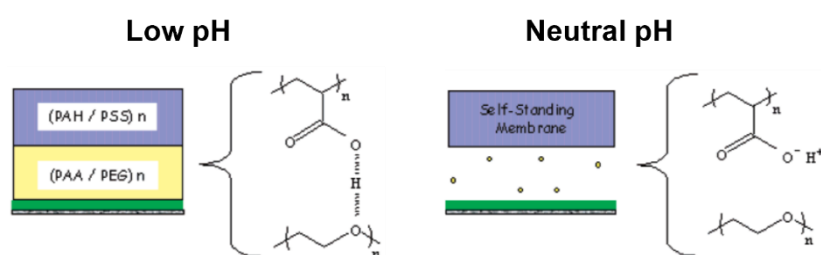


Figure 2.31. Application of H-bonded LbL film as a sacrificial layer to produce self-standing LbL film.^[253]

2.3.4 LbL assembly in porous membranes

LbL assembly in membranes with cylindrical pores provides a methodology to obtain functional tubes. Figure 2.32 shows the procedure of preparing polyelectrolyte tubes.^[256]

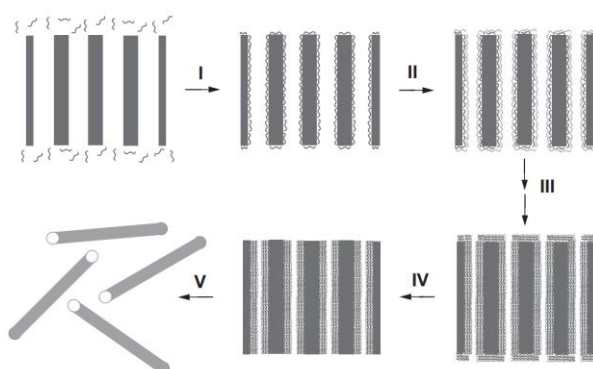


Figure 2.32. Scheme illustrating the procedure of preparing polyelectrolyte tubes by the LbL coating of porous membrane templates: (I) adsorption of a single polyelectrolyte layer, (II) adsorption of a second polyelectrolyte layer, (III) adsorption of multiple polyelectrolyte layers of opposite charge, (IV) removal of the polyelectrolyte film from the top and bottom surface of the membrane, (V) membrane dissolution, which results in the liberation of LbL assembly tubes. The scheme shows a cross-sectional view of the membrane.^[256]

The membrane is alternately dipped in solutions to adsorb polyelectrolytes on the pore walls (steps I to III). The polyelectrolytes simultaneously adsorb on the top and bottom surfaces of the membranes should be removed mechanically or etched by air plasma (step IV). Then, polyelectrolyte tubes are released by dissolving the template (step V) either in a phosphoric acid aqueous solution or in an organic solvent, depending on the membrane type. LbL assembly in a templating membrane offers a facile way to get polymer or hybrid tubes with diverse compositions. The outer diameter and length of the tubes are easily tuned, and the thickness and properties of the tubes can be controlled by the type of used materials and the deposition cycle. These features make LbL assembly in porous membranes an attractive approach to build tubes for multiple applications.^[257]

Anodic aluminum oxide (AAO) and PC membranes are two widely-used templates for LbL assembly. Small pores restrict polymer entrance into them, forcing perturbed chain conformations, leading to thinner tube walls per

bilayer. In contrast, when the pore size is large enough, the thickness growth of tube walls is similar to the one of films grown on a flat surface.^[258] An AAO membrane was alternately exposed to protein (glucose oxidase or hemoglobin) solutions and subsequently cross-linked by glutaraldehyde. The protein NTs were released by immersing the membrane in a 5% phosphoric acid solution. These glucose oxidase-loaded NTs showed an increasing enzymatic activity with wall thickness.^[259] To prevent the adsorption of polyelectrolytes on the membrane surfaces, an ultrathin layer of Au was sputtered on both surfaces of the AAO membrane. This method supplies a way to restrict LbL assembly over the flat surfaces of the AAO membrane, preventing the possible blockade of pores during deposition.^[260] Bicompartimentalized tubes were fabricated from a PC membrane of 3 μm pore diameter. The interaction of released tubes with cells was studied, showing an end-on attachment when HA/CHI bilayers were deposited as the internal compartment and a side-on attachment when HA/CHI bilayers were deposited as the external compartment.^[258]

LbL-assembled tubes were also synthesized for the general delivery or encapsulation of various compounds.^[261] LbL PAH/PAA tubes were shown to transform from cylindrical to ellipsoidal above the glass transition temperature of the PAH/PAA system. This temperature-triggered shape transformation of tubes can be used to load and release drugs or molecules.^[262] Human serum albumin (HSA), which has a strong affinity to zinc(II) protoporphyrin IX (ZnPP), was LbL assembled with poly-L-arginine (PLA). This (PLA/HSA)₃ tube demonstrated an ability to load and release ZnPP. A specific modification of (PLA/HSA)₃ tubes was then performed by depositing a single layer of avidin, providing a capability to capture biotin-labeled nanoparticles.^[263] Interestingly, incorporating an Fe₃O₄ layer in (PLA/HSA) tubes led to magnetic tubes for the capture of bacteria.^[125] In addition to the popular encapsulation and delivery applications, LbL tubes were also used as ion separation systems because of the selective electrostatic exclusion of ions by LbL films.^[264]

After dissolving the porous membrane, LbL tubes can be filtered and sedimented on a filter, leading to nanopapers with a random distribution of LbL tubes.^[265] As shown in Figure 2.33, a filter covered by a fructose cake was used to obtain a self-standing nanopaper after fructose dissolution. Since multiple materials can be used for LbL assembly, nanopapers with a variety of functional properties can be realized. After crosslinking and fluorosilanation, strongly oleophobic and hydrophobic nanopapers made from core-shell (PAH/PAA)₆/(PAH/PSS)₁₂ tubes were obtained. Enzymatic nanopapers were also fabricated by using PEI and glucose oxidase as building blocks, leading to porous nanopapers of tunable thickness and activity, which are promising filters for bio-transformations.^[266]

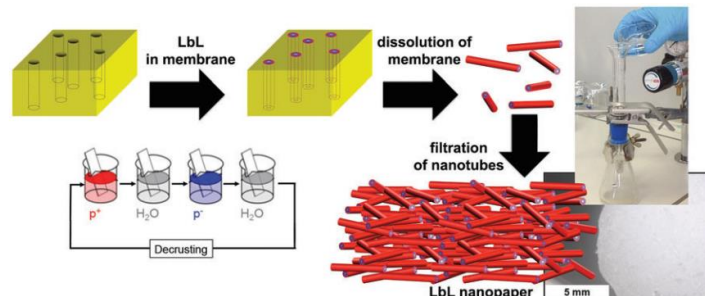


Figure 2.33. Fabrication of LbL nanopapers. Depending on whether a simple filter or a fructose cake resting over a filter is used during the filtration process, supported or self-standing nanopapers are obtained. The picture of a self-supporting nanopaper is shown in the bottom right image.^[266]

As demonstrated in previous research, the hollow structure of tubes can be used for loading materials. Therefore, porous membranes modified by LbL multilayers provide a way for encapsulation and delivery of molecules. Materials of varying properties and size, including enzymes, drugs, dyes, particles and even cells, can be loaded in LbL tubes by changing the composition and properties of LbL multilayers and pore size. Such modified membranes can either be directly used as a stiff matrix to protect cargos, or be treated to release the LbL tubes, leading to a fibrous and porous mat of tubes. According to the benefits of LbL-assembled multilayers and the two uses of LbL-modified membranes, we propose to use the encapsulation of bacteria in pores of PC membranes.

2.3.5 LbL antibacterial films

Bacteria can attach to surfaces and proliferate and form biofilms. Therefore, the prevention of bacteria colonization is an essential issue in many fields, such as medical implants, textiles, contact lenses, aquatic flow systems, and petroleum pipelines.^[267, 268] Bactericidal components are thus used to inhibit bacteria growth on surfaces. Heavy metals such as silver and copper have been applied for decades as bactericidal agents. Antibiotics are also frequently used, since they lead to bacterial cell death by inhibiting DNA, RNA, cell wall, or protein synthesis. However, antibiotic use can cause bacterial resistance. Cationic molecules have also been reported to kill bacteria by damaging cell membranes.^[267] LbL assembly supplies a versatile way to incorporate these bactericidal compounds to obtain antibacterial films.

Based on action mechanisms, antibacterial films are classified into three catalogs: anti-adhesive, contact-killing, and release-killing (Figure 2.34). Anti-adhesive films can prevent the adhesion of pathogens on surfaces.

Increasing film hydrophilicity by the incorporation of highly hydrophilic polymers is the simplest way to obtain anti-adhesive films because of the formation of a hydration layer on the top surface which prevents bacterial adhesion. Chemical modification of polyelectrolytes with hydrophilic chains or moieties such as PEG was demonstrated to enhance the anti-adhesive properties of LbL films.^[269] The adhesion of *E. coli* was reduced by 92% when PLL/PGA-*g*-PEG bilayers was coated on a glass substrate.^[270] Superhydrophobic coatings provide another way to prevent bacterial adhesion.^[267] The stiffness of LbL films is another parameter that may impact bacterial adhesion. The adhesion of viable *S. epidermidis* and *E. coli* were demonstrated to positively correlate with film stiffness.^[24] The stiffness of LbL films can be easily tuned by modulating the assembly pH, salt concentration, and temperature. For instance, the elastic modulus of PAH/PAA films varies from 1 to 100 MPa by changing assembly pH.^[24] Cross-linking also significantly increases film rigidity and stability. However, the effect of film stiffness on surface adhesion is still controversial and requires further investigation.

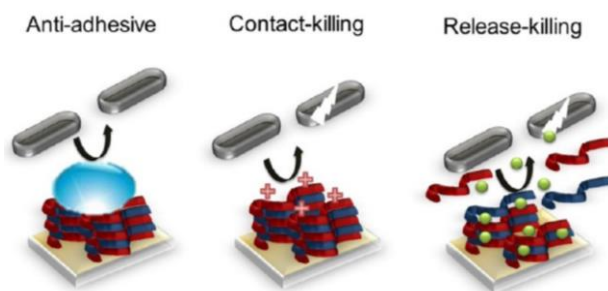


Figure 2.34. Three main strategies to design antimicrobial coatings: anti-adhesive films inhibit the close approach of pathogens; contact-killing films expose antimicrobial agents on the surface; release-killing films deliver antimicrobial agents to the supernatant.

Release-killing LbL films kill bacteria by liberating antibacterial agents in the environment. There are two strategies to develop release-killing films. The first one is the outward diffusion of antibacterial agents from loaded LbL films, and the second one is the dissolution of LbL films to release the active agents. A broad-spectrum antibacterial agent, triclosan, was loaded into an LbL film composed of cyclodextrins. The release of triclosan combined with the intrinsic contact-killing activity of the LbL film proved to inhibit the growth of bacteria.^[271] The most commonly-used antibacterial materials are silver or silver ions, which have long been taken as bactericidal agents. Silver

nanoparticles were incorporated in LbL films either by direct LbL assembly or by reducing silver ions. Fibers exhibiting 80% *S. aureus* inhibition were obtained by coating them with PMAA-coated silver nanoparticles.^[272] Several cationic antibiotics, such as tobramycin, gentamicin, and polymyxin B were assembled with TA. The diffusion of antibiotics from LbL films was controlled by the solution pH and assembly conditions. These films demonstrated a strong inhibition of bacteria growth both on surfaces and in the surrounding medium.^[273]

Contact-killing films only kill bacteria when they are into contact with the surface, displaying a constant antibacterial efficiency when unspoiled. Bacterial cells are negatively charged because of the presence of acidic polysaccharides on bacterial cell membranes. Therefore, positively-charged molecules are generally considered to be contact-killing materials, which can disrupt cell membranes and lead to cell death. CHI is a well-known weak polycation that possesses an intrinsic antibacterial property. CHI/ALG LbL capsules have been demonstrated to inhibit the growth of both Gram-positive *S. aureus* and Gram-negative *E. coli* in liquid medium, showing a higher inhibition rate with an increase of bilayer number.^[274]

PLA/HA multilayers drew interest for biological applications because of their powerful immunomodulatory properties and strong antibacterial effect against *S. aureus*. Mutschler and co-authors found that the diffusion capability of polycations played a key role in the function of films. The impact of PLA molar mass on antimicrobial activity was studied using PLA grades with 10, 30, 100, and 200 residues. The PLA 30/HA LbL film presented the most powerful contact-killing ability. However, the molar mass of HA did not impact the bioactivity of the film.^[275] This polycation chain-length dependency supplies a way to tune the functionality of antibacterial films.^[276] Experimental studies on PLL/HA LbL films also showed that the higher mobility of PLL in the films led to higher contact-killing efficiency.^[277] Quaternary ammonium compounds have also been incorporated in antibacterial films since they are permanently charged.^[268] Quaternized PEI/PAA films prevented the growth of not only bacteria but also virus replication.^[278]

In addition to the use of polycation-terminated LbL films, the incorporation of bactericidal molecules or particles is also a possible way to obtain contact-killing films. Single wall carbon NTs, which can damage bacterial membranes, were added in PLL/PGA LbL films to enhance their antibacterial activity against *E. coli* and *S. epidermidis*.^[279, 280]

Comparing the three types of LbL antibacterial films, contact-killing films are the most suited for our objective to encapsulate bacteria in a specific system and to kill them when they escape from the system.

2.4 Objectives and strategy of the thesis

S. epidermidis, a commensal skin bacterium with numerous benefits to hosts, is selected to develop a bioactive system than can be used for future skin treatment. In order to maintain the activity of bacteria while preventing its dispersal in the human body, encapsulation of bacteria is proposed. ATCC 12228, a generally-used non-pathogenic and non-biofilm-forming strain is used in this thesis to optimize the encapsulation conditions, and to study the bacterial activity and the release of bacteria from the developed encapsulating systems.

After screening various encapsulation methods, LbL assembly, a biocompatible technique that allows to tune the thickness and properties of multilayers, is selected to encapsulate bioactive bacteria. This surface modification technique also provides a way to prevent the escape of bacteria from the encapsulating systems, by adding antibacterial multilayers.

A PC membrane with cylindrical pores is modified by LbL assembly multilayers to entrap bacteria, which supplies two ways to obtain encapsulated bacteria. LbL-modified PC membrane is directly used to entrap bacteria, playing as a matrix. On the other hand, the released LbL tubes are used to produce a fibrous mat of tubes loaded with bacteria. Both ways can lead to bacterial-containing systems for skin applications. And different from previous LbL encapsulation approaches involving the entrapment of individual cell, bacterial colonies are encapsulated in PC membranes.

Polymers, including PVCL, PMAA, PVPON, PEI, ALG, and CHI, are selected in this thesis for the specific objectives of using them for the fabrication of erasable compartments, sustained protection compartments or antibacterial coatings.

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Chapter 3 Hydrogen-bonded LbL multilayers for the release of nanostructures

In a first approach, we propose to encapsulate bacteria in a MT mat (Scheme 1.3). In order to build a bi-compartmentalized tube with an external sacrificial compartment, we had to find an erasable LbL system; after a literature screening and preliminary experiments, the PVCL/PMAA H-bonded system was selected. In this chapter, we focus on studying the growth and the properties of PVCL/PMAA films, in order to get a comprehensive understanding of this H-bonded system before moving to apply it for LbL tube release.

PVCL/PMAA film is grown on a flat surface to monitor the thickness change. The effect of PVCL molar mass and NaCl concentration on film growth is also investigated. The stability of PVCL/PMAA LbL films in aqueous solutions with varying pH, NaCl concentration, and PVCL molar mass is discussed, demonstrating the disaggregation of film at close to physiological pH. Based on the pH sensitivity of this H-bonded LbL system, the liberation of nanostructures, especially LbL tubes, using PVCL/PMAA system as a sacrificial compartment is finally investigated. This chapter was a published article in ACS Applied Polymer Materials.^[1]

3.1 Introduction

LbL assembly is an efficient methodology to create functional coatings on a variety of substrates.^[2-12] When performing LbL inside the pores of a nanoporous membrane, LbL assembly provides access to in-template nanostructures replicating the porosity of the membrane.^[13-17] Dissolution of the membrane then leads to free nanostructures of cylindrical shape, *i.e.*, NTs^[18] and nanowires,^[19] which can be used for a variety of applications. Among these, biological applications such as delivery carriers,^[20-23] porous enzymatic NT mats,^[24-27] or bacterial patches^[28] have been proposed. For applications that involve sensitive biomacromolecules or living organisms, the dissolution step of the templating membrane is a significant issue, as it often occurs in conditions that may denature or kill the biological components.

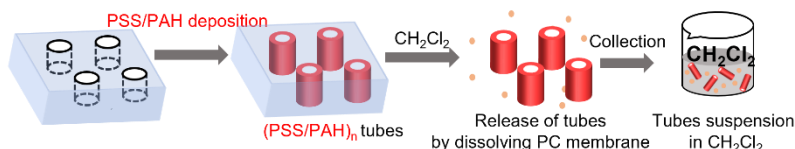
For instance, when using nanoporous track-etched PC membranes as templates, the dissolution step is typically done in an organic solvent such as dichloromethane,^[29] which is especially adverse for biological applications and creates safety issues (Scheme 3.1a). Here, we propose and study an alternative methodology of tube liberation, based on the fabrication in the nanopores of PC membranes of bi-compartmentalized tubes having a sacrificial outer compartment that can be erased to liberate the inner tube compartment in biologically-compatible conditions (Scheme 3.1b). Various triggers can be used to deconstruct LbL systems for the release of molecules.^[30] For our objective of encapsulating bioactive cells, the less stressful, easily available, and biocompatible aqueous solution with tunable pH, temperature, and ionic strength will be used for tube release.

Previous work on erasable LbL compartments focused on H-bonded-flat multilayers, providing a good starting point for the development of a water-based liberation method of NTs.^[31-37] Such systems can be grown at pH values for which the hydrogen bond-donating polymeric component is protonated; at higher pH, the degree of ionization of this component is increased, resulting in a weakening of its hydrogen bond donating capability with a simultaneous increase of electrostatic repulsions, leading to the disaggregation of the multilayers. Different systems are characterized by different values of critical pH at which the multilayer starts to become unstable. In particular, Sukhishvili *et al.* extensively studied H-bonded LbL systems with critical pHs ranging from 3.3 to 9.5. For instance, PAA/PEO multilayers have a low critical pH of 3.3 because of the weak hydrogen bonds established between PAA and PEO.^[38] The use of polyacids of higher pKa value, for example PMAA, (pKa *ca.* 4.5) and poly(ethacrylic acid) (PEAA) (pKa *ca.* 7), and/or of more hydrophobic neutral polymers such as PNIPAAm, results in an increase of the critical pH of multilayers to *ca.* 6.0.^[39] These authors also succeeded in

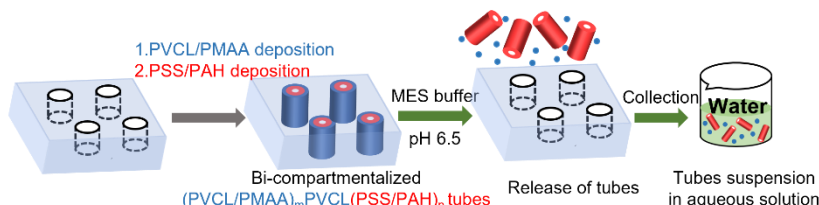
Hydrogen-bonded LbL multilayers for the release of nanostructures

reaching even higher critical pH values (8.0-9.5) by depositing TA, whose pKa is *ca.* 8.5, with several neutral hydrogen bond accepting polymers.^[40]

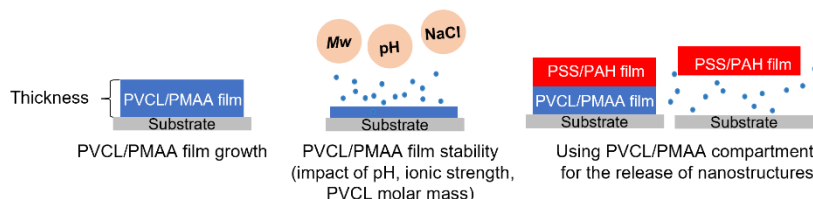
a. A standard LbL tube preparation and release process (non-biocompatible)



b. Our strategy: Adding of an erasable compartment (dark blue)



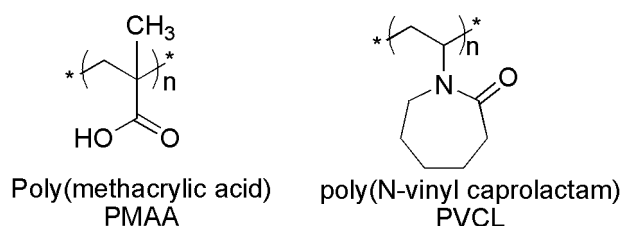
c. Work described in this chapter: properties of H-bonded PVCL/PMAA system



Scheme 3.1. (a) Scheme of the standard LbL process of tube fabrication in the pores of a membrane, requiring the dissolution of the templating membrane in non-biocompatible solvents to recover the tubes. (b) Schematic illustration of the fabrication of bi-compartmentalized $(\text{PVCL/PMAA})_m\text{PVCL}(\text{PSS/PAH})_n$ tubes, and of the release of the inner tubes in biologically-compatible conditions. (c) Summary of the work described in this chapter.

Considering our objective to encapsulate *S. epidermidis*, a NaCl concentration of 150 mM, corresponding to physiological conditions, was selected to test the release of LbL erasable compartments. We therefore screened the literature, aiming at identifying the most promising system for biocompatible release of LbL tubes, *i.e.*, a system that would be stable in 150 mM NaCl solution at *ca.* pH 6 and would dissolve at *ca.* pH 7, which would be optimal for biological applications. No system was exactly fitting these requirements, as was generally confirmed by rapid test experiments: H-bonded

PMAA/PVPON films were shown to disaggregate at pH 5.4 (in 150 mM NaCl) (Figure S3.1). TA/PVPON and TA/PNIPAAM LbL films were also tested, demonstrating no film disaggregation after 2h in a pH 7.8 solution (Figure S3.2). Nevertheless, we identified PMAA/PVCL systems as promising (Scheme 3.2), since they were reported to disintegrate at pH 6.5 in 10 mM phosphate buffer.^[39]



Scheme 3.2. Chemical structure of the H-bonding polymers of this study.

Additionally, the effect on multilayer stability of a biological concentration of NaCl was rarely considered in the literature. Previous work on polyphenol/neutral polymer systems such as TA/PEO, TA/PNIPAAM, and TA/PVPON showed that the critical pH for film disintegration decreases by 0.5 pH units in 150 mM NaCl for these systems.^[40] A similar phenomenon is to be expected for PMAA/PVCL, but was not studied so far.

In this chapter, bacterial encapsulation will not be discussed since we focus on the properties of the erasable PMAA/PVCL LbL system, as shown in Scheme 3.1c, including:

- The growth and disintegration of PMAA/PVCL LbL multilayers depending on pH, NaCl concentration and PVCL molar mass, parameters which have not been studied in depth before.
- The application of PMAA/PVCL multilayers to build sacrificial compartments below flat LbL films and as external compartments in templated LbL NTs, and demonstrate the floating-off of flat films and the release of NTs in biocompatible conditions.

3.2 Experimental section

3.2.1 Materials

Poly(methacrylic acid) (PMAA, M_w 100 kg/mol) was obtained from Polysciences, and poly(*N*-vinyl caprolactam)s (PVCL, M_w 1.8, 32 and 240 kg/mol) were purchased from Polymer Source Inc. Poly(vinyl

pyrrolidone) (PVPON, M_w 1 300 kg/mol), branched polyethylenimine (PEI, M_w 750 kg/mol), 2-(*N*-morpholino)ethanesulfonic acid (MES) and sodium alginate (ALG) were purchased from Sigma-Aldrich. Poly(styrenesulfonate) (PSS, M_w 70 kg/mol), and NaCl (ACS reagent) were obtained from Acros. Poly(allylamine hydrochloride) (PAH, M_w 120-200 kg/mol) was obtained from Alfa Aesar. Rhodamine-labeled PAH (PAH-Rh, M_w 15 kg/mol, 1:392 mol:mol monomer ratio in dye) was purchased from Surflay Nanotec GmbH. Chitosan (CHI, M_w 50-150 kg/mol, deacetylation degree > 90%) was purchased from NovaMatrix.

Polymer solutions of varying NaCl concentration were prepared at 1 mg/mL except for PAH-Rh (0.5 mg/mL) and PEI (0.5 mg/mL). Silicon wafers obtained from Topsil Germany were cleaned in acetone then isopropanol and MilliQ water and treated by air plasma (ETMITECH K1050X) before LbL deposition. PC membranes (800 nm pore diameter, 4×10^7 and 1×10^7 pores/cm², 24 μ m) were provided by it4ip S.A., Belgium. MilliQ water with a resistivity of 18.2 M Ω .cm was obtained from a Merck Millipore system and used in all experiments.

3.2.2 Fabrication of LbL films

PMAA/PVCL and PMAA/PVPON multilayers were deposited at pH 2.0, conditions in which PMAA is fully protonated, on Si wafers. To enhance polymer adsorption, a precursor layer was first deposited by dipping clean Si wafers in a PEI solution (pH 2.0; 0, 50 or 150 mM in NaCl) for 10 min followed by triple rinsing in solutions of same pH and NaCl concentration as the PEI solution (30 s each). Si wafers were then alternately immersed in PMAA and PVCL solutions for 5 min. Between each adsorption, Si wafers were triply rinsed (30 s each).

PSS/PAH deposition was carried out at pH 4.0 using a similar procedure as above, and 0.5 mg/mL PAH-Rh was used to label the films in the last five cycles. PSS/PAH multilayers were either directly deposited on Si wafers (with a precursor PEI layer), leading to PEI(PSS/PAH)_n films, or deposited on top of PEI(PMAA/PVCL)_m films to obtain bi-compartmentalized PEI(PMAA/PVCL)_m(PSS/PAH)_n films. The same procedure as for PSS/PAH was followed for ALG/CHI deposition at pH 4.0, leading to PEI(ALG/CHI)_n films and PEI(PMAA/PVCL)_m(ALG/CHI)_n films. For all LbL assemblies, no intermediate drying was performed until the desired number of bilayers were obtained, after which coated Si wafers were rinsed with MilliQ water (3 s), then carefully dried with a stream of nitrogen for thickness measurement.

3.2.3 Fabrication of LbL polyelectrolyte tubes

Track-etched PC membranes were used as received to prepare core-shell tubes comprising a (PVCL/PMAA)_mPVCL external compartment and a

(PSS/PAH)_n inner compartment (Scheme 3.1b), deposited at pH 2.0 and 4.0, respectively; the NaCl concentration was fixed at 150 mM for all steps. A 4×10^7 pores/cm² PC membrane was used to prepare the tubes unless otherwise indicated. Since most material surfaces are negatively-charged in water unless the pH is low, the deposition of the second compartment always started with the negatively-charged PSS to decrease the trend of liberated (PSS/PAH)_n tubes to stick on solid surfaces after dissolution of the sacrificial PVCL/PMAA compartment. Therefore, the deposition of the external PVCL/PMAA compartment always started and ended with PVCL. A 6×6 cm² membrane was dipped in polymer solutions for 20 min in the first PVCL/PMAA adsorption cycle, then for 10 min from the second deposition cycle on. Each adsorption was followed by two rinsing steps (2 min each) with a 150 mM NaCl solution of same pH as used for polymer adsorption. To label tubes, PAH and PAH-Rh (0.5 mg/mL) was alternately deposited in PSS/PAH deposition cycles. Both sides of the membrane were decrusted with a cotton swab every second cycle during PVCL/PMAA deposition and every fourth cycle during PSS/PAH deposition, to prevent nanopore blockade. When the desired number of bilayers was obtained, the membranes were decrusted, rinsed with MilliQ water and stored in a fridge.

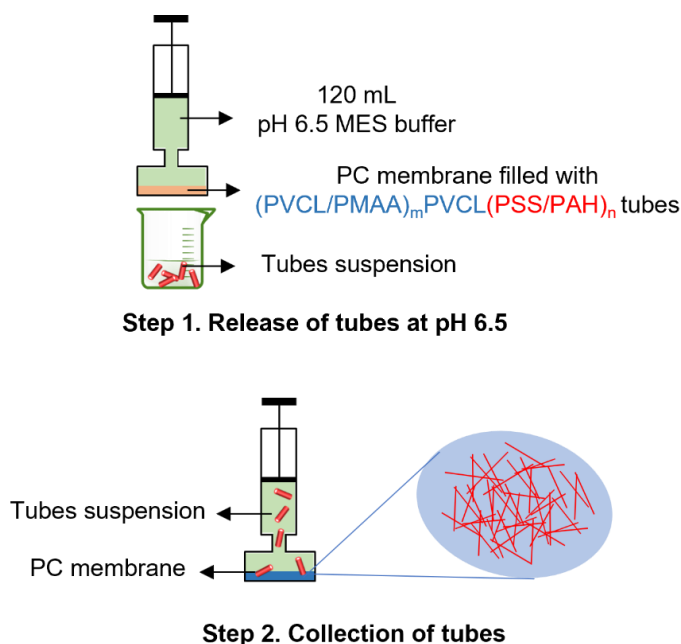
When ALG/CHI was used instead of PSS/PAH to prepare bi-compartmentalized (PVCL/PMAA)₅PVCL(ALG/CHI)₁₂ tubes, the same procedure was followed, except that the membranes were decrusted every second cycle and that PAH-Rh was used to form two ALG/PAH-Rh bilayers during the fifth and sixth cycles of deposition in order to fluorescently-tag the tubes.

3.2.4 Collection of polyelectrolyte tubes at pH 6.5

A circular (PVCL/PMAA)₅PVCL(ALG/CHI)₁₂ membrane (12 mm diameter) was placed in a Swinny filtration unit, and 120 mL of pH 6.5 buffer (20 mM MES and 150 mM NaCl) was pushed through the membrane (Scheme 3.3) to disaggregate the (PVCL/PMAA)₅PVCL external compartment and release the (PSS/PAH)₁₂ tubes which were collected in MES buffer. For microscopy observation, the released tubes were filtered onto a blank PC membrane covered with 20 nm of gold (for scanning electron microscopy), then triply rinsed with 4 mL MilliQ water to remove salt traces (Scheme 3.3). (ALG/CHI)₁₂ tubes were released and collected in the same way by treating (PVCL/PMAA)₅PVCL(ALG/CHI)₁₂ membrane with pH 6.5 MES, 150 mM NaCl buffer.

After pH 6.5 MES treatment, the tubes remaining in the pores were also collected. For this purpose, the MES-treated PC membrane was placed in 3 mL dichloromethane and shaken for 10 min to fully dissolve the PC template. The tubes were filtered onto a blank PET membrane (200 nm pore

diameter) and rinsed three times (5 mL each) with fresh dichloromethane to remove PC traces.



Scheme 3.3. The release and collection of the inner $(PSS/PAH)_n$ tubes compartments is performed by disaggregating the sacrificial outer $(PVCL/PMAA)_m PVCL$ compartment in a pH 6.5 aqueous solution while pushing the inner compartments outside the pores of the membrane.

3.2.5 Thickness measurement of LbL films

The thickness of dry LbL films was measured in air by spectroscopic ellipsometry (Jobin-Yvon UVisel) at an incidence angle of *ca.* 65° , with wavelengths ranging from 400 to 700 nm. The refractive index of the dry film (including the *ca.* 1.5 nm of native silicon oxide thickness) was fixed to 1.50, and the refractive index of Si was taken from the literature.^[41] To monitor the thickness change of the film in solution, we used spectroscopic in-situ ellipsometry at *ca.* 65° incidence angle and wavelengths varying from 400 to 700 nm. The film was placed in an Accurion temperature-controlled liquid cell (volume 0.7 mL) aligned on a home-made multi-axis sample stage. Then 20 mM MES buffer (20°C) at a fixed pH and NaCl concentration was added into the cell and circulated at *ca.* 1 mL/min. The film thickness was obtained by fitting the data with a two-layer model, using the tabulated refractive index

for Si and a fitted Cauchy transparent model for the film. The refractive index of water and its temperature-, wavelength-, and NaCl concentration-dependence were obtained from literature.^[42, 43]

3.2.6 Characterization of polyelectrolyte tubes

Gas flow porometry measurement. Measurement of the thickness of the compartments in pores. The thickness of (PVCL/PMAA)_mPVCL and (PSS/PAH)_n compartments deposited in the pores of PC membranes was determined by calculating the average pore diameter after gas flow porometry measurements performed before and after LbL assembly. For the measurement, a dry PC membrane was firmly held inside a membrane holder with an effective section area of 0.137 cm². Filtered air gas at room temperature was flown upstream with a pressure ranging from 0.35 to 4 bar. The gas flow rate (mL/min) downstream from the sample was measured with a calibrated GFM Pro Gas Flowmeter from Thermofisher Scientific. The inner diameter of the pores was calculated by using Knudsen diffusion and the Hagen-Poiseuille flow relationship, as described elsewhere.^[44]

Morphology of tubes in PC membrane. The membranes modified with LbL tubes were coated by 20 nm of sputtered gold and their flat faces were imaged with a scanning electron microscope SEM (JSM-7600F, JEOL Ltd) at 5 kV. Non-coated membranes containing fluorescently-tagged tubes were additionally imaged by epifluorescence microscopy (red channel, Olympus IX71). Since this only provides images of the tips of the tubes, the LbL-modified membranes were embedded in paraffin^[45] and microtomed, and 5 μm thick sections were imaged by a Zeiss LSM 710 confocal laser scanning microscope (CLSM).

Morphology of liberated tubes and trapped tubes. The tubes liberated under buffer treatment were collected over a gold-coated PC membrane, and observed by SEM (5 kV) and epifluorescence microscopy. The trapped tubes, *i.e.* tubes which remained in the nanopores of PC membranes after buffer treatment, were collected on a PET membrane after dissolution of the PC membrane template and observed directly by epifluorescence microscopy or SEM (in which case the samples were coated with 20 nm of gold).

Quantification of the amount of liberated polyelectrolyte tubes. The amount of rhodamine-labeled tubes liberated after buffer treatment was measured by fluorescence spectroscopy. The total amount of tubes was obtained by dissolving 0.5 cm² of LbL-modified membrane in 1 mL dichloromethane, and by measuring the fluorescence at 590 nm of the tubes suspension with a Varian Cary Eclipse fluorescence spectrophotometer (550 nm excitation wavelength). A blank was subtracted using as reference the same membrane LbL-modified with non-fluorescent tubes. The amount of tubes trapped in the membrane after disaggregation of the outer sacrificial compartment was

measured similarly, by dissolving the membrane obtained after pH 6.5 treatment and expulsion of the trapped tubes. The proportion of tubes liberated from the membrane was obtained by comparing the fluorescence intensity of the total number of tubes and the one of the trapped tubes.

3.3 Results and discussion

3.3.1 Effect of pH and salt concentration on PMAA/PVCL LbL films

PVCL is a biocompatible polymer with a LCST of 36 °C.^[46] LbL film is prepared and studied at ambient temperature (20 °C) unless otherwise indicated. The LCST of PVCL was previously shown to decrease in the presence of SO_4^{2-} , H_2PO_4^- or Cl^- in aqueous solution, suggesting strong salt effects on the hydrophobicity of the chain.^[47] Therefore, we monitored the growth and stability of (PMAA/PVCL)_m films depending on ionic strength, having in mind biological applications for which a background NaCl concentration of 150 mM is typical. (PMAA/PVCL)_m films were thus deposited on PEI-modified Si wafers at pH 2.0 in 0, 50 or 150 mM NaCl solutions, using a low molar mass PVCL (M_w 1.8 kg/mol). The dry thickness of PEI(PMAA/PVCL)_m films versus number of deposited bilayers is shown in Figure 3.1a. A linear growth is observed with an average thickness increment per bilayer of 4.1 nm and no significant influence of salt concentration on film growth, showing that PMAA/PVCL multilayers do not have a significant electrostatic contribution to stability at pH 2.0. This is in marked contrast compared to a weakly H-bonded LbL system such as PAA/PEO, whose film growth was reported to be completely inhibited in 500 mM NaCl.^[48]

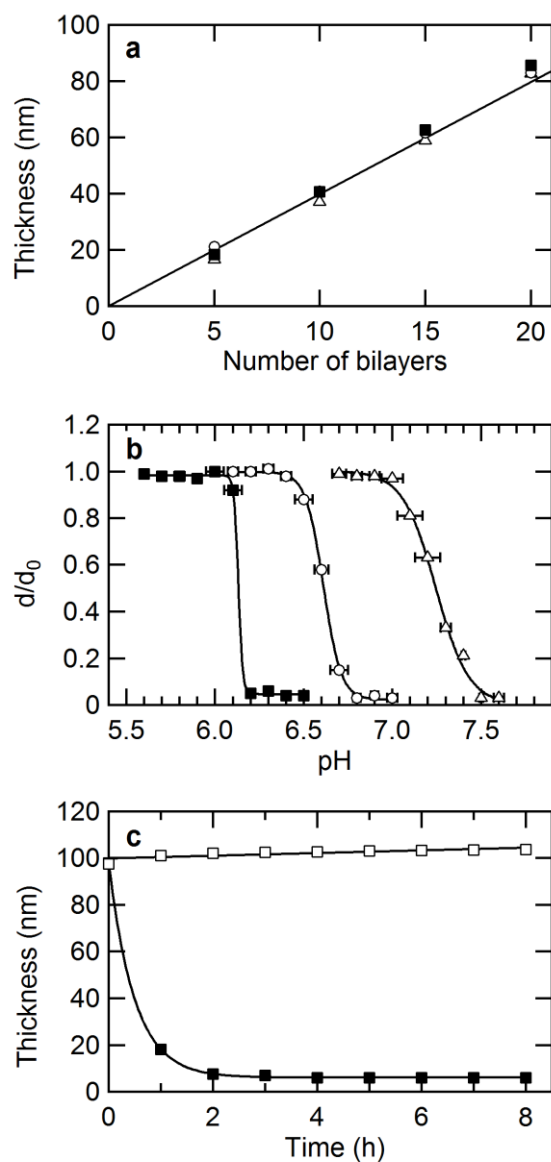


Figure 3.1. (a) Dry thickness of PEI(PMAA/PVCL)_m LbL films grown at pH 2.0, in 0 (triangles), 50 (circles), or 150 mM (squares) NaCl. The line is a fit to the data. (b) Relative dry thickness (d/d_0) of PEI(PMAA/PVCL)₂₀ films (d_0 ca. 85 nm, corresponds to the initial dry thickness of the film) grown in 0 (triangles), 50 (circles), or 150 mM (squares) NaCl and subsequently immersed for 1 min in 20 mM MES buffers of different pH as indicated. The curves are sigmoidal fits. (c) In-situ measurements of the thickness of

PEI(PMAA/PVCL)₂₀ films initially grown in 150 mM NaCl at pH 2.0, and subsequently immersed in 20 mM MES buffer (150 mM NaCl) at pH 5.7 (hollow squares) and 6.0 (filled squares). The MES buffer was flown at the constant rate of 1 mL/min during the measurements. The curves are drawn to guide the eye.

The stability of a thick PEI(PMAA/PVCL)₂₀ film (starting dry thickness $d_0 = 85$ nm) was then assessed in solutions of increasing pH. The films were immersed in 20 mM MES buffer at a given pH for 1 min, rinsed with water to remove the adsorbed salt, dried, and their dry thickness (d) was measured again by ellipsometry. For all experiments, the stability of LbL multilayers was tested at the same NaCl concentration as the one used for film growth. The relative film thickness, d/d_0 , is plotted versus immersion pH in Figure 3.1b, from which the critical pH for disintegration, pH_{crit} , can be obtained as the pH corresponding to half-disintegration. The pH_{crit} of PEI(PMAA/PVCL)₂₀ films decreased with the salt concentration, being 7.1, 6.6, and 6.1 for 0, 50, and 150 mM NaCl, respectively. Simultaneously, the disintegration curves were steeper at higher salt concentrations: for instance, at 150 mM NaCl, a 0.1 increment in pH units (from 6.0 to 6.1) resulted in the full disintegration in 1 min of the initially stable film, whereas an increment of 0.5 pH units (from 7.0 to 7.5) was required to have the same effect in the absence of salt. The differences of steepness of the disintegration curves depending on salt content probably arise from the more homogeneous electrostatic environment existing at higher ionic. pH_{crit} is decreased and hydrogen bonds are weakened in the presence of salts because of, *e.g.*, promotion of polymer ionization and change of polymer solubility and hydration.

The long-term stability of PEI(PMAA/PVCL)₂₀ films was also studied at pH 5.7 and 6.0 (just below the pH_{crit} measured for a 1 min immersion time) in 20 mM MES (150 mM NaCl) buffer (Figure 3.1c). At pH 5.7, a PEI(PMAA/PVCL)₂₀ film of 85 nm dry thickness swelled to 100 nm upon immersion; its thickness then remained essentially constant over the next 8 h, with only a very slight increase over time indicating limited reorganization. This film was then rinsed, dried, and its dry thickness was measured again by ellipsometry, providing a value of 86 nm, very close to its original dry thickness. At pH 6.0, the PEI(PMAA/PVCL)₂₀ film also rapidly swelled to 100 nm when immersed in MES buffer; however, contrasting with the case of pH 5.7, the film slowly disaggregated with 80% of the film removed in 1 h and a complete disintegration in 2 h. This illustrates that the pH_{crit} is a time-dependent parameter and that the kinetics of film disaggregation vary substantially around pH_{crit} . When farther below pH_{crit} as at pH 5.7, the film is stable for extended periods of time.

Similar experiments were performed on the PMAA/PVPON LbL system, in which the hydrogen bond acceptor component is of increased polarity compared to PVCL (Supporting Information, Figure S3.1). The pH_{crit} of these PMAA/PVPON multilayers was significantly lower than for the PMAA/PVCL system; in the sequel, we thus concentrate on PMAA/PVCL multilayers fabricated from 150 mM NaCl, since they meet our stability criteria for biological work.

As discussed in section 2.3.3, PVCL/PMAA films showed a slightly higher pH_{crit} at higher temperatures due to the presence of thermosensitive PVCL (Figure 2.30).^[39] Thus the impact of temperature on the film disintegration was also tested. pH_{crit} measured for PMAA/PVCL films at 37 °C is the same as pH_{crit} measured at 20 °C (data not shown), showing that the impact of temperature on pH_{crit} is not significant compared to the ionic strength.

3.3.2 Effect of PVCL molar mass on PMAA/PVCL films

Previous studies have shown that polymer molar mass as well as the difference of molar mass between components in H-bonded multilayers can lead to different film thickness.^[49] Weakly H-bonded LbL systems, such as PAA/PEO and PMAA/PEO, were especially shown to be very sensitive to PEO molar mass: when the PEO molar mass increased from 1.5 to 20 kg/mol, the film thickness increased by a factor of seven.^[48] Additionally, one might expect that larger molar masses would be conducive to more stable multilayers, due to the increased number of hydrogen bonds per chain. Therefore, films comprising PVCL of M_w 1.8, 32 and 240 kg/mol were grown in conditions identical to before (*i.e.*, pH 2.0 and 150 mM NaCl), and their stability was assessed versus pH. The average thickness increment per bilayer was 4.2 nm for the three types of PEI(PMAA/PVCL)_m films, independent of PVCL molar mass (Figure 3.2a). This molar mass independence again hints to strong hydrogen bonds in the PMAA/PVCL system. Accordingly, when the stability of $\text{PEI(PMAA/PVCL)}_{20}$ films deposited from PVCL of different molar masses was measured in solutions of increasing pH (Figure 3.2b), the pH_{crit} was found to be independent of molar mass (6.1, 6.1 and 6.2, for PVCL of M_w 1.8, 32 and 240 kg/mol, respectively).

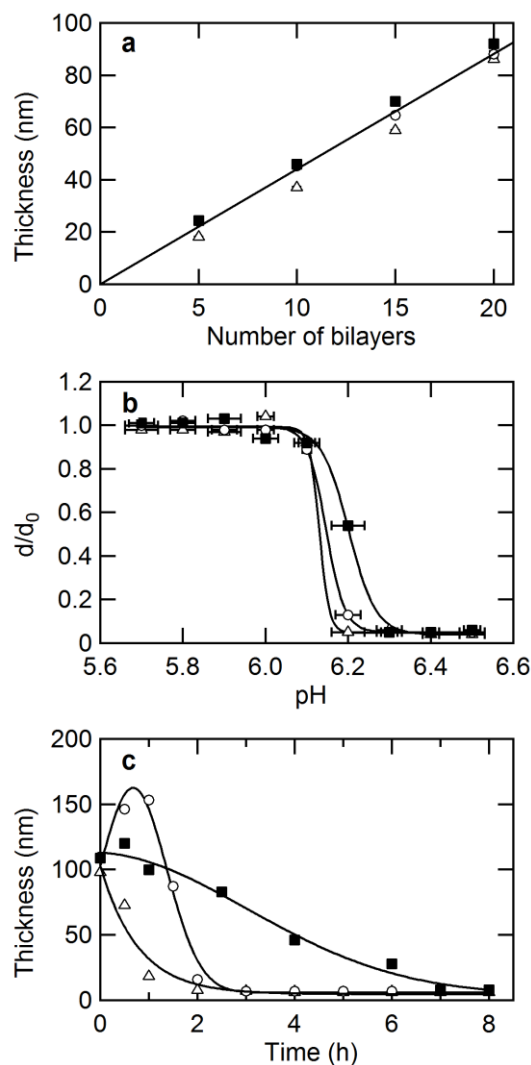


Figure 3.2. (a) Dry thickness of PEI(PMAA/PVCL)_m films comprising PVCL of different molar masses, grown at pH 2.0 in 150 mM NaCl. The lines are fits to the data. (b) Relative dry thickness (d_0/d) of $\text{PEI(PMAA/PVCL)}_{20}$ films grown at pH 2.0 then immersed in 20 mM MES buffer (150 mM NaCl) for 1 min. The curves are sigmoidal fits. (c) In-situ thickness of $\text{PEI(PMAA/PVCL)}_{20}$ films initially grown in 150 mM NaCl at pH 2.0, and subsequently immersed in pH 6.0, 20 mM MES buffer (150 mM NaCl). The curves are drawn to guide the eye. Molar mass of PVCL: 1.8 (triangles), 32 (circles), or 240 kg/mol (squares).

The long-term stability of these films was also measured at pH 6.0 (Figure 3.2c). The film comprising the low molar mass PVCL (M_w 1.8 kg/mol) directly swelled from 85 to 100 nm upon immersion in the MES buffer, and fully disaggregated in 2 h. By contrast, PEI(PMAA/PVCL)₂₀ films comprising PVCL of higher molar mass swelled more slowly, then gradually disaggregated at a slower rate. The higher the molar mass of PVCL, the longer the time for full film disaggregation (3 and 7 h for 32 and 240 kg/mol, respectively). Hence, although the three films have a similar pH_{crit} when measured for short immersion times (1 min), their disaggregation kinetics depends on the molar mass of the PVCL component when probed at a pH just below the critical one, probably resulting from the molar mass dependence of disentanglement kinetics. By increasing the PVCL molar mass, a slight increase of dynamic stability can thus be obtained.

Summing up, PMAA/PVCL multilayers growth is independent of NaCl concentration and PVCL molar mass, while for PMAA/PVPON multilayers, salt facilitates thickness growth. Such H-bonded films can be disaggregated by a pH trigger, with pH_{crit} strongly depending on salt concentration and very weakly on PVCL molar mass. The transition between stable and unstable films occurs over a narrow range of pH values, with the critical pH being generally compatible with sensitive biological components. In the sequel, we thus concentrate on utilizing PMAA/PVCL multilayers as sacrificial compartments for the release of flat LbL films and of LbL NTs, essentially using the lower molar mass PVCL (1.8 kg/mol) and films grown in 150 mM NaCl unless otherwise indicated.

3.3.3 (PMAA/PVCL)_m film for the release of LbL films

PEI(PMAA/PVCL)₂₀(PSS/PAH)_n bi-compartmentalized films were prepared by the LbL deposition at pH 4.0 of (PSS/PAH)_n films over a PEI(PMAA/PVCL)₂₀ compartment of 85 nm dry thickness, initially grown at pH 2.0. As shown in Figure 3.3a, the thickness of the (PSS/PAH)_n film was insensitive to whether it was grown over a PEI-coated Si wafer or over a PEI(PMAA/PVCL)₂₀ compartment, with an average thickness increment per bilayer of 2.5 nm. The long-term stability of this PEI(PMAA/PVCL)₂₀(PSS/PAH)₂₀ film was then monitored at pH 6.0 in 20 mM MES buffer (150 mM NaCl) by in-situ ellipsometry. Single PEI(PMAA/PVCL)₂₀ and PEI(PSS/PAH)₂₀ films were also tested in the same conditions (Figure 3.3b). The single PEI(PMAA/PVCL)₂₀ film fully disaggregated in 2 h, whereas the single PEI(PSS/PAH)₂₀ compartment was stable at pH 6.0 for 8 h. By contrast, the bi-compartmentalized film immediately swelled from 125 nm (dry film total thickness) to 150 nm upon immersion, then gradually swelled to 200 nm and started to erode after 4 h, until the film fully detached after 8 h. Obviously, the upper pH-stable

(PSS/PAH)₂₀ compartment acted as a barrier delaying but not preventing the disaggregation of the buried PMAA/PVCL compartment.

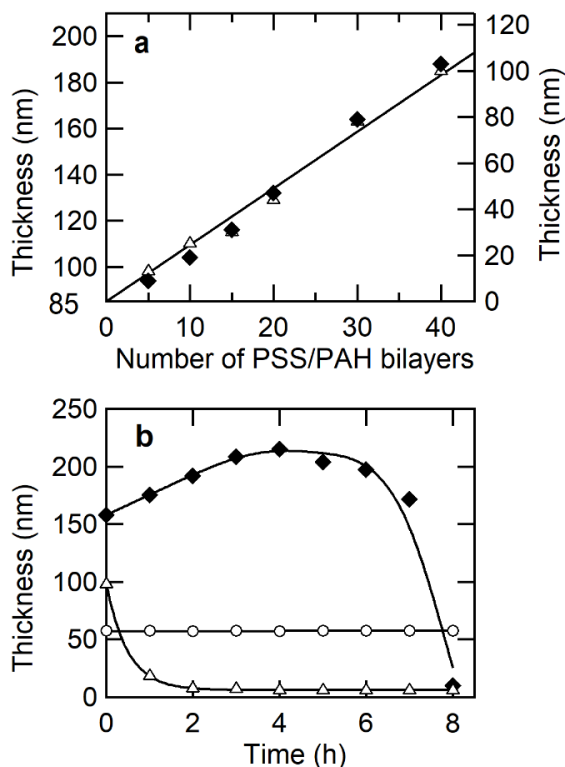


Figure 3.3. (a) Dry thickness of bi-compartmentalized PEI(PMAA/PVCL)₂₀(PSS/PAH)_n films (squares, left axis) and of PEI(PSS/PAH)_n films (triangles, right axis) versus number of PSS/PAH bilayers. The (PSS/PAH)_n films were deposited at pH 4.0 in 150 mM NaCl. The line is a fit to the data. (b) In-situ thickness of PEI(PMAA/PVCL)₂₀(PSS/PAH)₂₀ (squares), PEI(PMAA/PVCL)₂₀ (triangles) and PEI/(PSS/PAH)₂₀ (circles) films immersed in pH 6.0, 20 mM MES buffer (150 mM NaCl). The MES buffer was flown at the constant rate of 1 mL/min during the measurements. The curves are drawn to guide the eye.

We then tested whether the upper (PSS/PAH)_n films could be freely released from Si wafers by disaggregation of the underlying PMAA/PVCL compartments in such PEI(PMAA/PVCL)_m(PSS/PAH)_n films. Three bi-compartmentalized films comprising $m = 5, 10$, and 20 PMAA/PVCL bilayers were prepared, followed by $n = 40$ bilayers of PSS/PAH, with LbL deposition

performed at pH 2.0 and 4.0 for (PVCL/PMAA)_m and (PSS/PAH)₄₀ compartments, respectively. The floating-off of the (PSS/PAH)₄₀ films was tested at pH 6.5 in 20 mM MES buffer (150 mM NaCl). After immersion for 10 min, the films were fully released from the Si wafers for all the systems tested (Supporting information, Figure S3.3), but broke into several smaller pieces because of the low mechanical strength of (PSS/PAH)₄₀ films. The floating film fragments were collected on clean Si wafers, rinsed and dried to check their thickness, which was *ca.* 100 nm, corresponding to the initial dry thickness of the (PSS/PAH)₄₀ compartment. This demonstrates that H-bonded (PMAA/PVCL)_m films are proper sacrificial compartments for the release of overgrown electrostatic LbL multilayers.

3.3.4 (PVCL/PMAA)_mPVCL for the release of LbL tubes

Encouraged by these results on flat films, we then evaluated the possibility to release by the same mechanism LbL tubes grown in templating track-etched PC membranes. For our objective to load bacterial cells in LbL tubes, a pore size in the micron range should be used. However, in this chapter, a PC membrane with nanopores of 800 nm was chosen to test the tube release by disaggregation of PVCL/PMAA compartment because (1) the gas flow porometry measurement, a method to monitor the LbL deposition in pores, can only be conducted with pores smaller than 1 μm ; (2) when depositing polyelectrolytes in smaller pores, a hydrogel rather than LbL film was obtained.^[44] Therefore, a PC membrane with 800 nm nominal pore diameter (750 nm measured pore diameter) and 4×10^7 pores/cm² pore density was used for LbL assembly. The PVCL/PMAA growth was linear after two bilayers, with an average thickness increment per bilayer of 35 nm (Supporting information, Figure S3.4). This is much larger than the growth increment measured for flat multilayers (4.1 nm/bilayer, Figure 3.1a), which is typical for LbL assembly in nanopores as extensively reported before for electrostatic multilayers;^[24, 28, 44, 50-52] apparently, the same phenomena (crowding of chains leading to gel formation, bridging of chains between opposite pore walls, etc.) happen for H-bonded multilayers as well. Since this hints at a possible change of multilayer structure, we checked that the disaggregation of the nano-confined (PVCL/PMAA)_mPVCL multilayers was not hindered at physiological pH. A PC membrane modified with (PVCL/PMAA)₈PVCL multilayers of 210 nm residual average dry pore diameter was thus immersed in 20 mM MES buffer (150 mM NaCl) at pH 6.5, rinsed, dried, and the pore diameter of the membrane was measured again by gas flow porometry. A value of 750 nm was found, which is identical to that of the starting unmodified PC membrane, indicating quantitative disintegration of the LbL NTs in the membrane.

Therefore, bi-compartmentalized (PVCL/PMAA)₅PVCL(PSS/PAH)₁₂ NTs were grown in the same type of PC membrane, at pH 2.0 and 4.0 for

(PVCL/PMAA)₅PVCL and (PSS/PAH)₁₂ compartments, respectively. The thickness of the two compartments was 65-75 nm and 210-240 nm, respectively. Unfortunately, subsequent immersion of the bi-compartmentalized NTs-modified PC membrane in pH 6.5 MES buffer did not lead to any collection of NTs. We suspected that this resulted from the (PSS/PAH)₁₂ inner NTs compartment adhering on the nanopore walls after dissolution of the sacrificial compartment. Therefore, we used a forced expulsion process to release and collect the NTs (Scheme 3.3). 120 mL of pH 6.5, 20 mM MES buffer (150 mM NaCl) was flown through the LbL-modified membrane to disaggregate the sacrificial compartment and expel the liberated (PSS/PAH)₁₂ inner NT compartment. The liberated NTs (Figure 3.4a, b) and trapped NTs (Figure 3.4c, d) were collected by filtration and imaged by microscopy. The SEM image shows that the liberated NTs are *ca.* 24 μ m long, which corresponds to pore length, and 1 μ m wide, which is larger than the expected outer diameter of the (PSS/PAH)₁₂ compartment and is due to the swelling and flattening of the NTs during collection. This demonstrates the possibility to directly release NTs in bio-compatible aqueous solution without using organic solvent or dissolving the template. The NTs still trapped in the membrane after the treatment at pH 6.5 were also collected on a porous PET membrane, by dissolving the PC membrane in dichloromethane (Figure 3.4c, d); these trapped NTs have the same size as liberated ones.

The PC membranes were also observed by SEM, epifluorescence microscopy and before and after the liberation of (PSS/PAH)₁₂ NTs (Figure 3.5). Although many pores were opened by the release process as shown in the SEM images (Figure 3.5c), a substantial amount of NTs was not liberated (Figure 3.5f). The 3D reconstruction of CLSM images of microtomed membranes also demonstrates the partial liberation of NTs at pH 6.5 (Figure 3.5i). Since rhodamine-tagged PAH (PAH-Rh) was used to label NTs, the fluorescence of the collected suspensions of NTs can be used to quantify their amount. The fluorescence of PAH-Rh solutions of varying concentration was measured in pH 4.0/6.5 MES buffer (Figure S3.5), showing that the fluorescence intensity at 590 nm is proportional to PAH-Rh concentration regardless of the pH value. In order to quantify the fraction of liberated NTs, the fluorescence of suspensions containing all NTs or only trapped NTs was measured, by dissolving the initially LbL-modified membrane and the membrane after pH 6.5 treatment in dichloromethane, respectively. An average fraction of liberated NTs of 51 $\pm$ 6% was thereby obtained (Figure S3.6a).

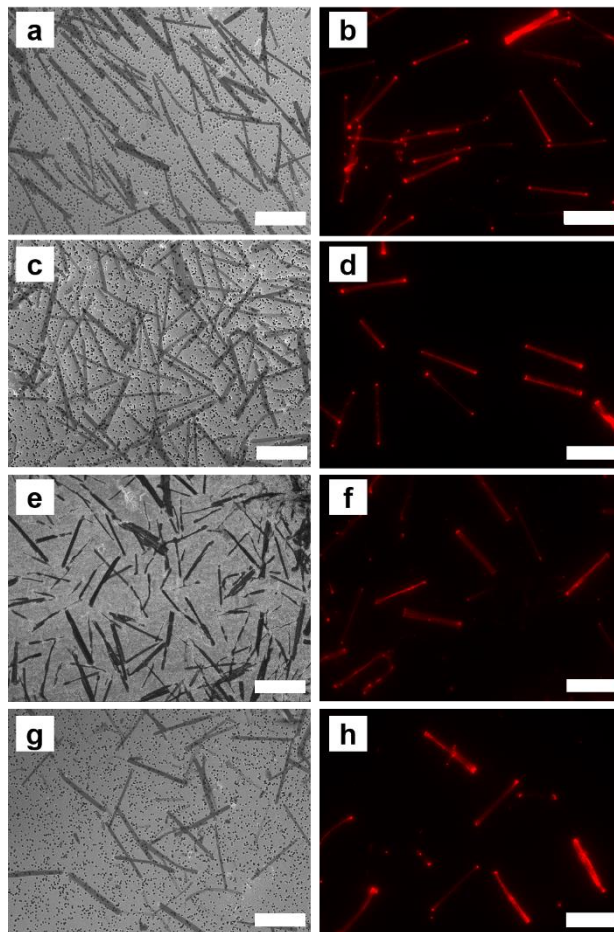


Figure 3.4. SEM (left column) and epifluorescence microscopy images (right column) of NTs. (a, b) Liberated (PSS/PAH)₁₂ NTs obtained by dissolving at pH 6.5 the sacrificial compartment of bi-compartmentalized (PVCL/PMAA)₅PVCL(PSS/PAH)₁₂ NTs in membranes. (c, d) The remaining NTs trapped in the membrane after pH 6.5 treatment, recovered by dissolving the membrane in dichloromethane. (e, f) Same as (a, b), except for the molar mass of PVCL which was 240 kg/mol. (g, h) Liberated (ALG/CHI)₁₂ NTs obtained by dissolving at pH 6.5 the sacrificial compartment of bi-compartmentalized (PVCL/PMAA)₅PVCL(PSS/PAH)₁₂ NTs in membranes. Scale bars: 20 μm.

The reason why some NTs are not liberated is that overlapping pores in the membrane create fused NTs that are physically blocked in the membrane (as shown in Figure 3.6). According to the manufacturer, the proportion of

overlapping pores is *ca.* 55%, close to the 49% of membrane-trapped NTs found by fluorescence spectroscopy. In order to confirm this hypothesis, we performed a similar experiment on a PC membrane of four times lower pore density (1×10^7 pores/cm²), corresponding to a lower proportion of overlapped pores of 18% according to the manufacturer. The proportion of collected NTs logically increased to $83 \pm 5\%$ (Figure S3.6b). Note that the trapping of fused NTs in the membranes can be seen as a positive outcome of our methodology, since it results in the exclusive collection of single NTs, contrarily to what happens when dissolving the membranes.

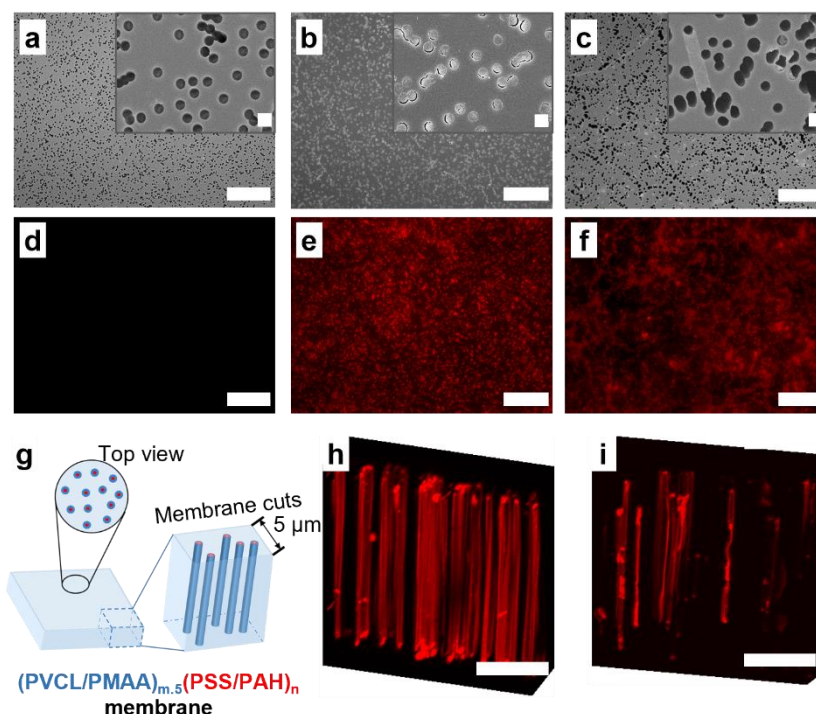


Figure 3.5. Microscopy images of (a,d) the virgin unmodified PC membrane, (b,e,h) a membrane modified with (PVCL/PMAA)₅PVCL(PSS/PAH)₁₂ NTs, and (c, f, i) a membrane after the liberation of the NTs at pH 6.5. (a-c) Top-view SEM images; scale bars: 20 μm, scale bars of insets: 1 μm. (d-f) Top-view epifluorescence microscopy images; scale bars: 20 μm. (g, h, i) Scheme of the sample and tridimensional reconstruction of CLSM images of microtomed membrane cuts (5 μm thickness); scale bars: 10 μm.

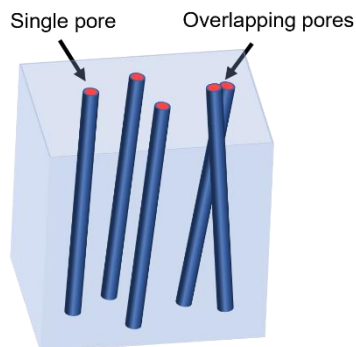


Figure 3.6. Single pore and overlapping pores in PC membrane.

The effect of PVCL molar mass and PVCL/PMAA compartment thickness on the efficiency of the release process were also studied. Bi-compartmentalized $(\text{PVCL/PMAA})_5\text{PVCL}(\text{PSS/PAH})_{12}$ NTs were prepared from 32 and 240 kg/mol PVCL and their inner $(\text{PSS/PAH})_{12}$ NTs were liberated at pH 6.5 (Figure 3.4e, f). The released NT fraction was $60 \pm 11\%$ and $55 \pm 13\%$, respectively, non-significantly different from the $51 \pm 6\%$ obtained with 1.8 kg/mol PVCL. This is in agreement with the almost insignificant influence of PVCL molar mass on the critical pH of PVCL/PMAA flat films. The effect of the thickness of the sacrificial PVCL/PMAA compartment on tube release was also studied for a PC membrane of larger pore diameter (Figure S3.7). Although thicker sacrificial compartments resulted in a slightly higher fraction of liberated tubes, the amount of liberated tubes always remained close to the fraction of non-overlapping pores in the membrane.

Finally, in order to test the release of more biologically-compatible NTs, ALG/CHI LbL multilayers were deposited instead as PSS/PAH for the inner compartment. The measurement of the growth of ALG/CHI multilayers over a flat surface provided a thickness increment per bilayer of 2.7 nm, close to the 2.5 nm of the PSS/PAH system. The floating-off of the ALG/CHI film was also observed when immersing a flat $\text{PEI}(\text{PVCL/PMAA})_{20}(\text{ALG/CHI})_{40}$ film in pH 6.5 MES/NaCl buffer for 1 h. However, compared with PSS/PAH films, the higher swelling degree of ALG/CHI films (70% as measured by in-situ ellipsometry) resulted in a lower rigidity and mechanical strength which prevented to collect the film after floating-off. The liberation of $(\text{ALG/CHI})_{12}$ NTs was then carried out on a $(\text{PVCL/PMAA})_5\text{PVCL}(\text{PSS/PAH})_{12}$ -modified membrane according to the same procedure as for PSS/PAH-based NTs. Fluorescence spectroscopy provided a fraction of liberated $(\text{ALG/CHI})_{12}$ NTs of $60 \pm 8\%$, close to what was obtained for PSS/PAH-based NTs. Additionally, the liberated $(\text{ALG/CHI})_{12}$ NTs were intact and exhibited the expected morphology (Figure 3.4g, h). This demonstrates that our biocompatible

liberation conditions can be applied to different types of systems, and are well-adapted for the production of polysaccharide NTs at close-to-neutral saline condition. It is therefore suited to the encapsulation of sensitive biological components such as enzymes or even living organisms.

3.4 Conclusions

Summing up, we have studied the growth and stability of H-bonded multilayers based on PMAA and PVCL. PMAA/PVCL multilayer growth at low pH was independent of NaCl concentration and PVCL molar mass, due to strong H-bonding between this pair of polymers. The disaggregation of the films was then performed at higher pH values. The critical pH for disaggregation was found to depend strongly on salt concentration, whereas PVCL molar mass did not play an important role. Interestingly, the PVCL/PMAA system is compatible with biologically-sensitive components, as it provides multilayers which are stable just below pH 6.0 and can be disaggregated in 150 mM NaCl neutral pH conditions.

We then demonstrated the liberation of flat PSS/PAH films by disaggregating a sacrificial PMAA/PVCL compartment and implemented the methodology to the liberation of PSS/PAH NTs grown in templating PC membranes, then to the liberation of biocompatible ALG/CHI NTs. We showed for the first time the possibility to liberate such tubes without having to dissolve the membrane, a methodology that could easily be generalized to other types of templating methods. The liberated NTs were directly obtained in benign conditions (pH 6.5, 150 mM NaCl) that are fully bio-compatible and promising for biological applications. The liberation process was close to quantitative with respect to the liberation of non-crossing NTs and offers the supplementary advantage to eliminate crossing NTs from the collection process.

Supporting information

Effect of pH and salt concentration on the growth and disintegration of PMAA/PVPON films

Similar experiments as for PMAA/PVCL were performed on the PMAA/PVPON LbL system, in which the hydrogen bond acceptor component is of increased polarity compared to PVCL. PEI(PMAA/PVPON)_m films were thus prepared at pH 2.0 in 0, 50, and 150 mM NaCl, using the same assembly protocol as before. The dry thickness of PEI(PMAA/PVPON)_m films versus number of bilayers is shown in Figure S3.1a. The average thickness increment per bilayer is 3.5 nm for films deposited in 0 and 50 mM NaCl, increasing to 4.1 nm for films deposited in 150 mM NaCl. The pH_{crit} is 6.3, 5.6, and 5.4 for PEI(PMAA/PVPON)₂₀ films deposited in 0, 50 and 150 mM NaCl, respectively (Figure S3.1b). These results are compatible with a previous study on this specific pair of polymers, which provided a 10% increase of film thickness and 1.4 pH units of reduction of critical pH in a 500 mM NaCl solution.^[30]

The pH_{crit} of these PMAA/PVPON multilayers is significantly lower than for the PMAA/PVCL system, which hints to the stabilization of PVCL-based multilayers by hydrophobic interactions, thereby requiring a higher degree of ionization of PMAA to destabilize the layers.

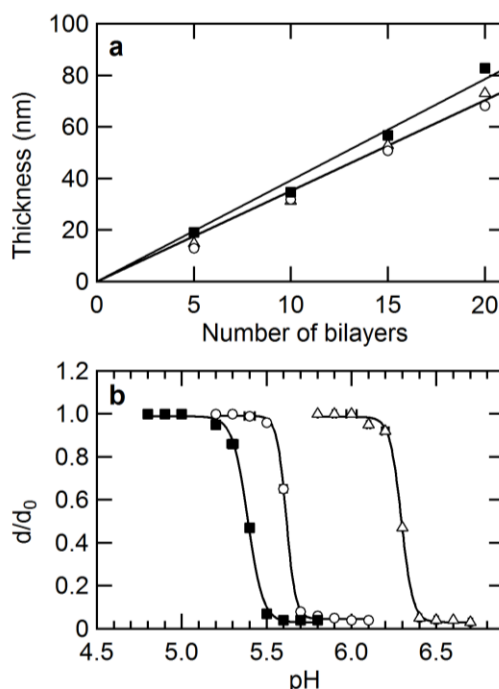


Figure S3.1. (a) Dry thickness of PEI(PMAA/PVPON)_m LbL films grown at pH 2.0, in different NaCl concentrations. The lines are fits to the data. (b) Relative dry thickness (d/d_0) of PEI(PMAA/PVPON)₂₀ films (*ca.* 80 nm starting dry thickness) grown at pH 2.0 and different NaCl concentration and immersed for 1 min in 20 mM MES buffers of different pH values as indicated. The curves are sigmoidal fits. NaCl concentration used for film grown in (a) and (b): 0 (triangles), 50 (circles) and 150 mM (squares).

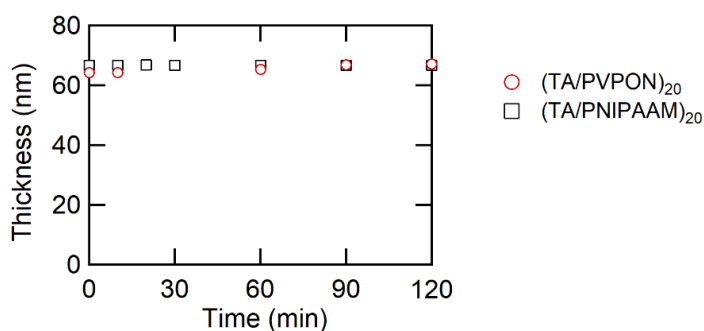


Figure S3.2. Variation of thickness of LbL multilayers immersed at pH 7.8 in 150 mM NaCl, versus time.

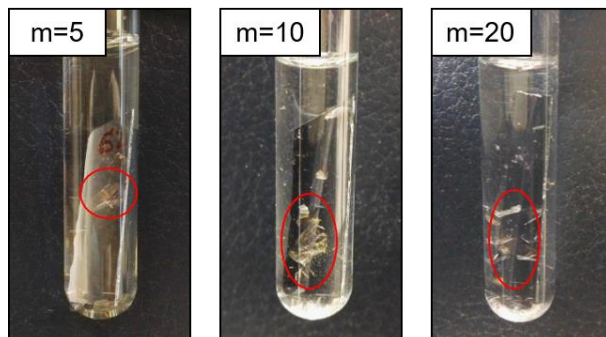


Figure S3.3. Pictures of $(\text{PSS/PAH})_{40}$ films released upon immersion of $\text{PEI(PMAA/PVCL)}_m(\text{PSS/PAH})_{40}$ films in pH 6.5, 20 mM MES (150 mM NaCl) buffer; $m = 5, 10$ and 20 .

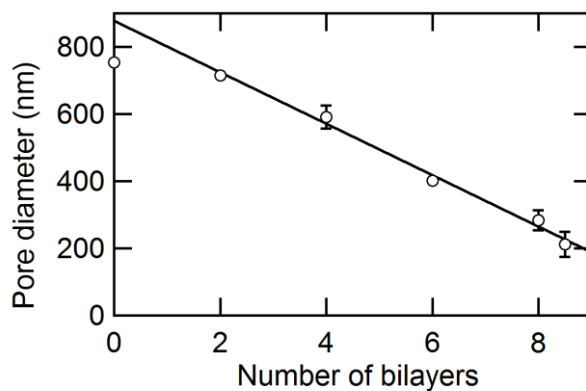


Figure S3.4. Average dry pore diameter of a PC membrane versus number of PVCL/PMAA bilayers deposited in the pores. The deposition was performed at pH 2.0 in 150 mM NaCl solution.

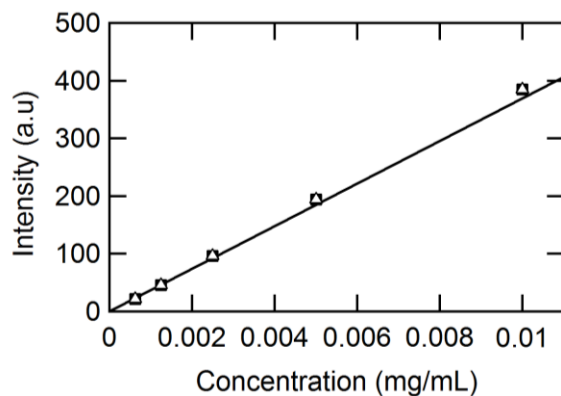


Figure S3.5. Fluorescence intensity of PAH-Rh versus solution concentration, in pH 4.0 (triangles) and pH 6.5 (squares) MES buffer (20 mM, 150 mM NaCl). The line is a fit to data.

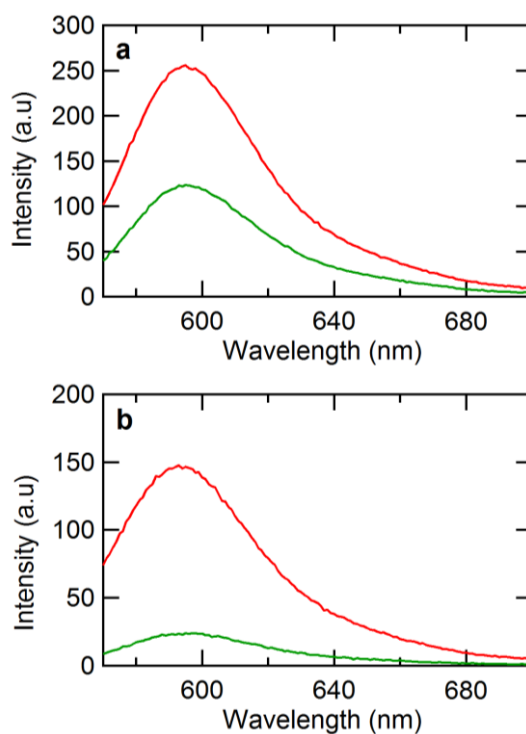


Figure S3.6. Quantification of the amount of liberated (PSS/PAH)₁₂ NTs obtained by disaggregating at pH 6.5 the PVCL/PMAA compartment of (PVCL/PMAA)₅PVCL(PSS/PAH)₁₂ bi-compartmentalized NTs. The NTs were built in (a) 4×10^7 and (b) 1×10^7 pores/cm² PC membranes. The amount

of total NTs (red lines) and NTs trapped in membrane after pH 6.5 treatment (green lines) was obtained by dissolving the corresponding membranes in dichloromethane and measuring the fluorescence intensity of the suspensions (excited at 550 nm).

Effect of the number of sacrificial bilayers on the fraction of released tubes

Bi-compartmentalized $(\text{PVCL}/\text{PMAA})_m\text{PVCL}(\text{PSS}/\text{PAH})_{12}$ tubes were built in a PC membrane of 3 μm pore diameter using the same LbL procedure as for NTs built in pores of 800 nm diameter. The proportion of overlapping pores was 36% for this membrane. Sacrificial compartments of different thickness ($m=2, 5$ and 8) were fabricated, and the inner $(\text{PSS}/\text{PAH})_{12}$ tubes were liberated at pH 6.5. The fraction of released tubes was evaluated by comparing the fluorescence intensity of the as-prepared tubes and the one of the tubes still trapped in the membrane after pH 6.5 treatment (Figure S3.7). Thicker sacrificial compartments contribute to a slight increase of the fraction of released tubes (67%, 69% and 75% for $m=2, 5$ and 8 , respectively).

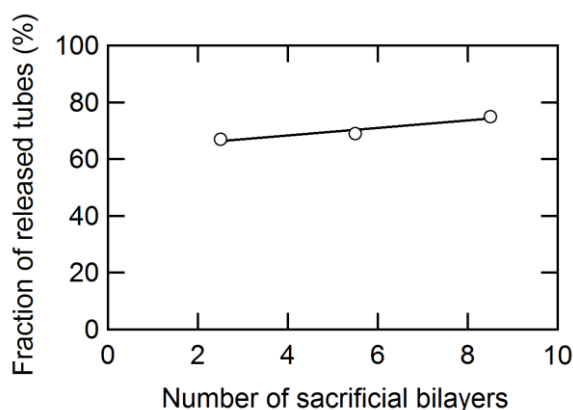


Figure S3.7. Fraction of tubes released from $(\text{PVCL}/\text{PMAA})_m\text{PVCL}(\text{PSS}/\text{PAH})_{12}$ grown in a PC membrane of 3 μm pore diameter, for $m=2, 5$ and 8 .

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Chapter 4 Encapsulation of bacteria in microtube mats

After having developed bicompartimentalized LbL tubes that can be released in neutral aqueous solutions, we tried to use these tubes for the entrapment of bacteria. In this chapter, we focus on improving the loading efficiency of bacteria in the tubes of the PC membrane, and study the activity of bacteria in MT mats after release from the membrane (Scheme 1.3). Latex microbeads, which have a similar shape and size as *S. epidermidis*, are used as model particles to optimize the loading conditions, including the diameter of pores of the PC membranes, the composition of the last layer of LbL MTs, and the dilution factor of microbead suspension. We also tried to close the pore by decrusting to maintain more microbeads in LbL MTs.

4.1 Introduction

Commensal skin bacteria are essential components of the skin microbiota that play a role as “physical”, “chemical”, and “biological” barriers to protect their hosts.^[1-3] *S. epidermidis*, a Gram-positive bacterium, has been shown to benefit human body in several aspects. It is known to regulate immune response,^[4-7] secrete phenol-soluble modulins^[8] and antibacterial peptides able to reduce bacterial colonization,^[3, 8-10] act against influenza virus infections, and even kill tumor cells.^[11, 12] The short-chain fatty acids produced by *S. epidermidis* fermentation have been demonstrated to control the growth of *Cutibacterium acnes*,^[13, 14] and of *P. acnes*,^[15-18] both of which are important acne triggers. Therefore, there is an increasing interest of using *S. epidermidis* to regulate skin homeostasis and treat skin disorders. However, some strains of *S. epidermidis* are demonstrated as accidental pathogens when entering the blood stream.

Encapsulation to maintain bacterial activity is a way to take their benefits and eliminate their uncontrolled spreading. Biocompatible and porous hydrogels have been made *via* a 3D printing technique to entrap bacteria for wound healing.^[19] A thermal-sensitive hydrogel formula that hardens upon skin administration was developed to entrap bacteria for antifungal applications.^[20] Fibrillar mats are also popular in skin treatment due to their high loading surface and easy topical application. Electrospinning and microfluidics are two methods that efficiently lead to bacterial fibrillar mats of tunable porosity.^[21, 22] Another attractive mat fabrication route was reported by our group *via* the liberation and collection of LbL assembly tubes.^[23, 24] Based on the biocompatibility of this fabrication process, LbL tubes have been developed for biological applications.^[25-27] Additionally, the challenging tube liberation step was improved by adding an external sacrificial compartment, as shown in chapter 3.

In this chapter, a non-biofilm-forming and non-pathogen *S. epidermidis* strain, ATCC 12228, is used. We study the encapsulation of *S. epidermidis* in MT mat, which is the first bacterial encapsulation method proposed in Scheme 1.3, including the following steps:

- Fabrication of two compartmentalized MTs, composed of the external sacrificial PVCL/PMAA compartment and the inner ALG/CHI compartment in porous PC membranes;
- Modification of the LbL assembly process to entrap as many bacteria as possible during bacteria loading and MT release. We show first the possibility of controlling pore closure and opening by optimizing the conditions used to decrust the modified membrane;

- Investigation of the loading conditions depending on pore size, microbead concentration and the type of inner layer in the MTs, intending to improve the entrapping efficiency;
- Fabrication of bacteria-containing MT mats according to the process shown in Scheme 1.3, and investigation of their bioactivity.

4.2 Experimental section

4.2.1 Materials

Poly(methacrylic acid) (PMAA, M_w 100 kg/mol) was obtained from Polysciences and poly(*N*-vinyl caprolactam) (PVCL, M_w 1.8 kg/mol) was purchased from Polymer Source Inc. NaCl (bioXtra), sodium alginate (ALG), polyethylenimine (PEI, M_w 750 kg/mol) and fluorescent latex microbeads (carboxylate-modified polystyrene, 1 μ m particle size) were purchased from Sigma-Aldrich. Biochemical grade 2-(*N*-morpholino)ethanesulfonic acid (MES) was obtained from Acros. Rhodamine-labeled PAH (PAH-Rh, M_w 15 kg/mol, 1:392 mol:mol monomer ratio in dye) was purchased from Surflay Nanotec GmbH. Chitosan (CHI, M_w 50-150 kg/mol, deacetylation degree > 90%) was purchased from NovaMatrix. Glycerol (bioreagent), Live/Dead bacterial viability kit and AlamarBlue™ cell viability reagent were purchased from Fisher Scientific. Freeze-dried *S. epidermidis* was purchased from ATCC (ref. ATCC 12228). Nutrient broth (BD 234000) was purchased from BD Bioscience.

Polymer solutions were prepared in 150 mM NaCl at a concentration of 1 mg/mL, except for PAH-Rh (0.5 mg/mL) or unless otherwise specified. Track-etched polycarbonate PC membranes (PC membranes of 1.6 μ m pore diameter, 24 μ m thickness, 1×10^7 pores/cm²; 2 μ m pore diameter, 23 μ m thickness, 2×10^6 pores/cm²; 3 μ m pore diameter, 22 μ m thickness 1.6×10^6 pores/cm² and polyethylene terephthalate membranes (PET membranes, 200 nm pore diameter, 23 μ m thickness, 5×10^8 pores/cm²) were provided by it4ip S.A., Belgium and used as received. Milli-Q water with a resistivity of 18.2 M Ω .cm was obtained from a Millipore system.

4.2.2 Fabrication of two compartment MTs

PVCL/PMAA and ALG/CHI were deposited at pH 2.0 and pH 4.0, respectively, as the external and internal compartments, respectively, into porous PC membranes. A 6×6 cm² PC membrane was dipped in polymer solutions for 20 min for the first PVCL/PMAA deposition cycle, then for 10 min from the second deposition cycle on. Between each polymeric deposition, PC membranes were twice rinsed (2 min each) with 150 mM NaCl at the same pH as used for polymer adsorption. The bottom surface of the PC membranes was decrusted every second bilayer in a 150 mM NaCl solution.

The top surface of the membranes was quickly decrusted in a 3M NaCl solution at the end of the LbL deposition process. This decrusting step was completed in 2 min to prevent damaging LbL MTs inside the PC membranes. The PC membranes were triply rinsed with MilliQ water then dried for storage. Scheme 4.1c illustrates the process of MTs fabrication. To fluorescently tag MTs, PAH-Rh was used instead of CHI at the last two ALG/CHI deposition cycles.

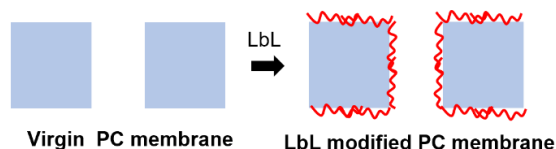
4.2.3 Surface modification of latex microbeads

Green fluorescent latex microbeads (1 μm particle size) were modified with (PEI/ALG)₁ to create a soft shell, increasing the interaction between microbeads and LbL MTs. PEI and ALG solutions were prepared in the absence of NaCl because we found that centrifugation of microbeads (used as model of bacteria cells) was inhibited in the presence of 150 mM NaCl. 14 μL stock solution of microbeads was dispersed in water (486 μL , pH 4.0) and 500 μL PEI solution was added into the microbead suspension. Vortex and sonication (30s) were performed to disperse the microbeads in the PEI solution. After deposition for 10 min, the microbeads were centrifuged at 12 krpm for 10 min. Precipitated microbeads were twice rinsed by dispersing them in 1 mL water (pH 4.0) for 2 min, followed by centrifugation. Then ALG was similarly coated on the microbeads. Finally, microbeads were dispersed in water and stored in the fridge. The fluorescence intensity of microbeads suspension was measured with a microplate reader (TECAN Infinite 200 PRO) to fix the concentration of (PEI/ALG)₁-modified microbeads at $1.5 \times 10^8/\text{mL}$.

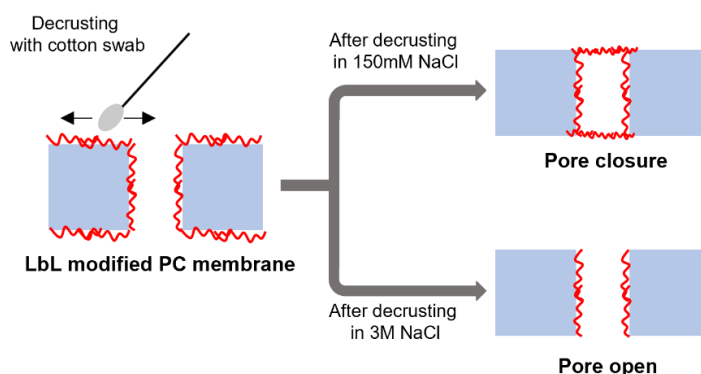
4.2.4 Loading of microbeads in MTs

A LbL-modified PC membrane cut to a diameter of 12 mm was placed in a Swinny filtration unit. The top surface of the PC membrane (open side) was selected as the upper face and 1.5×10^8 microbeads were loaded into MTs by filtration (Scheme 4.2). PEI/ALG-modified microbeads were diluted with 150 mM NaCl by a factor of 10, and 50 before being loaded into the PC membrane. The loading time of not diluted and 10x-diluted microbeads was 1 and 10 min, respectively. For 50x-diluted microbeads, 10 mL of suspension was loaded into the PC membrane in 2 min. This step was repeated until 50 mL of microbead suspension were filtered. After encapsulation, PC membranes were decrusted with cotton swabs to remove unencapsulated microbeads sticking to the outer surfaces.

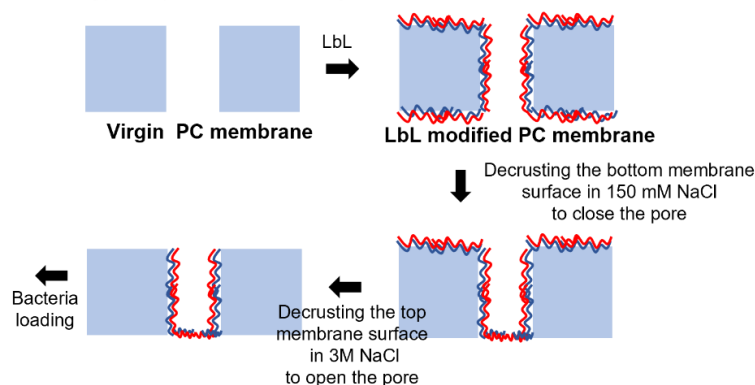
a. LbL deposition in PC membrane



b. Decrusting to remove the multilayers on surfaces in 150mM NaCl or 3M NaCl

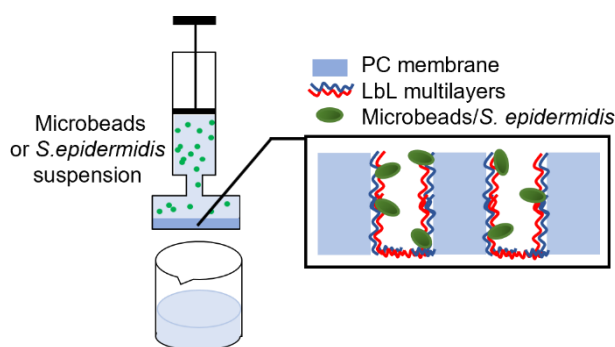


c. Our strategy: stepwise decrusting on membrane surfaces



Scheme 4.1. Fabrication of LbL MTs in a porous membrane and their closure/opening pores by decrusting. (a) LbL multilayers are deposited not only in pores but also on membrane surfaces. (b) Decrusting to remove LbL multilayers present on membrane surfaces, using a cotton swab. Closure or opening of the tubes can be obtained by decrusting the LbL film accumulated on the membrane surface, using 150 mM NaCl or 3 M NaCl, respectively. (c) Our strategy to fabricate LbL MTs for bacterial loading: before bacteria loading, the LbL-modified membrane was decrusting in 150 mM NaCl to close

pores on the bottom surface, then decrusted in 3 mM NaCl to open pores on the top surface.



Scheme 4.2 Loading of PEI/ALG-modified microbeads or *S. epidermidis* by forced filtration into LbL multilayers-modified PC membranes.

4.2.5 Revival of *S. epidermidis*

Freeze-dried *S. epidermidis* was revived overnight in 8 mL broth (8 g/L) 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 (OD) of the broth was measured at 540 nm (OD_{540}) until it reached *ca.* 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 *ca.* 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.

4.2.6 Encapsulation of *S. epidermidis* in MTs

A (PVCL/PMAA)_x(ALG/CHI)_n-modified PC membrane cut to a diameter of 12 mm was disinfected with 70% ethanol for 2 min and triply rinsed with water (30 s) to remove ethanol traces. Then, the modified membrane was placed under UV light for 1 min and dried overnight in a safety cabinet. A *S. epidermidis* suspension of 0.042 OD_{540} was prepared, corresponding to 1.5×10^8 bacteria/mL. 1 mL of this suspension was diluted by a factor of 50

with 150 mM NaCl (pH 6.0). Then, 10 mL of the diluted *S. epidermidis* suspension was filtered through the PC membrane for 2 min (Scheme 4.2). This step was repeated until having filtered 50 mL of the diluted bacterial suspension. The pH of NaCl solution used for this filtration process is close to the pH_{crit} of PVCL/PMAA multilayers (Figure 3.1b). However, the bacteria loading process takes only 10 min so that the filtration conditions used (*i.e.*, pH 6.0 and 150 mM NaCl) do not lead to the disaggregation of the erasable compartment. 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 cotton swabs to remove unencapsulated *S. epidermidis* according to the process described in Scheme 4.1c.

To close the pores after bacteria loading, (ALG/CHI)₉ALG multilayers were deposited on the top surface of the membrane, followed by decrusting. To avoid immersion, the PC membrane was floated over ALG and CHI solutions. After 1 min polymer deposition, the PC membrane was twice rinsed with a NaCl solution (2×15 s) and was decrusted every second bilayer.

4.2.7 Fabrication of MT mats

LbL MTs were released from the PC membrane by filtering a pH 6.5 MES buffer (120 mL, 20 mM MES, 150 mM NaCl). As shown in Scheme 3.3, the released MTs were then collected over a porous membrane (pore size 2 μ m, 2×10^6 pores/cm²). The MTs were rinsed with fresh MES buffer (3×10 mL) to remove traces of disaggregated PVCL/PMAA compartments and free bacteria escaped from MTs during the release process, thereby obtaining a MT mat encapsulating *S. epidermidis*. We checked by epifluorescence microscopy that most free bacteria were removed from the MT mat.

4.2.8 Metabolic activity of *S. epidermidis* in MT mats

A MT mat was placed in a vented culture tube containing 2 mL broth, and cultured at 37 °C, 120 rpm. A positive control of free bacteria was prepared by adding 10 μ L 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. AlamarBlueTM cell viability reagent (200 μ L) was added in each tube at the starting time. The fluorescence intensity of the supernatant was measured to evaluate the metabolic activity of bacteria. For each test, 200 μ L 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 vented tube in the safety cabinet to maintain a constant volume of broth. In case of water evaporation, 10 μ L water was added into the broth every 4 h culture.

4.2.9 Characterization of MTs

The PC membranes modified with LbL MTs, and MTs released at pH 6.5 by disaggregating the sacrificial compartment were coated by a 20 nm-thick layer of sputtered gold then imaged with a scanning electron microscope SEM (JSM-7600F, JEOL Ltd) at 5 kV. (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs deposited in PC membrane were liberated by dissolving the PC membrane in CH₂Cl₂. Then (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs were collected on a PET membrane, coated by 20 nm-thick layer of sputtered gold then imaged on SEM at 5 kV.

Fluorescently-tagged PC membranes encapsulated with microbead or *S. epidermidis* were imaged by confocal laser scanning microscopy (CLSM, Zeiss LSM 710) using Z-stack scanning from the top and the bottom surfaces, respectively. It is worth noting that CLSM allowed to observe only a part of the MT, as shown in Figure S4.3. Released MTs tagged with rhodamine were observed by epifluorescence microscopy (Olympus IX71).

4.3 Results and discussion

4.3.1 Closure of MTs by decrusting

During LbL in a porous membrane, polyelectrolyte deposition occurs not only inside the pores, which leads to MTs, but also on the top and bottom surfaces of PC membranes, resulting in LbL films on membrane surfaces (Scheme 4.1a). The SEM images in Figure 4.1a and b illustrate the change of membrane surface after LbL deposition. The LbL films deposited on the PC membrane surface will impact the encapsulation of *S. epidermidis*. Although decrusting in 150 mM NaCl solution can remove the unexpected LbL films, pores appear to be closed when several cycles of decrusting are used (Scheme 4.1b). A lot of stimuli, including chemical stimuli (pH, ionic strength, electrochemical), physical stimuli (temperature, laser light, ultrasound, magnetic field, etc.) and biological stimuli have been investigated to disaggregate polyelectrolyte multilayers.^[28] In chapter 3, we studied the disaggregation of PVCL/PMAA compartment upon variation of pH and NaCl concentration.

A 3 M NaCl solution was used to remove the PAH/PSS film deposited on the top and bottom surfaces of the LbL-modified PC membrane.^[29] Therefore, the decrusting process was attempted with a highly concentrated saline solution. A (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membrane was dipped in 3 M NaCl solution then decrusted to remove the LbL films. After decrusting in 3 M NaCl, >90% of pores are open (Figure 4.1d). By contrast, >90% of pores are closed when the PC membrane is decrusted every second bilayer in a 150 mM NaCl solution (Figure 4.1c). This demonstrates a method to control

the closure and opening of pores by decrusting either in 150 mM or in 3 M NaCl solution, respectively.

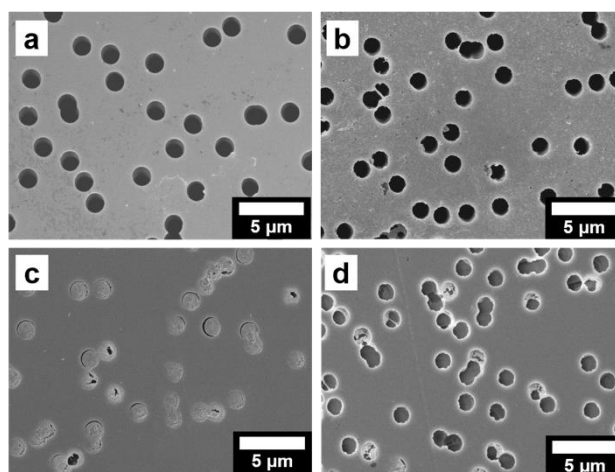


Figure 4.1. The morphology of a PC membrane (1.6 μm pore diameter) observed by SEM. (a) Starting virgin PC membrane; (b) (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membrane without decrusting; (c) (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membrane decrustated every second bilayer in a 150 mM NaCl solution of the same pH as the deposition solution; (d) (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membrane decrustated in 3 M NaCl at pH 6.0.

A stepwise decrusting method was used when fabricating LbL multilayers-modified PC membranes for encapsulation. As shown in Scheme 4.1c, the bottom surface of PC membrane was decrustated every second bilayer in 150 mM NaCl solution during LbL deposition. The top surface of the PC membrane was then decrustated in 3 M NaCl when finishing LbL deposition. Using this method, PC membranes with a pore size of 1.6 μm could be functionalized with (PVCL/PMAA)₅PVCL(ALG/CHI)₆ multilayers. As shown in Figure 4.2, the top pores were fully open. By contrast, >90% of the bottom pores were closed.

Figure 4.3a shows the morphology of (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs deposited in a PC membrane (1.6 μm pore diameter). The width of the MTs was measured to be 2 μm , which is slightly larger than the pore diameter because of flattening upon deposition. One side of the MTs shows a spherical morphology (red circle) with a diameter of 1.3 μm , similar to the real diameter of pores. The spherical blob at the end is most probably made of the crust

pushed in the pore during decrusting; it is rigid and thus maintains a spherical shape after collection. The MTs decrusted in 150 mM NaCl for both surfaces showed a similar morphology of tube ends (Figure S4.1a, red circles). Hence, we surmise that the spherical ends of tubes are the bottom side of MTs. The other side appears to be flattened, showing a similar morphology to MTs decrusted in 3 M NaCl on both surfaces (Figure S4.1b, triangles). The (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs decrusted in 3 M NaCl preserve their integrity, demonstrating the innocuity of this treatment on MTs. The liberation of only the internal (ALG/CHI)₆ compartments from (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs grown in PC membranes was also performed, showing that (PVCL/PMAA)₅PVCL compartment is still efficient for releasing MTs after the top and bottom specific decrusting processes (Figure 4.3b and c).

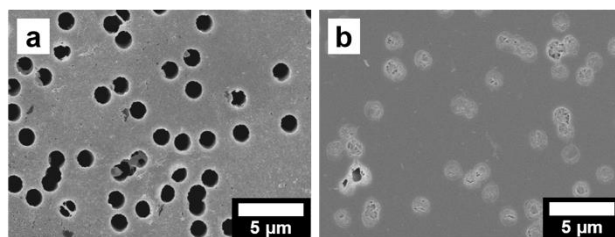


Figure 4.2. The morphology of a (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membrane (1.6 μm pore diameter) observed by SEM. (a) The top surface of the PC membrane was decrusted in 3 M NaCl when finishing LbL deposition. (b) The bottom surface of the PC membrane was decrusted in 150 mM NaCl every second bilayer during LbL deposition.

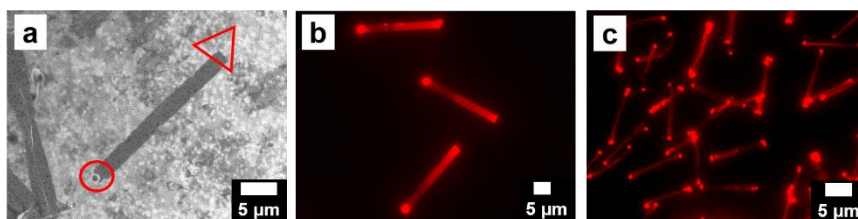


Figure 4.3. Morphology of MTs fabricated in a PC membrane (1.6 μm pore diameter). (a) SEM image and (b) epifluorescence microscopy image of (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs. The (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membrane was decrusted in 3 M NaCl at the top surface at the end of the LbL deposition, and was decrusted every second bilayer in 150 mM NaCl at the bottom surface. Then MTs were released by dissolving the PC membrane in CH₂Cl₂ and were

collected on a PET membrane for imaging. (c) Epifluorescence microscopy image of liberated MTs at pH 6.5, by disaggregating the PVCL/PMAA compartment of (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs.

4.3.2 Optimization of encapsulation efficiency

Bacteria entrapment in PC membrane results from the attachment of bacteria onto the surface of the pores. The efficiency of this attachment depends on electrostatic and hydrophobic interactions between cell surface and pore surface, as discussed in chapter 2. Considering the complexity of encapsulating living bacteria into LbL MT, a model particle was used to optimize the loading process. Study shows that the adhesion efficiency of latex beads on polyelectrolyte films is identical to *S. epidermidis* under flow.^[30] Therefore, fluorescently-tagged latex microbead of similar size (1 μm) as *S. epidermidis* was used in this thesis. The latex microbeads are different from *S. epidermidis* in shape (spherical and ellipsoidal), size dispersion (monodisperse and 0.5~1.5 μm size), surface charge density, hydrophobicity, etc. We are well aware that the microbead is a very simplistic model of *S. epidermidis*; however, they nevertheless offer a convenient and fluorescence-visible model to screen rapidly encapsulation conditions before turning to real bacteria. The latex microbeads were surface-modified with a PEI/ALG bilayer at pH 4.0, in order to get a negatively charged and soft surface, which very roughly mimics the characteristics of bacterial cell membranes. Microscopy experiments indicated that 85% of microbeads were single particles, while the remaining fraction was doubly- or triply-aggregated particles. The concentration in microbeads was adjusted to 1.5×10^8 beads/mL, with 1 mL of microbeads used for loading.

Pore diameter. The internal diameter of LbL tubes can affect the loading efficiency, as shown by Fan and co-authors.^[31] These authors demonstrated that larger internal diameters unsurprisingly lead to an increased number of particles loaded in LbL tubes. Therefore, (PVCL/PMAA)₅PVCL(ALG/CHI)₆ modified PC membranes of 1.6 μm , 2 μm and 3 μm pore size were studied in the present study. As shown in Figure S4.2, the top surface of 2 μm and 3 μm LbL-modified PC membranes showed a similar morphology to 1.6 μm LbL-modified PC membranes, with fully open top pores. By contrast, the bottom surfaces of the modified PC membranes differed depending on initial pore diameter. Compared with the >90% closed pore of 1.6 μm PC membranes, fewer pores of the 2 μm and 3 μm PC membranes were closed. The pore closure thus highly depends on pore size, due to more crust being required to fill-in the opening of larger pores. The proportion of closed pores in membranes of larger pore diameter can be increased by increasing the number of LbL deposition cycle, leading to a thicker crust. However, the thickness of the compartments of the LbL tubes also increases by this process, leaving less

space for encapsulation. In addition, the time for LbL modification of the PC membrane is longer.

PEI/ALG-modified microbeads were loaded in PC membranes at a dilution factor of 10. With more microbeads entrapped in MTs, less space is left for solution passage, leading to higher filtration stresses, especially for a 1.6 μm PC membrane. Orthogonal images of (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membranes of 1.6 μm , 2 μm , and 3 μm pore size, with PEI/ALG-modified microbeads encapsulated, are shown in Figure 4.4. Red fluorescent MTs with homogenous shape and size can be observed, showing the proper LbL modification of pores of PC membranes. To show the encapsulation of microbeads into LbL MT due to filtration, without contribution by the post-decrusting process, the PC membranes were observed without decrusting.

A microbead mat was formed on the top surface of the membranes, its thickness being larger for the 1.6 μm PC membrane due to its small pore size. Quantitative counting of microbeads entrapped in LbL MTs is not easy. Though the CLSM images do not show the whole length of pores, they nevertheless provide a way to roughly evaluate the encapsulation efficiency in modified PC membranes. About 600 pores on top and bottom surfaces of the LbL-modified PC membrane were counted to evaluate the proportion of pores filled with microbeads, as shown in Table S4.1. Comparing the orthogonal images of the top surfaces of PC membranes of increasing pore diameter with the increase of pore size, the proportion of MTs trapping microbeads decreases. By contrast, the encapsulation of microbeads at the bottom surface shows an opposite trend (Figure 4.4b, d, and f). More microbeads are loaded at the bottom of 2 μm and 3 μm PC membranes. Due to the forced loading process by filtration (Scheme 4.2), the smaller the diameter of MTs, the quicker the microbeads will be trapped; this leads to more microbeads trapped at the top end of the MTs in 1.6 μm membrane. However, the immobilization of microbeads at the top surface also leads to less space for the passage of microbeads, resulting in a decrease of the number of trapped microbeads at the bottom side for membranes of smaller pore diameter. The pore density is another parameter impacting the encapsulation capability of a PC membrane. The average number of microbeads encapsulated in a 52×52 μm^2 PC membrane area (*i.e.*, the area of the XY view shown in Figure 4.4) was also counted and it was found to be 120, 89, and 54 for 1.6, 2 and 3 μm PC membranes, respectively. Therefore, 1.6 μm PC membranes show the best encapsulation efficiency.

In addition, microbeads in 2 μm and 3 μm PC membranes tend to gather more at the end of MT, showing hetero-distribution along the pore. Thus, 1.6 μm PC membranes were chosen for encapsulation.

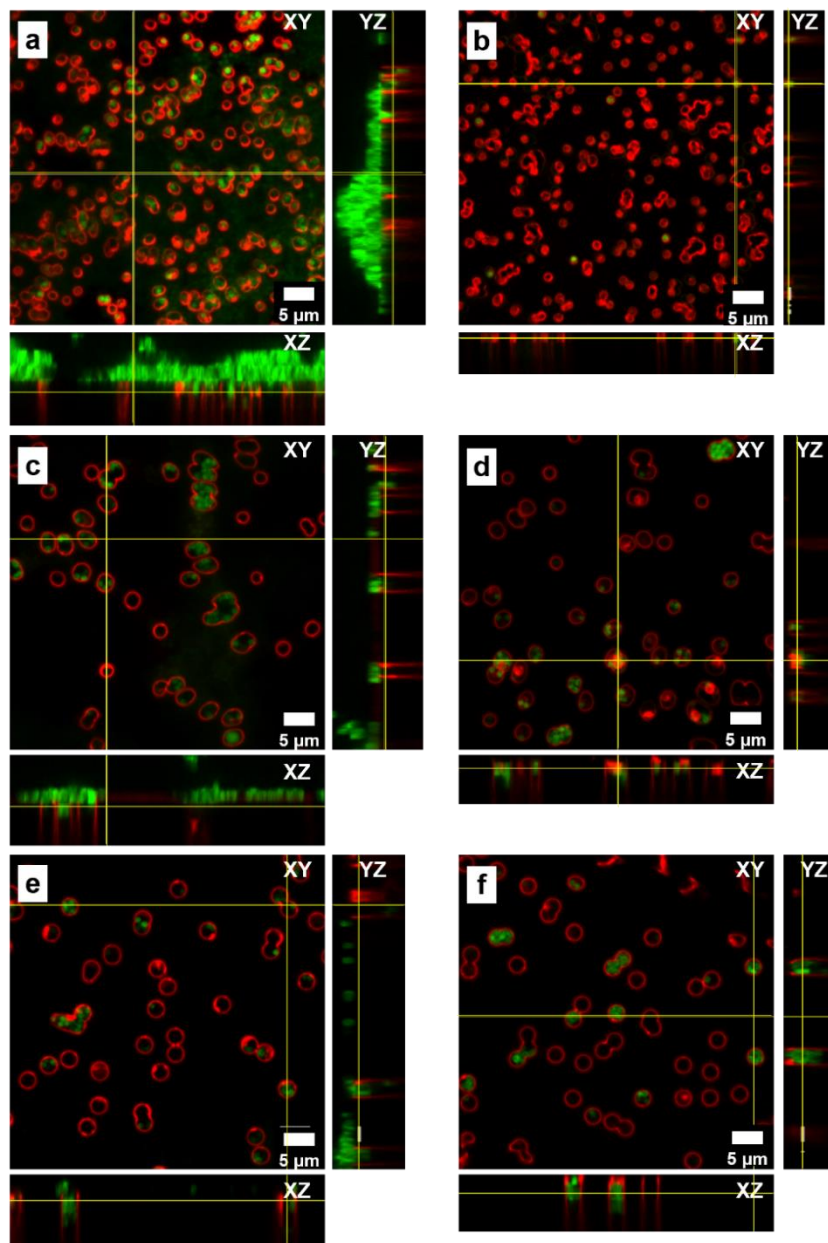


Figure 4.4. Orthogonal images of fluorescently-tagged (red) (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membranes loaded with PEI/ALG-modified microbeads (green), without decrusting. XZ and YZ images share the same scale bar as XY image. PC membranes of (a, b) 1.6 μm , (c, d) 2 μm , and (e, f) 3 μm pore diameter were loaded with 10x-diluted PEI/ALG-modified microbead suspensions. PC membranes were observed

from (a, c, e) the top surface and (b, d, f) the bottom surface by CLSM using Z-stack scanning. Orthogonal XZ and YZ images only show a part of the length of LbL MTs, as illustrated in Figure S4.3.

Microbead concentration. The rapid trapping of PEI/ALG-modified microbeads at the top side of MTs is one reason for decreased microbead encapsulation efficiency. Therefore, the effect of the concentration of microbeads was studied. 1 mL of PEI/ALG-modified microbeads (1.5×10^8 microbeads/mL) was either diluted by a factor of 10 and 50, or not diluted, before loading into 1.6 μm (PVCL/PMAA)₅PVCL(ALG/CHI)₆-modified PC membranes.

The orthogonal images of PC membranes loaded with not diluted and 50x-diluted PEI/ALG-modified microbead are shown in Figure 4.5. The proportion of pores filled with microbead is summarized in Table S4.1. When a not diluted suspension of microbeads was used for loading, fewer pores were filled with microbeads, compared to the 10x-diluted microbead. By contrast, 76% and 17% of pores were filled with microbeads on the top and bottom surface, respectively at a dilution factor of 50. In addition, microbeads can be loaded deeper in pores when a dilution factor of 50 was used (Figure 4.5c and d). The use of high diluted microbead suspensions thus prevents the blockade of MTs at an early stage of the encapsulation. Therefore, more microbeads can be immobilized in MTs and can go deeper in MTs. As demonstrated in Figure 4.5 and Table S4.1, a high dilution factor improves the encapsulation efficiency of microbeads and the dispersion of microbeads along MTs. Therefore, a dilution factor of 50x was used for microbead encapsulation in the sequel.

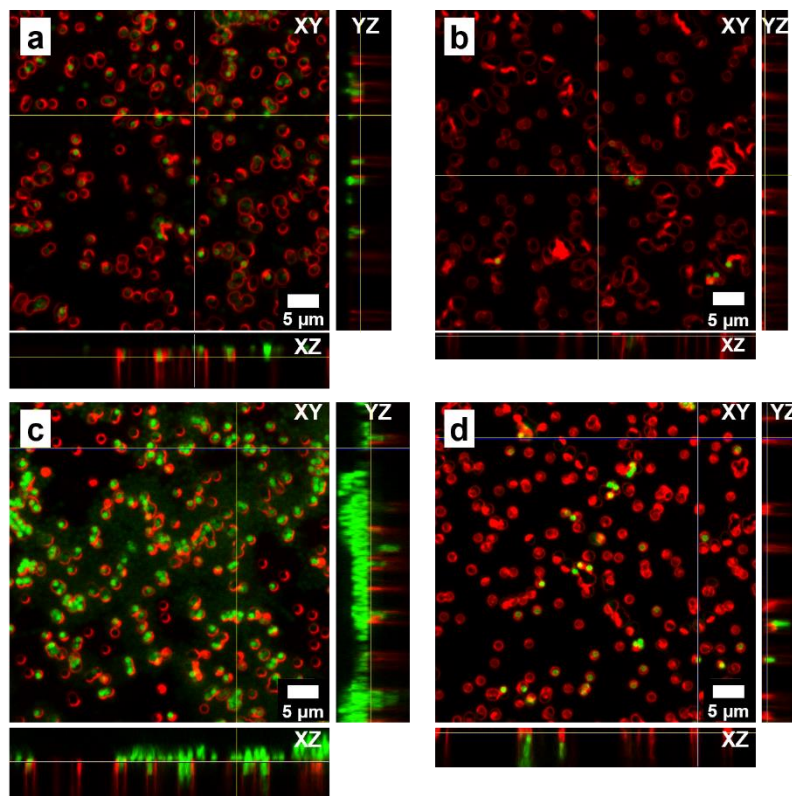


Figure 4.5. Orthogonal images of fluorescently-tagged (red) $(\text{PVCL/PMAA})_5\text{PVCL}(\text{ALG/CHI})_6$ -modified PC membranes ($1.6\ \mu\text{m}$ pore diameter) loaded with (a, b) not diluted and (c, d) 50x-diluted PEI/ALG-modified microbead (green) suspensions. PC membranes without decrusting on both surfaces were observed from (a, c) the top surface and (b, d) the bottom surface by CLSM using Z-stack scanning. Orthogonal XZ and YZ images share the same scale bar as XY image; XZ and YZ images only show a part of the length of LbL MTs, as illustrated in Figure S4.3.

Charge of the last layer in LbL MTs. Microbeads were modified with PEI/ALG, leading to negatively charged surfaces (as are on average bacterial surfaces). We investigated whether the charge type of the last layer inside the LbL MT can impact microbead loading. Following the same LbL assembly process, $(\text{PVCL/PMAA})_5\text{PVCL}(\text{ALG/CHI})_6\text{ALG}$ -modified PC membranes were fabricated. Figure 4.6a and b show that MTs are open on their top end and closed on their bottom end, similar to $(\text{PVCL/PMAA})_5\text{PVCL}(\text{ALG/CHI})_6$ -modified PC membranes.

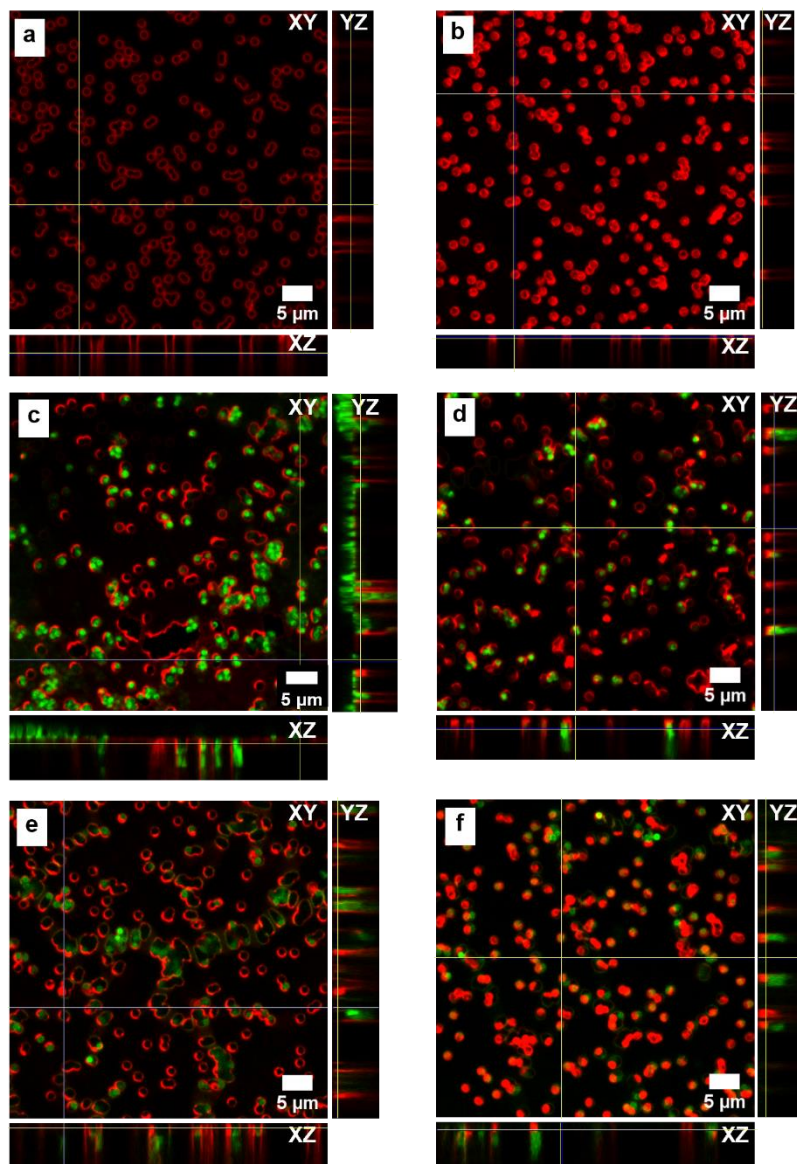


Figure 4.6. Orthogonal images of fluorescently-tagged (red) (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membranes (1.6 μm pore diameter). (a, b) LbL-modified PC membrane; (c, d) PC membrane after encapsulation of 50x-diluted PEI/ALG-modified microbeads, without decrusting; (e, f) PC membrane after encapsulation of microbeads and decrusting. PC membranes were observed from (a, c, e) the top surface and (b, d, f) the bottom surface by CLSM using Z-stack scanning. Orthogonal XZ and

YZ images share the same scale bar as XY image. XZ and YZ images only show a part of the length of LbL MTs, as illustrated in Figure S4.3.

By contrast, (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG MTs could immobilize more microbeads over the whole Z-scanning depth on both surfaces (Figure 4.6c and d). 73% and 44% of pores were filled with microbeads from the inspection of the top and bottom surfaces, respectively, showing the highest proportion (Table S4.1). We speculate that the electrostatic interactions between (PVCL/PMAA)₅PVCL(ALG/CHI)₆ MTs and PEI/ALG-modified microbeads promote the quick trapping of microbeads at the entrance of the pores, leading to blockade of MTs. (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membranes show a higher encapsulation capability because the repulsion between beads and LbL compartments is higher and therefore decreases pore blockade.

After microbead loading, the top surface of the membrane was also observed by SEM (Figure S4.4a). After decrusting (Figure S4.4c), the microbeads adsorbed at the top surface were efficiently removed, and no visible increase in the number of encapsulated microbeads was observed by CLSM (Figure 4.6e and f). In the following study, 1.6 μm (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membranes will thus be used in combination with 50x-diluted microbead/bacteria suspensions.

4.3.3 MT Mats

After loading of microbeads, MT mats were prepared by liberation and collection of MTs from the PC membrane. However, many microbeads were released at this stage (Figure S4.5). Closure of the top side of MTs is one possible way to prevent the free diffusion of microbeads. Inspired by the closure of MTs by decrusting, ALG/CHI LbL multilayers deposition followed by decrusting is promising for the quick closure of MTs on their top side. The ALG/CHI LbL system was chosen for the following reasons. First, ALG/CHI can be deposited at pH 6.0, at which bacteria remain viable (pH 5.5-6.5) and (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG MTs are stable for short times. Second, the thickness of ALG/CHI films increases by a factor of two in a 0.15 M NaCl solution, leading to a quicker blockade of pores by decrusting.

To decrease the impact of this supplementary LbL step on the integrity of MTs and microbeads/bacteria, the polymer adsorption time was shortened to 1 min; additionally, the PC membranes were floated over solutions during LbL assembly rather than immersed in them, in order to deposit the multilayers only on the top surface. After deposition and decrusting of (ALG/CHI)₉ALG multilayers, 90% of the pores on the top surfaces were closed, as shown in Figure 4.7a. The pores on the bottom surface remained closed (Figure 4.7b).

A SEM image of the liberated MTs is displayed in Figure 4.7c. The microbeads remain covered by films, illustrating the encapsulation of microbeads in MTs. A few rare free microbeads were also observed on MTs mat (Figure 4.7d).

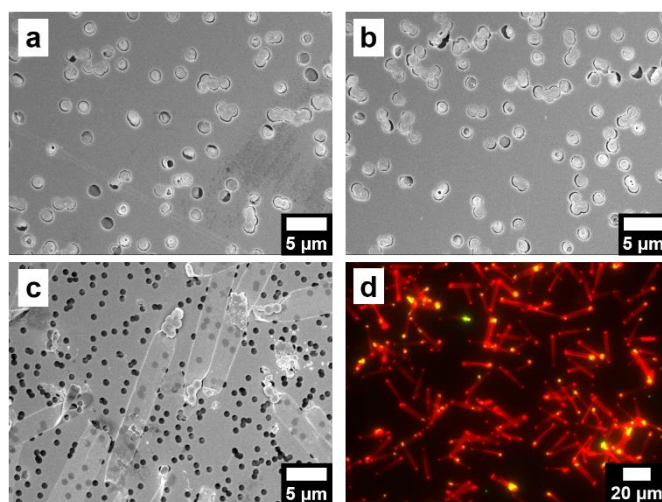


Figure 4.7. Closure of the pores of a PC membrane by deposition and decrusting of (ALG/CHI)₉ALG multilayers. A (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membrane was loaded with PEI/ALG-modified microbeads. Then (ALG/CHI)₉ALG multilayers were deposited on the top surface and the PC membrane was decrusting every second bilayer. The PC membrane was observed by SEM on its (a) top and (b) bottom surfaces. (c) SEM image and (d) epifluorescence microscopy image of MTs released at pH 6.5. Red signal: MTs tagged with rhodamine; Green signal: PEI/ALG-modified microbeads.

4.3.4 Encapsulation of *S. epidermidis* into LbL MTs

ATCC 12228 is a biofilm-negative and non-pathogen bacterial strain widely used to investigate the beneficial effect of *S. epidermidis*. Fresh *S. epidermidis* was prepared and a bacteria suspension of OD₅₄₀=0.042, corresponding to 1.5×10⁸ bacteria/mL (Figure S4.6), was used for encapsulation. *S. epidermidis* is roughly spherical and has an average diameter of 0.5-1.5 μm.^[32] The viability of fresh *S. epidermidis* was checked by staining bacteria with Live/Dead kit. As shown in Figure S4.7, ca. 80% of bacteria were alive. To visualize bacteria, *S. epidermidis* was stained with SYTO 9, then loaded in (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membranes. CLSM images of the PC membrane (Figure 4.8) show that *S. epidermidis* was

encapsulated in LbL MTs and about 17% of pores is filled with bacteria (by counting 1800 pores); each pore is filled with 1 to *ca.* 3 bacteria cells with most bacteria trapped at the top and bottom sides of MTs. However, the number of bacteria encapsulated in the PC membrane was fewer compared to microbeads.

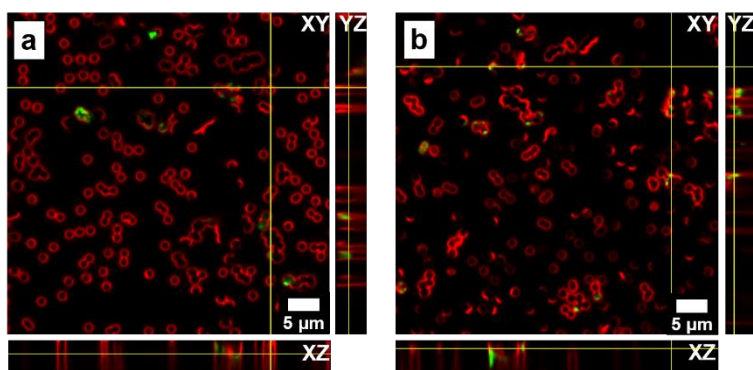


Figure 4.8. Orthogonal images of fluorescently-tagged (red) (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membranes (1.6 μm pore diameter) encapsulated with *S. epidermidis*. *S. epidermidis* was stained with SYTO 9 for 20 min before being loaded into a rhodamine-tagged LbL-modified PC membrane. The PC membrane was observed from (a) the top surface and (b) the bottom surface by CLSM using Z-stack scanning. Orthogonal XZ and YZ images share the same scale bar as XY image. XZ and YZ images only show a part of the length of LbL MTs, as illustrated in Figure S4.3.

(PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membranes encapsulating bacteria were cultured in broth. AlamarBlue™ reagent was added to the broth and the emission fluorescence intensity (at 585 nm) was used to evaluate the metabolic activity of bacteria; 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 lag time and the time to reach the stationary phase were tested. Free bacteria were cultured as positive control. The CLSM observation of PC membranes encapsulating bacteria (Figure 4.8) showed that the encapsulation efficiency is quite low. For this reason, a bacterial concentration corresponding to only 1% of the suspension used to load bacteria in the membrane (*ca.* 5×10^6 bacterial cells), was used to prepare the positive control to perform activity tests. To study

whether the presence of (ALG/CHI)₉ALG shell and MES treatment impact bacteria, the metabolic activity of bacteria in LbL-modified PC membranes was measured.

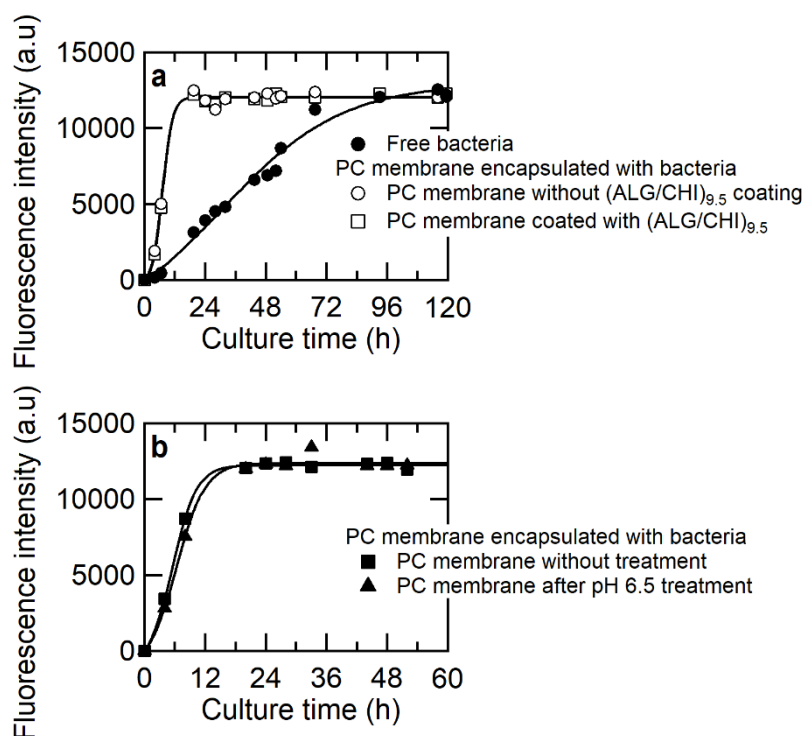


Figure 4.9. Integrated metabolic activity of *S. epidermidis* in PC membranes. (a) The effect of (ALG/CHI)₉ALG deposition on bacteria. (ALG/CHI)₉ALG multilayers were deposited and decrusted on the top surface of the PC membrane at pH 6.0. As a positive control, free bacteria were cultured in identical conditions by adding $ca. 5 \times 10^6$ bacterial cells in a broth/alarmarBlue™ mixture. (b) The effect of MES treatment. A PC membrane was dipped in pH 6.5 MES buffer for 1 h. Then, a PC membrane without treatment and the PC membrane treated by pH 6.5 MES were placed in 2 mL broth and 200 μ L alamarBlue™, then cultured at 37 °C, 180 rpm in the incubator. 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.

As shown in Figure 4.9a, the activity of the free bacteria displays a typical exponential increase due to bacterial growth. Bacteria encapsulated in a PC

membrane coated with/without (ALG/CHI)₉ALG show a quick growth of the fluorescence intensity, plateauing after only 20 h culture, showing that (ALG/CHI)₉ALG deposition has no impact on bacterial activity. Compared to free bacteria, the bacteria in PC membrane showed a shorter time to reach the stationary phase. There may be two reasons for this, either the number of encapsulated *S. epidermidis* was larger than 1.5×10^6 (indeed a shorter time to reach the stationary phase is obtained when the number of inoculated bacteria increases), or the bacterial proliferation was promoted when adhering on the inner surface of LbL MTs, compared to free bacteria.

For MT mat fabrication, MTs were released from the PC membrane by disaggregation of the PVCL/PMAA compartment at pH 6.5. The impact of MES buffer at this pH on bacteria activity was therefore studied. After encapsulation of *S. epidermidis*, the PC membrane was dipped in MES buffer for 1 h then cultured in broth. As shown in Figure 4.9b, MES treatment does not significantly delay bacterial growth, showing that MES treatment has no impact on encapsulated *S. epidermidis*.

4.3.5 Metabolic activity of *S. epidermidis* in MT mats

After encapsulation of *S. epidermidis*, (ALG/CHI)₉ALG was deposited on the top surface of a membrane to close the top openings of the MTs. Then, the MTs were released at pH 6.5 and were collected on a 2 μm PC membrane (2×10^6 pores/ cm^2). As shown in Figure 4.10, a MT mat was obtained with *S. epidermidis* dispersed inside the tubes. The yellow circle in Figure 4.10a illustrates a free bacterium, showing some release of bacteria during the pH 6.5 treatment. However, the number of free bacteria remains low compared to bacteria encapsulated in MTs.

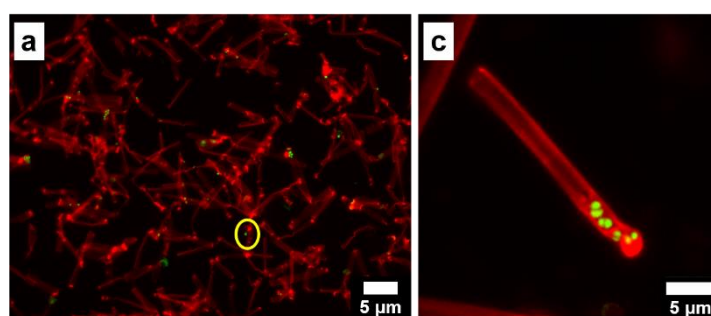


Figure 4.10. Epifluorescence microscope image of a MT mat. A (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membrane was loaded with STYO 9-tagged *S. epidermidis*. Then, the MTs were released at pH 6.5 and were collected on a 2 μm PC membrane, (a) leading to a MT mat. (b) An enlarged image of one liberated MT.

Before culturing the bacteria, the stability of the MT mats was studied. As shown in Figure S4.8b, MTs maintained their integrity, and the number of MTs collected on a PC membrane did not decrease after 5 days incubation in the broth. The metabolic activity of bacteria in MT mats was tested by culturing them in broth. Free bacteria were tested in the same conditions. As shown in Figure 4.11a, free bacteria show a typical exponential growth due to bacterial growth. Figure 4.11b shows the slight fluorescence intensity increase in an bacteria-loaded MT mat, until reaching a quasi-plateau at 48 h culture. However, the absolute fluorescence intensity of bacteria trapped in the MT mat was much lower than free bacteria, indicating a very weak activity of *S. epidermidis*. The number of bacteria in the MT mat was tested. A MT mat was cut into two pieces; one was cultured in broth for 72 h and the other was used as reference. Bacteria were stained with SYTO 9 to be able to observe the dispersion of bacteria in the MT mat. The results of microscopy imaging (Figure S4.8f) and the metabolic activity test both demonstrate that bacteria can only slightly proliferate inside the MT mat.

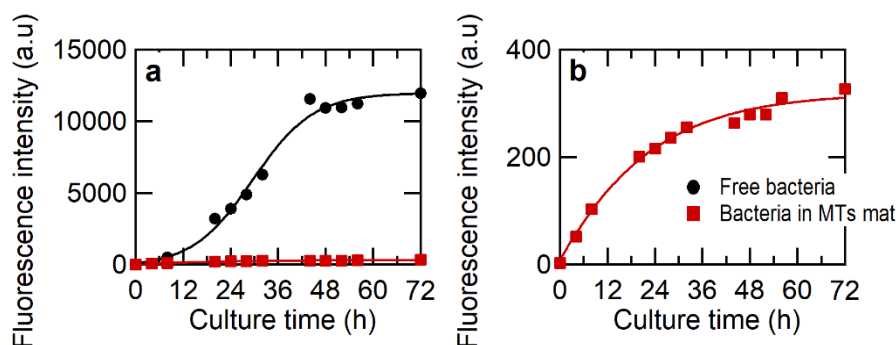


Figure 4.11. Integrated metabolic activity of free bacteria and bacteria in MT mats. The MT mats were placed in 2 mL broth with 200 μ L alamarBlueTM, then cultured at 37 °C in the incubator. As a positive control, free bacteria were cultured in identical conditions by adding *ca.* 5×10^6 bacterial cells in a broth/alamarBlueTM 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. Panel b is an enlargement of the red curve of panel a.

There might be three reasons for the negligible activity of bacteria in MT mats: (1) a low bacterial loading in MT mats because of the escape of bacteria during MT release; (2) In comparison to bacteria entrapped in modified PC membranes, bacteria in the MT mat are fully covered by (ALG/CHI)₆ALG multilayers (Figure 4.7c). Additionally, the two sides of MTs are closed by a

crust containing ALG/CHI; therefore, the physical barrier due to the polyelectrolyte shell may impact the nutrient, oxygen, and metabolic waste diffusion; (3) the presence of bactericidal CHI in the polyelectrolyte shell can impact the activity of encapsulated bacteria.

The ALG/CHI LbL film has been reported as a bactericidal material, showing a higher inhibition rate when the bilayer number increases.^[33] Furthermore, the viability and growth of *S. epidermidis* coated by LbL ALG/CHI bilayers were studied by our group. It was demonstrated that the growth of bacteria is controlled by coating thickness. The lag time was *ca.* 30 h when bacteria were coated with (ALG/CHI)₃. However, bacteria coated with (ALG/CHI)₆ALG showed no growth, and almost all bacteria were dead.^[34] The viability of bacteria in MT mats was tested by staining them with Live/Dead bacteria viability kit. As shown in Figure S 4.8d, 84% of bacteria were alive (green) after 48 h incubation, which is similar to the viability of bacteria before culture (80%) (Figure S 4.8c). The inner (ALG/CHI)₆ALG compartment of MTs has an ALG layer in contact with bacteria whereas an (ALG/CHI)₆ALG film directly deposited on bacteria has a CHI layer in contact with the bacteria. This might be the reason why the MTs are less bactericidal and maintain some (quite limited) bacterial activity.

4.4 Conclusions

In this section, bicompartimentalized (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG MTs were used to entrap *S. epidermidis* and MT mats were fabricated. We showed that it is possible to control the closure or opening of MT by decrusting the LbL films on PC membrane surfaces in gentle (150 mM) or strong (3 M) saline solutions, respectively. PEI/ALG-modified microbeads were used as rough model particles to study entrapment in the LbL MT while still in the membrane. A slower microbead loading speed is preferred to get a higher encapsulation efficiency by avoiding pore blockade at the top. To increase the encapsulation efficiency, the encapsulation parameters were studied, including the pore size of PC membrane, the microbead concentration, and the charge type of the last layer of the MTs. A PC membrane of 1.6 μm pore size showed the best trapping efficiency. A 50x dilution factor of microbead suspension (3×10^6 microbeads/mL) could prevent the quick blockade of pores, leading to more microbeads being loaded in MTs. The LbL MTs ended by negatively charged ALG were superior to CHI-ended ones, which can be explained by the fact that when the MTs are ended by a positively charged CHI, the interaction with negatively-charged particles is stronger and results in aggregation and rapid pore blockade.

S. epidermidis was then encapsulated in (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified MTs under optimized

conditions. A MT mat entrapping bacteria was obtained in biocompatible aqueous conditions by disaggregating the PVCL/PMAA compartment at pH 6.5. However, *S. epidermidis* in MT mats showed a low activity during culture. The (ALG/CHI)₆ALG MT mats maintained the viability of bacteria but cells showed negligible metabolic activity.

Bacterial encapsulation in MT mat provides a way to entrap *S. epidermidis* in gentle aqueous and biocompatible conditions. Different from previously introduced encapsulation methods, in which the production of encapsulating matrix and the bacterial encapsulation are often carried out simultaneously, bacterial entrapping in MTs is separated in two steps. Firstly the shell encapsulating the bacteria is fabricated, then bacteria are loaded. Therefore, the loading process is compatible with living cells. The MT mat, produced by release then collection of bacteria-loaded MTs, might also be used for encapsulation of other type of cells or molecules since the size, length and pore density of pores used are tunable depending on the requirements. By increasing the number of MTs used for collection, a thicker MT mat could be obtained, with a higher number of encapsulated cells. In addition, a multilayered MT mat could also be produced by gradually filtering different MTs.

However, bacteria release from MT is a critical issue during MT mat preparation. To increase the encapsulation efficiency, pore closing steps are introduced in this chapter, leading to a complex MT mat preparation process. Moreover, the measured activity of entrapped *S. epidermidis* was negligible. There might be several reasons for this, including the low bacterial encapsulation efficiency, the physical barrier due to the polyelectrolyte shell or the antibacterial effect of (ALG/CHI)₆ALG compartment. Considering the complexity of this encapsulation process, we did not continue in this way.

In contrast to the very weak metabolic activity observed for bacteria in MT mat, *S. epidermidis* in PC membranes had a high activity, with the fluorescence of supernatant reaching a maximum after 20 h culture, which is even higher than what is measured for 1% free bacteria in broth. This result suggests entrapping then keeping bacteria in the PC membranes, which is the second encapsulation method proposed in chapter 1. This approach is described and discussed in chapter 5.

Supporting information

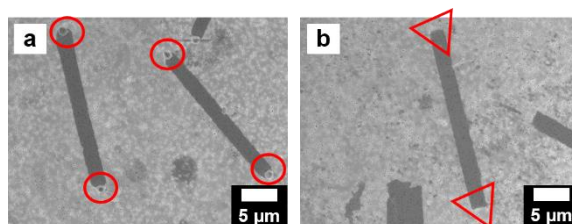


Figure S4.1. The morphology of $(\text{PVCL/PMAA})_5\text{PVCL}(\text{ALG/CHI})_6$ MTs observed by SEM. $(\text{PVCL/PMAA})_5\text{PVCL}(\text{ALG/CHI})_6$ was deposited in $1.6\ \mu\text{m}$ PC membrane, and MTs were released by dissolving the PC template in CH_2Cl_2 then collected on a PET membrane. (a) PC membrane was decrusted every second bilayer in $150\ \text{mM}$ NaCl on both surfaces. (b) PC membrane was decrusted in $3\ \text{M}$ NaCl on both surfaces when finishing LbL deposition.

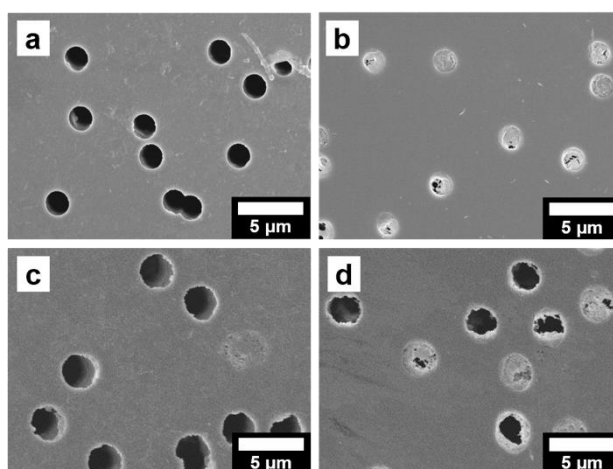


Figure S4.2. The morphology of $(\text{PVCL/PMAA})_5\text{PVCL}(\text{ALG/CHI})_6$ -modified PC membrane observed by SEM. Pore size of PC membrane: (a, b) $2\ \mu\text{m}$ and (c, d) $3\ \mu\text{m}$. (a, c) The top surface of the PC membrane was decrusted in $3\ \text{M}$ NaCl when finishing LbL deposition. (b, d) The bottom surface of PC membrane was decrusted in $150\ \text{mM}$ NaCl every second bilayer.

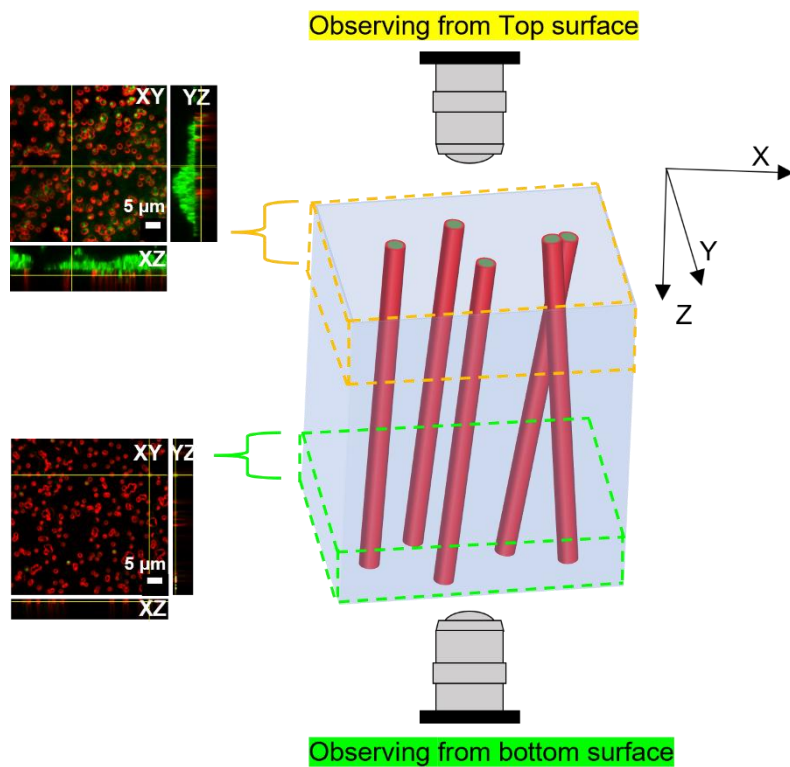


Figure S4.3. LbL MTs deposited in PC membrane were observed by CLSM from top (yellow) and bottom (green). Orthogonal XZ and YZ images only show a part of the length of LbL tubes.

Table S 4.1 Summary of the proportion of pores filled with microbeads by counting ~600 pores.

Description of the LbL-modified membrane	Dilution factor of bacterial suspension	Proportion of filled pores on top surface	Proportion of filled pores on bottom surface
1.6 μm PC membrane modified with (PVCL/PMAA) ₅ PVCL(ALG/CHI) ₆	10	53%	4%
2 μm PC membrane modified with (PVCL/PMAA) ₅ PVCL(ALG/CHI) ₆	10	46%	39%
3 μm PC membrane modified with (PVCL/PMAA) ₅ PVCL(ALG/CHI) ₆	10	38%	22%
1.6 μm PC membrane modified with (PVCL/PMAA) ₅ PVCL(ALG/CHI) ₆	Not diluted	32%	4%
1.6 μm PC membrane modified with (PVCL/PMAA) ₅ PVCL(ALG/CHI) ₆	50	76%	17%
1.6 μm PC membrane modified with (PVCL/PMAA) ₅ PVCL(ALG/CHI) ₆ ALG	50	73%	44%

*The initial concentration of bacteria suspension is 1.5×10^8 bacteria/mL.

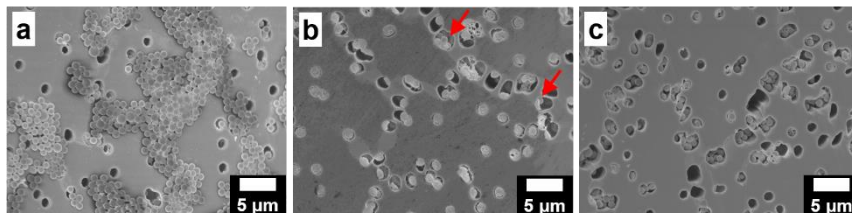


Figure S4.4. SEM images of 1.6 μm (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membrane encapsulating PEI/ALG-modified microbeads. (a) The top surface and (b) bottom surface of PC membrane after loading of PEI/ALG-microbeads. (c) The top surface of PC membrane after triple decrusting to remove microbeads. The red arrows show the microbeads inside the pores.

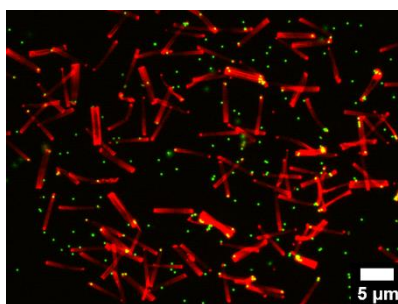


Figure S4.5. Epifluorescence microscopy image of a MT mat. (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membrane was loaded with microbeads (green). Then, the MTs were released from the PC membrane at pH 6.5 and collected, getting a MT mat.

Fresh *S. epidermidis* was prepared and the concentration of bacteria was determined by counting the number of *S. epidermidis*. A bacteria suspension was increasingly diluted by factors of 2, and the OD₅₄₀ of these bacteria suspensions was tested. A drop of each bacteria suspension (0.5 μL) was deposited on a glass slide and covered with a cover slip. Then, bacteria were counted by optical microscopy over three images. The average number of bacteria is noted as “ n ”, the size of the image is noted as “ S_0 ” and the area of bacteria solution on glass slide is noted as “ S ”. The concentration of bacteria “ c ” (bacteria/mL) can be calculated by formula S4.1, valid when $S \gg S_0$ as was the case here. The OD₅₄₀ of *S. epidermidis* vs bacteria concentration is shown in Figure S4.6. OD₅₄₀=0.042 corresponds to 1.5×10^8 bacteria/mL.

$$c = \frac{nS}{S_0(5 \times 10^{-4} \text{ mL})} \quad \text{Formula S4.1}$$

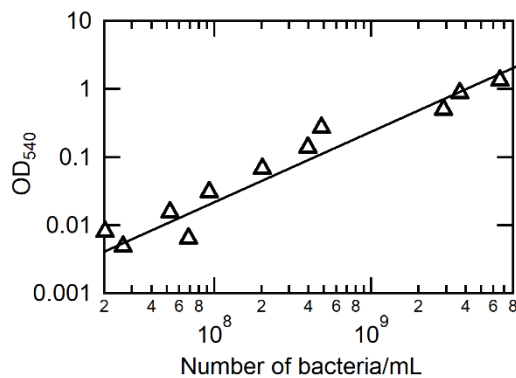


Figure S4.6. Optical density of *S. epidermidis* suspension at 540 nm (OD_{540}) vs bacteria concentration. An $OD_{540} \sim 0.042$ corresponds to 1.5×10^8 bacteria/mL.

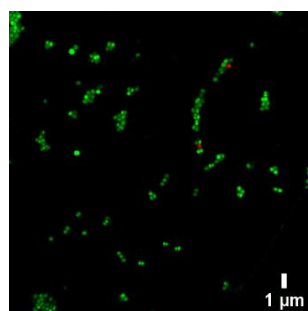


Figure S4.7. CLSM image of *S. epidermidis* stained with Live/Dead Kit for 20 min. The live and dead bacteria show green and red fluorescence, respectively.

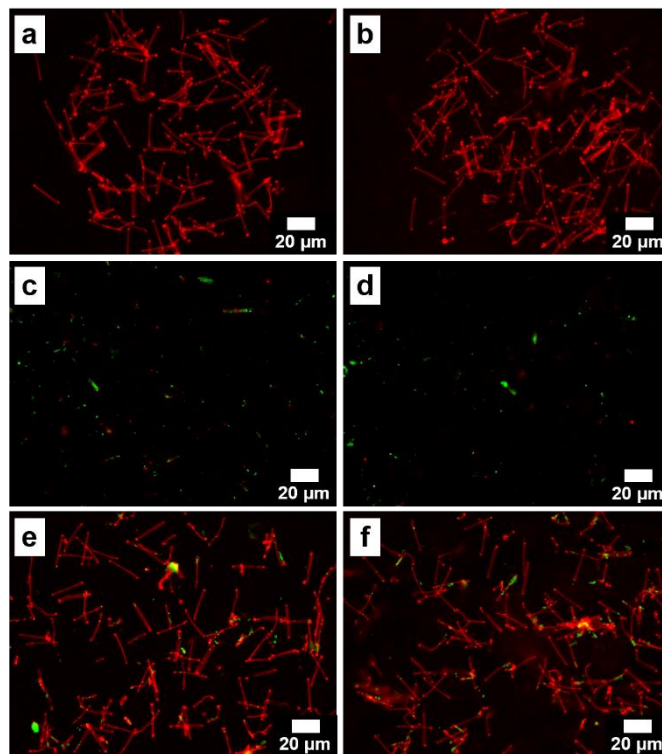


Figure S4.8. Epifluorescence microscopy images of MT mats. (a, b) The stability of MT mat in broth. (a) Freshly-prepared MT mat and (b) MT mat after 5 days incubation. (c, d) The viability of bacteria encapsulated in MT mat. *S. epidermidis* was encapsulated in a (PVCL/PMAA)₅PVCL(ALG/CHI)₆ALG-modified PC membrane and MT mats were prepared. (c) Freshly-prepared MT mat and (d) MT mat after 48 h incubation. Bacteria were stained with Live/Dead bacteria viability kit. (e, f) The growth of bacteria in MT mat. (e) Freshly-prepared MT mat and (f) MT mat after 72 h culture in broth. Bacteria in MT mat were stained with SYTO 9. All MT mats were incubated in 2 mL broth at 37 °C, 180 rpm.

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Chapter 5 Encapsulation of bacteria in membrane-in-gel patches

According to the results of chapter 4, *S. epidermidis* entrapped in MT mats cannot maintain a high bacterial activity. However, *S. epidermidis* entrapped in LbL-modified PC membranes maintained a high activity. Therefore, we propose a second method to encapsulate bacteria in a membrane-based patch.

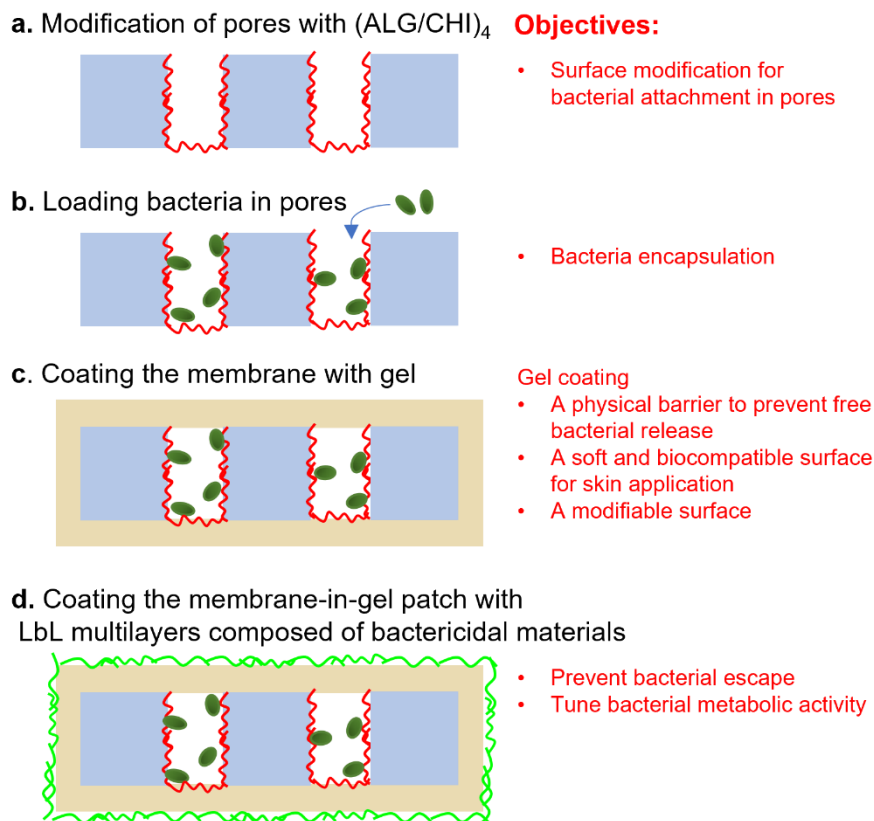
In this chapter, *S. epidermidis* is encapsulated in a membrane-in-gel patch (Scheme 1.4). For this, bacteria are loaded in LbL-modified pores of a PC membrane; then agarose gel is coated on PC membrane to prevent the free release of bacteria. Adding agarose gel coating also provides a soft surface for topical applications; to prevent the release of bacteria entrapped in the patch, LbL multilayers containing antibacterial materials are coated on the patch surface. The metabolic activity and the release of bacteria from the membrane-in-gel patch are studied in one broth, in refreshed broth, or on refreshed agar plates. Two bactericidal polycations PEI and CHI, having a high density of ammonium groups in their chains are chosen to prepare LbL multilayers for preventing the release of encapsulated bacteria. The impact of the two types of LbL multilayers, containing CHI or PEI, on the release of bacteria and their activity are tested. The effect of CHI or PEI-containing LbL multilayers on free bacteria growth is also studied to demonstrate their antibacterial activity.

Note: a part of this chapter is accepted for publication in *Advanced Materials Interfaces*.

5.1 Introduction

Skin together with its microbiota play an essential role in human health, being a critical interface between human body and the environment.^[1, 2] 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.^[3, 4] The environmental, interpersonal and temporal diversity of skin microbiota has been proved, a composition rich in the bacterial genera *Staphylococcus*, *Propionibacterium*, *Micrococcus* and *Corynebacterium*.^[1, 5] 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 acne considered to arise at least partly from an alteration of these bacterial communities.^[3, 6]

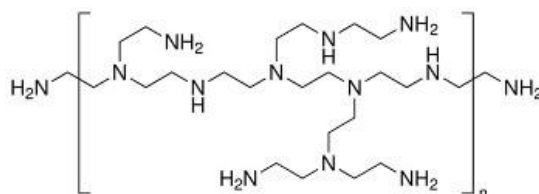
S. epidermidis, a permanent member of the human skin microbiota, has been extensively studied because of its prevalence on skin sites and its numerous benefits to hosts. On the one hand, *S. epidermidis* has been shown to promote the production of AMPs by keratinocytes,^[7] and induce T-cell development,^[8] resulting in the enhancement of the innate immune protection. The extent and duration of the immune response has been reported to be controlled by *S. epidermidis* which diminishes excessive inflammation after injury.^[9, 10] On the other hand, diverse molecules, such as phenol-soluble modulins,^[11] serine proteases,^[12] and nucleobase analogs,^[13, 14] can be produced by *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.^[15, 16] Therefore, instead of using antibiotics, which has the potential to cause bacterial resistance, bacteriotherapy has been advocated as a promising alternative to prevent skin colonization by pathogenic bacteria.^[17-19] The introduction of *S. epidermidis* on patients' skin has been shown to significantly reduce the colonization by *S. aureus* and group A *Streptococcus*.^[11, 12, 16, 20] The short-chain fatty acids produced by *S. epidermidis* fermentation have been demonstrated to control the growth of *Cutibacterium acnes*,^[15, 21] and of *P. acnes*,^[22-25] which are both important acne triggers. However, *S. epidermidis* is also claimed to "live at the edge between commensalism and pathogenicity";^[26] it is indeed a frequent cause of hospital-acquired infections when entering the bloodstream, notably due to its high ability to form biofilms on implanted devices. Thus it is also considered as an opportunistic pathogen.^[27-29] Therefore, it is critical to develop methods allowing us to reap the benefits of living *S. epidermidis* while keeping in check bacteria proliferation and migration for skin application.^[15, 30, 31]



Scheme 5.1. Schematic illustration of the process used to prepare a membrane-in-gel patch encapsulating bacteria and objectives of each step.

Encapsulation has emerged as an attractive method to incorporate bacteria into matrices that not only preserve the metabolic activity of bacteria,^[32-34] while preventing their proliferation but also offer a way for the modification of the surface of bacteria cells.^[35-37] For example, surface modification of *S. epidermidis* with biocompatible multilayers has been demonstrated to control bacteria growth depending on multilayer thickness.^[30] In contrast to the extensively studied probiotics encapsulation for food industry and gastrointestinal therapy,^[33, 38-43] the encapsulation of commensal skin bacteria was only considered recently due to a growing interest for bacteriotherapy. For instance, the thermo-responsive triblock polymer Pluronic F127, which hardens upon administration on skin, was selected to encapsulate *B. 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.^[44] *B. subtilis*-encapsulating wound healing agarose hydrogel was built by 3D printing, offering on-demand production of patches for

individual patients.^[45] Electrospinning was also tested for the encapsulation of bacteria in the fibers of patches, which can be directly applied on skin.^[19, 46-48] 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.^[31] 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 long periods of time, which is a prerequisite for efficient bacteriotherapy.



Scheme 5.2 Chemical structure of branched PEI

According to the results described in chapter 4, *S. epidermidis* entrapped in LbL-modified PC membranes maintain a high activity. Therefore, in this chapter, the second encapsulation method described in chapter 1 is presented: encapsulation of bacteria in a membrane-in-gel patch with the objective to get a bacteria-containing system, which is permeable to small molecules, maintains the metabolic benefits of bacteria, and is biocompatible for topical applications. Bacterial escape from the patch is also studied to prevent penetration of bacteria from the patch to the human body.

Scheme 5.1 shows the process to prepare the membrane-in-gel patch: (a) the inner surface of the pores of a track-etched PC membrane is modified with LbL assembled multilayers, providing an anchoring surface for *S. epidermidis*; (b) loading of bacteria in modified pores; (c) the PC membrane is then embedded in a thick layer of agarose gel, in order to prevent the free release of bacteria from PC membrane, leading to a membrane-in-gel patch, which can be applied on skin; (d) to prevent the release of viable bacteria, LbL multilayers composed of bactericidal components are coated on the membrane-in-gel patch. Among the antibacterial materials investigated, polycations bearing ammonium groups have emerged as potential antibacterial materials because of their sustained inhibitory effect and biocompatibility. Two polycations, branched PEI and CHI, as shown in Scheme 5.2 and Figure 2.6, respectively, have a high density of amine groups in their chains, especially PEI, with primary, secondary and tertiary amines.

Protonated CHI and PEI can interact and destroy bacterial membranes, resulting in antibacterial activity against a broad-spectrum of bacteria.^[49-52]

In this chapter, we study the impact of multilayer composition (films containing PEI or CHI), 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 play a key role in regulating bacterial release, which can be fully prevented with a proper design.

5.2 Experimental section

5.2.1 Materials

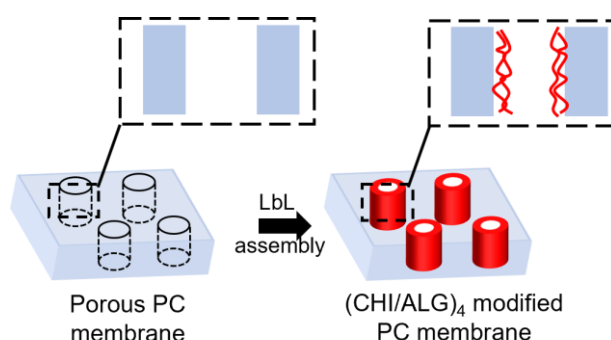
NaCl, polyethylenimine (PEI, M_w 750 kg/mol), fluorescent latex microbeads (carboxylate modified polystyrene, 1 μ m particle size), sodium alginate (ALG), rhodamine B, FITC-dextran (M_w 3-5 kg/mol, 10 kg/mol and 40 kg/mol) and agarose were purchased from Sigma-Aldrich. Poly(styrene sulfonate) (PSS, M_w 70 kg/mol) was obtained from Acros. Rhodamine-labeled poly(allylamine hydrochloride) (PAH-Rh, M_w 15 kg/mol, 1:392 mol:mol monomer ratio in dye) was purchased from Surflay Nanotec GmbH. Chitosan (CHI, M_w 50-150 kg/mol, deacetylation degree > 90%) was provided by NovaMatrix. Glycerol (bioreagent), Live/Dead™ bacteria viability kit and alamarBlue™ Cell Viability Reagent were purchased from 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 in 150 mM NaCl except for PAH-Rh (0.5 mg/mL). Track-etched PC membrane (10⁷ pores/cm², pore diameter 1.6 μ m, thickness 24 μ m) was provided by it4ip S.A., Belgium and was used as received. Milli-Q water with a resistivity of 18.2 M Ω .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 μ m filter. All containers were sterilized or autoclaved before use.

5.2.2 LbL modification of PC membranes

(CHI/ALG)₄ multilayers were deposited on the inner surface of the pores of PC membranes at pH 4.0 (Scheme 5.3). A 6×6 cm² 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.



Scheme 5.3. Modification of the pores of a PC membrane with LbL assembled (CHI/ALG)₄ multilayers.

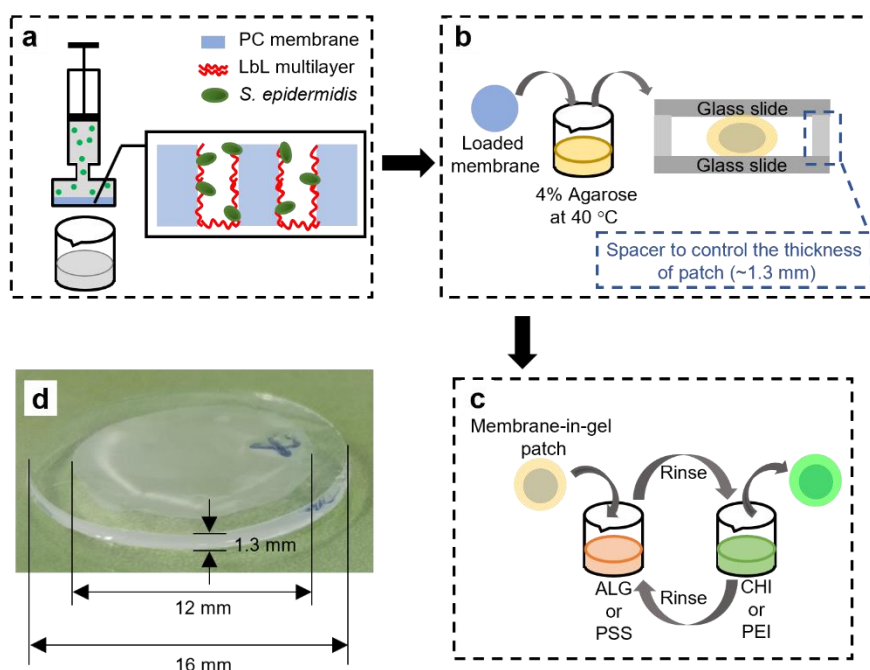
5.2.3 Revival of *S. epidermidis*

Freeze-dried *S. epidermidis* was revived overnight in 8 mL broth (8 g/L) 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 (OD₅₄₀) until it reached *ca.* 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 OD₅₄₀ reached *ca.* 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.

5.2.4 Fabrication of membrane-in-gel patches

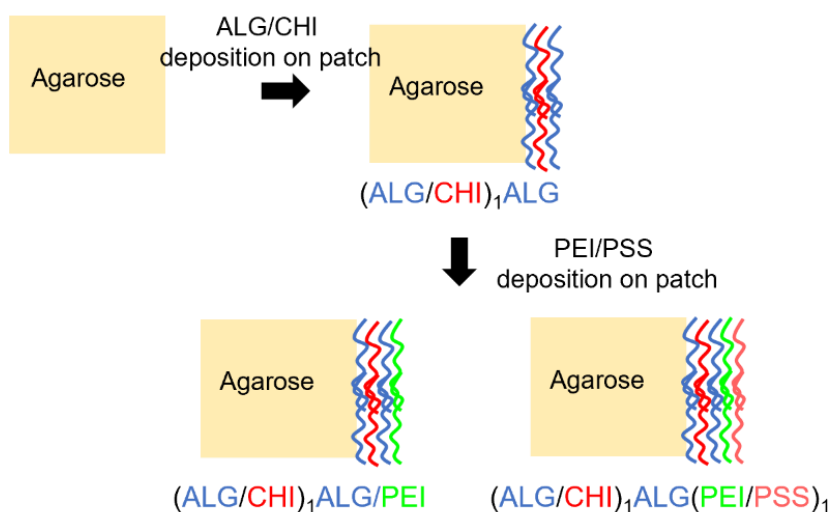
A *S. epidermidis* suspension of 0.042 OD₅₄₀ was prepared, corresponding to 1.5×10^8 bacteria/mL (Figure S4.6). 1 mL of this suspension was diluted by a factor of 50 with 150 mM NaCl (pH 6.0). A circular sample of (CHI/ALG)₄-modified membrane with a diameter of 12 mm was placed in a Swinney filter unit. Then, 10 mL of the diluted *S. epidermidis* suspension was filtered through the PC membrane for 2 min (Scheme 5.4a). 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.



Scheme 5.4. (a-c) Fabrication of a membrane-in-gel patch: (a) encapsulation by filtration of *S. epidermidis* in the (CHI/ALG)₄-modified membrane; (b) 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. (c) Surface modification of the membrane-in-gel patch with (ALG/CHI) or (PSS/PEI) multilayers at pH 6.0. (d) A digital image of the membrane-in-gel patch.

Chapter 5

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 *ca.* 1.3 mm to obtain a well-defined membrane-in-gel patch as shown in Scheme 5.4b. 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 5.4b and 5.4c show the process to coat the membrane with agarose gel and the subsequent surface modification of the patch with LbL multilayers. Scheme 5.5 illustrates the patch after ALG/CHI or PEI/PSS multilayer deposition. A patch denoted as (ALG/CHI)₁ALG/PEI indicates that the last layer of the coating is a PEI layer. By contrast, (ALG/CHI)₁ALG(PEI/PSS)₁ indicates a coating ended with a PSS layer.



Scheme 5.5. Description of the LbL deposition of antibacterial coatings on agarose gel. Deposition of (ALG/CHI)₁ALG/PEI and (ALG/CHI)₁ALG(PEI/PSS)₁ on agarose is described as an example. By continuing the LbL process, more polyelectrolyte layers can be deposited, getting patches coated with (ALG/CHI)₁ALG(PEI/PSS)_n or (ALG/CHI)₁ALG(PEI/PSS)_nPEI.

5.2.5 Culture of membrane-in-gel patches

Culture of membrane-in-gel patch in broth. 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 in the safety cabinet to maintain a constant volume of broth. In case of water evaporation, 10 µL water was added into the broth every 4 h culture.

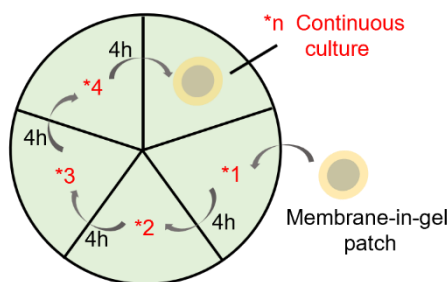
A single plate serial dilution spotting technique was used to determine the number of viable bacteria escaped from the membrane-in-gel patch. For each time point (4 h), the supernatant was taken from the well plate then serially diluted by a NaCl solution (150 mM, pH 6.0). Thus, six suspensions with 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.

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

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™.

Culture of membrane-in-gel patch on an agar plate. An agar plate is divided into several sectors that a membrane-in-gel patch was put on sector *1 and

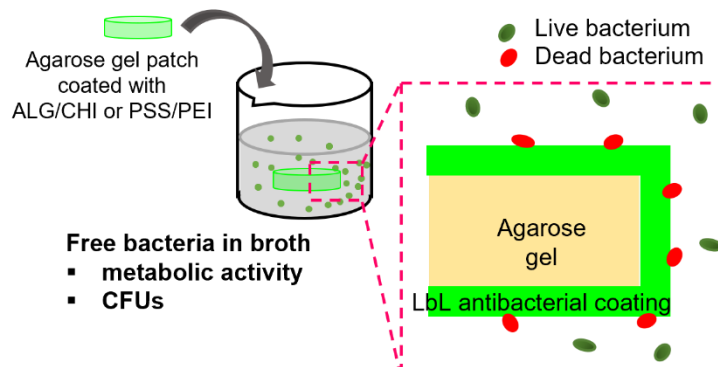
incubated at 37 °C (Scheme 5.6). After every 4 h culture, the membrane-in-gel patch was transferred to a new sector. Eventually, the membrane-in-gel patch was left on sector *5 and was continuous cultured.



Scheme 5.6. Schematic illustration of culture of a membrane-in-gel patch on an agar plate. Membrane-in-gel patch was put on sector *1 to start the culture. After every 4 h culture, membrane-in-gel patch was transferred to a new sector. Eventually, the membrane-in-gel patch was leaved on sector *5 and continuously cultured at 37 °C.

5.2.6 Effect of patches coated with polyelectrolyte multilayers on free suspended bacteria

As shown in Scheme 5.7, due to the contact-killing effect of LbL multilayers, bacteria suspended in broth can be killed upon attaching to the antibacterial coating, causing CFUs decrease and reduced metabolic activity of bacterial community. Therefore, 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 PSS/PEI multilayers were deposited on these patches, following the previously-described protocol. These patches were placed in 2 mL broth containing 10 μ L of bacterial suspension ($OD_{540} = 0.042$) and 200 μ L 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 μ L water was added into the broth every 4 h culture.



Scheme 5.7. Test of metabolic activity and CFUs of free bacteria in broth in the presence of an agarose gel patch coated with an antibacterial coating.

5.2.7 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. 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 turbidities were 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.

5.2.8 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-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.

5.3 Results and discussion

5.3.1 Encapsulation of bacteria in membrane-in-gel patches

The LbL assembly technique, which is one of the most studied approaches for creating functional surfaces, allows to cover a wide range of substrates of variable shape (planar or not), size (nano to millimeters), and chemical composition.^[53-55] A microchamber array made up of LbL-assembled multilayers showing a similar structure as pores in PC membrane was demonstrated for loading cargos and programmed release of cargo from individual chambers in a site-specific manner.^[56] Therefore, to anchor bacteria in the pores of PC membrane, the inner surface of the pores was first modified with LbL-assembled multilayers based on ALG and CHI (Scheme 5.3). Subsequently, bacteria were filtered through these surface modified pores to trap them in the membrane (Scheme 5.4a). 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. As shown in chapter 4, microbeads are used as model particles to screen and optimize trapping conditions. A PC membrane of 1.6 μm pore size and 10^7 pores/ cm^2 (Figure S5.1b) showed the best trapping efficiency. It was demonstrated that multilayers ended with a polyanionic ALG layer were conducive to an increased and more homogeneous microbead trapping in the pores; this counter-intuitive result can be explained by the fact that, when the pore coating is ended by a positively-charged polyelectrolyte, the interaction with negatively-charged particles is strong and results in aggregation and rapid pore blockade. A suspension concentration of 3×10^6 microbeads/mL was found to improve encapsulation efficiency, higher concentrations leading to pore blockade, whereas lower ones were too dilute for efficient trapping.

Four bilayers of CHI/ALG were finally 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,^[57] the reduction of pore size due to the surface modification is negligible. 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 is 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 S5.1c), which is favorable for bacteria loading while limiting adsorption of the bacteria on the top surface. The bottom surface of membrane is 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 S5.1d), which may improve the encapsulation efficiency.

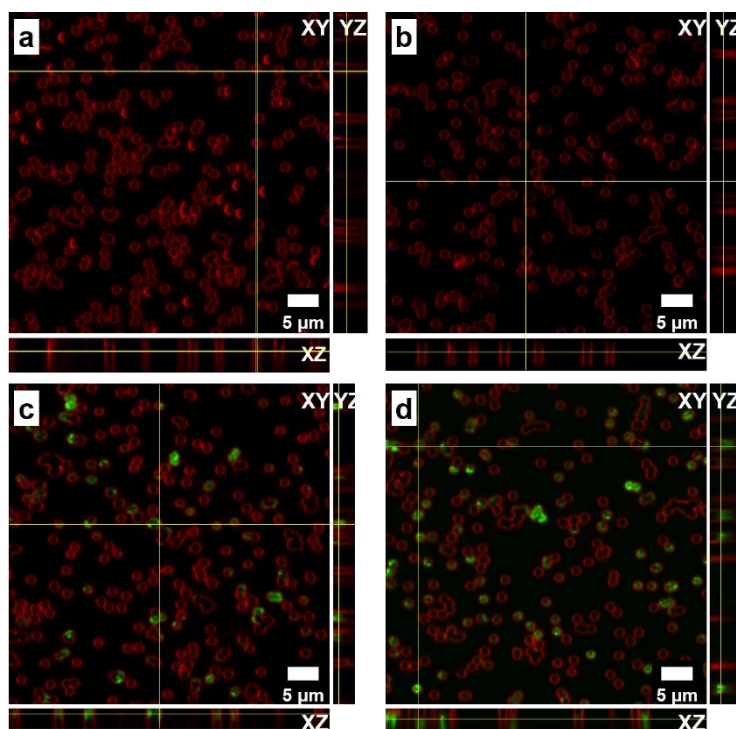


Figure 5.1. Orthogonal images of (a, b) a fluorescently-tagged (red) (CHI/ALG)₂(PAH-Rh/ALG)₂-modified membrane and (c, d) a membrane trapping SYTO 9-stained (green) *S. epidermidis*. The membranes were observed by CLSM with Z-stack scanning from (a, c) the top surface and (b, d) the bottom surface. In (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. Orthogonal XZ and YZ images share the same scale bar as XY image; XZ and YZ images only show a part of the length of LbL MTs, as illustrated in Figure S4.3.

To visualize the modification of the pores, PAH 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 CLSM. As shown in Figure 5.1a and 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 5.1c and 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.

Agarose gel was cast around the bacteria-containing membrane to create a flexible and permeable layer, leading to a membrane-in-gel patch (Scheme 5.4d). The agarose gel acts as a physical barrier to prevent bacteria to freely disperse, which could easily happen since the membrane pores are 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.^[58] Here, we simply checked that migration of fluorescent microbeads does not occur in the agarose layer (Figure S5.2), which should be the same for our flagella-lacking bacteria.

Bulk hydrogels have been widely applied for cell encapsulation because they allow an easy exchange of nutrients and water with the environment.^[59, 60] The diffusion of small and large molecules was tested to evaluate their selective penetration into the patch (Scheme S5.1). Rhodamine B was used as a model molecule to test the outward diffusion of small molecules such as nutrients through the agarose gel. As shown in Scheme S5.1b, 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 S5.3, 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 (Scheme S5.1c). The diffusion of FITC-dextran in the gel layer was determined with CLSM (Figure S5.4 and S5.5). After a dipping time of 2 h, the 3-5 kg/mol 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* ATCC 12228 strains such as bacteriocins have a molar mass lower than 5 kDa and are heat-stable class I antibiotics,^[61, 62] our membrane-in-gel patch should thus be permeable to them. By contrast, the diffusion thickness of 10 kg/mol and 40 kg/mol FITC-dextran macromolecules was small. After a dipping time of 4 h, patches dipped in 3-5 kg/mol and 10 kg/mol FITC-dextran showed a similar profile. However, no change was observed for the patch dipped in 40 kg/mol FITC-dextran, demonstrating that the diffusion of molecules through the patch depends on their size.^[63]

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. AlamarBlueTM 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 alamarBlueTM 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 5.2a). 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.

As observed on CLSM images (Figure 5.1), most bacteria disperse at the top or bottom sides of the pore. Therefore, they may colonize the gel. After growing into the gel, they can reach the gel/medium interface then be released in the surrounding broth. To check this hypothesis, the release of bacteria from the membrane-in-gel patch was measured using a single plate serial dilution spotting technique.^[64] As shown in Figure 5.2b, 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.

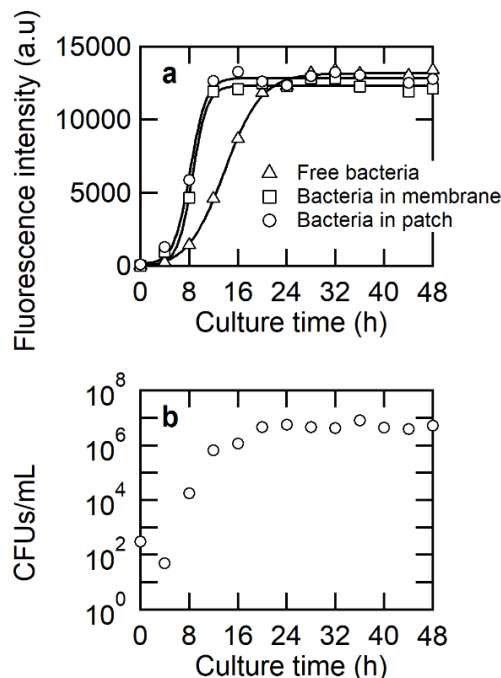


Figure 5.2. (a) Integrated metabolic activity of free bacteria, bacteria trapped in a (CHI/ALG)₄-modified membrane and bacteria encapsulated in a membrane-in-gel patch. The samples were placed in 2 mL broth and 200 μ L alamarBlue™, then cultured at 37 °C in the incubator. As a positive control, free bacteria were cultured in identical conditions by adding *ca.* 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) were calculated and reported vs the culture time of the patch.

5.3.2 Control of bacterial escape with LbL coatings

Therefore, the patch was coated by LbL multilayers comprising bactericidal components (Scheme 5.4c). LbL assembly has been used early on to prepare antimicrobial coatings,^[65-69] for applications in biomedicine, healthcare, and marine engineering.^[52, 70] Antimicrobial LbL coatings generally act via three main mechanisms: adhesion-resistance,^[71] release-killing,^[72, 73] and contact-

killing.^[74-76] These mechanisms can work synergistically to protect a surface against infections.^[51, 77] In antibacterial contact-killing LbL coatings, polycations such as PEI and CHI are often used since they can kill bacteria by disrupting cell membranes.^[51, 52] Here, ALG/CHI and PSS/PEI multilayers were explored as a supplementary way to control the release of bacteria from the membrane-in-gel patches.

The possibility to deposit LbL multilayers on the surface of an agarose gel patch was first investigated by dipping a 4% agarose gel in a PAH-Rh solution, followed by an observation of the cross-section of the gel. A fluorescent layer was observed on the surface of the gel (Figure S5.6); the average diffusion thickness of a single layer of PAH-Rh (Table S5.1) is *ca.* 20 μm over 2 min of adsorption, which is much smaller than the thickness of membrane-in-gel patch (*ca.* 1.3 mm) (Scheme 5.4d). Similar observations were made when a complete (PAH-Rh/ALG)₅ multilayer was deposited onto the agarose gel (Figure S5.7). These results demonstrate that LbL multilayers can be successfully deposited on an agarose gel, with a penetration limited to the external skin of the gel.

CHI is a frequently used antibacterial polysaccharide, which can be assembled with ALG. The antibacterial effects of ALG/CHI multilayers have been studied before, showing a higher bacterial inhibition when increasing the number of bilayers.^[78, 79] Therefore, (ALG/CHI)₅ALG multilayers were deposited on the membrane-in-gel patch, and the resulting patches were cultured in broth implemented with alamarBlueTM. First, we tried to culture the patch in refreshed broth (Scheme S5.2, Figure S5.8). However, this method could not quantitatively show the release of bacteria from patch and is complicated on operation. Therefore, membrane-in-gel patch was cultured in one broth and the escape of encapsulated bacteria from the LbL coated membrane-in-gel patch was tested by the single plate serial dilution spotting technique.

As shown in Figure 5.3b, no bacterial colony was observed in the supernatant for the first 4 h of culture, showing that the (ALG/CHI)₅ALG 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 5.3a), showing that the encapsulated bacteria are metabolically-active. After 4 h of culture, living bacteria start to escape and grow in the broth (Figure 5.3b), which explains the strong increase of integrated metabolic activity seen in Figure 5.3a.

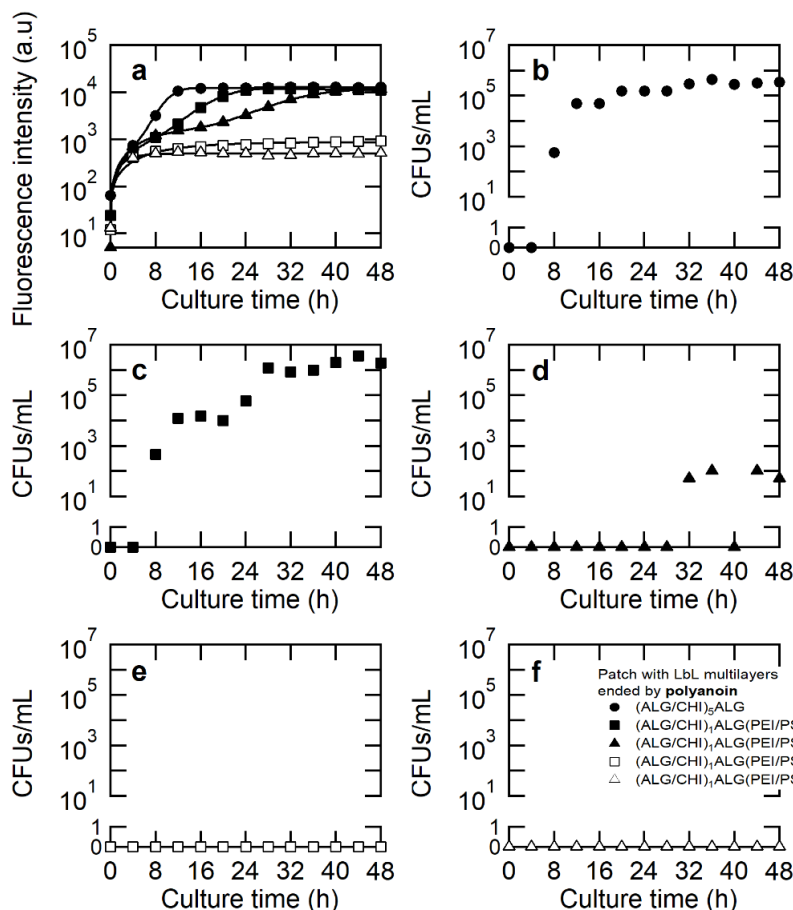


Figure 5.3. (a) Integrated metabolic activity of *S. epidermidis* encapsulated in membrane-in-gel patches with different LbL coatings vs culture time. Bacteria were loaded in a (CHI/ALG)₄-modified PC membrane followed by agarose gel coating. Then, LbL multilayers incorporating antibacterial polycations 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 μ L 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 was calculated.

Compared to the porous agarose gel, LbL multilayers have a less open and permeable structure,^[80, 81] and therefore may play the role of slight physical barrier to delay bacteria release. The bactericidal effect of (ALG/CHI)₅ALG cannot be ruled out either at this stage. However, with culture time increasing, bacteria seem quickly proliferate in the membrane-in-gel patch and the (ALG/CHI)₅ALG 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 was measured to be 2 µg/mL, much lower than the MIC of CHI which was found to be 8 µg/mL (results not shown). To prevent killing the bacteria in the patch, (ALG/CHI)₁ALG multilayers were first deposited on the patch, acting as a primer film for the 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)₁ALG(PEI/PSS)_n-coated membrane-in-gel patch, with *n* varying from 1 to 4, are shown in Figure 5.3c-f. For the membrane-in-gel patch coated with (ALG/CHI)₁ALG(PEI/PSS)₁, no bacterial colony was observed in the broth at 0 and 4 h culture times (Figure 5.3c). 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)₁ALG(PEI/PSS)₂ until 28 h culture (Figure 5.3d), demonstrating that the presence of (ALG/CHI)₁ALG(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 remained below 10². For the membrane-in-gel patch coated with (ALG/CHI)₁ALG(PEI/PSS)₃ or 4, no bacteria colony was observed during culture (Figure 5.3e and 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)₁ALG(PEI/PSS)₁₋₄ are shown in Figure 5.3a. These curves are best explained by comparing them with the data on bacterial escape and growth (Figure 5.3c-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 escaped from the patch, and the fluorescence intensity rapidly increased

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 increased for times at which bacteria did not grow in the broth (first 28 h). It then increased 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.

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.^[82] Therefore, membrane-in-gel patches coated with multilayers ended with a PEI layer, namely, $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_n\text{PEI}$ were also prepared to study the metabolic activity of encapsulated bacteria as well as their release (Figure 5.4).

No release and growth of bacteria in the broth were detected for patches coated with $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_{1-3}\text{PEI}$ (Figure 5.4c-e). The fluorescence intensity measurements performed in presence of these patches clearly indicate a behavior similar to what was observed for patches coated with $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_3$ 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 $(\text{PEI/PSS})\text{PEI}$, $(\text{PEI/PSS})_2\text{PEI}$ and $(\text{PEI/PSS})_3\text{PEI}$, respectively). However, for the membrane-in-gel patch coated with $(\text{ALG/CHI})_1\text{ALG/PEI}$ (Figure 5.4b), 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 $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_2$ (Figure 5.3).

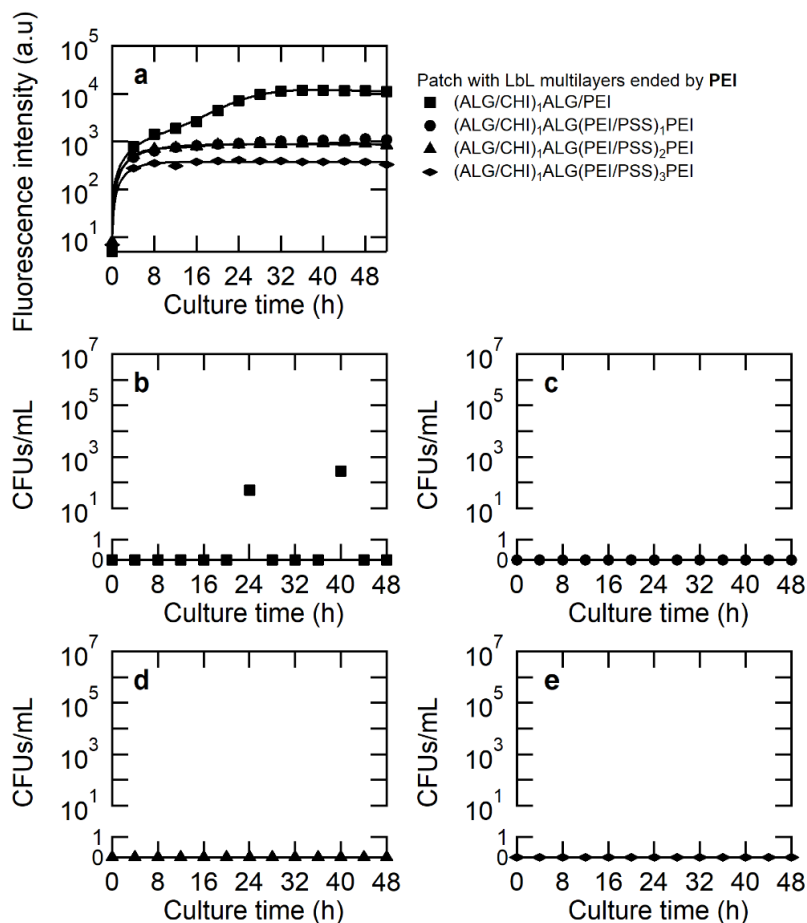


Figure 5.4. (a) Integrated metabolic activity of *S. epidermidis* encapsulated in membrane-in-gel patches coated with (ALG/CHI)₁ALG(PEI/PSS)_nPEI ($n = 0-3$). Bacteria were encapsulated in a (CHI/ALG)₄-modified membrane, followed by agarose gel coating. The LbL multilayers were then deposited on the membrane-in-gel patch, which was cultured at 37 °C in 2 mL broth with 200 μ L 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)₁ALG(PEI/PSS)_nPEI 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 was calculated.

The comparison between patches coated either by $(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_n\text{PEI}$ or $(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_{n+1}$ 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.

Other polycations have also been tested to modify membrane-in-gel patches. PLL, a common polypeptide whose cationic nature imparts an intrinsic antibacterial functionality,^[83] has the same MIC as CHI. $(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PLL}/\text{HA})_4$ had a similar effect on bacterial activity and release as $(\text{ALG}/\text{CHI})_4\text{ALG}$ multilayers. Increasing PLL/HA bilayer number to 20 demonstrated to delay bacteria release for 4 h. However, this greatly increased the surface modification time. Antibacterial properties of lysozyme^[84] was also tested against *S. epidermidis*, showing no antibacterial effect below a concentration of 128 $\mu\text{g}/\text{mL}$.

5.3.3 Bacterial escape on agar plates

Table 5.1. Bacterial release from patches tested on agar plates. An agar plate was divided into several sectors (*n) that membrane-in-gel patch was put on sector *1 to start culture. Then the patch was transferred to a new sector every 4 h, until the continuous culture on sector *5. The bacterial colonies on agar plates were counted after 48 h culture at 37 °C.

Membrane-in-gel patches coated with LbL multilayers	Sector				
	*1	*2	*3	*4	*5
$(\text{ALG}/\text{CHI})_5\text{ALG}$	50	>100	>100	>100	Yes
$(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_1$	27	>100	>100	>100	Yes
$(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_2$	2	4	19	>100	Yes
$(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_3$	0	0	0	>100	Yes
$(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_4$	0	0	0	0	No
$(\text{ALG}/\text{CHI})_1\text{ALG}(\text{PEI}/\text{PSS})_5$	0	0	0	0	No

Since we have in mind the use of membrane-in-gel patch on skin, the release of bacteria was also tested on agar plates. Membrane-in-gel patch was transferred to a new sector on agar plate every 4 h during culture until continuous culture on sector *5 (Scheme 5.6). The bacterial colonies on sector *1 to *4 were counted. As shown in Table 5.1, bacterial colonies were observed from patch coated with $(\text{ALG}/\text{CHI})_5\text{ALG}$ and

(ALG/CHI)₁ALG(PEI/PSS)_{1, 2, and 3}. This demonstrates the release of viable bacteria from patches. The number of bacterial colonies increased from sector *1 to sector *4, showing that bacteria release increases with culture time. By contrast, no bacterial colony is growing on sector *1 to *4 for patch coated with (ALG/CHI)₁ALG(PEI/PSS)_{4 and 5}, illustrating no viable bacteria release. This result corresponds to what we obtained in section 5.3.2. Comparing patch coated with antibacterial materials, PEI/PSS is more efficient than CHI/ALG on controlling bacteria release. Bacterial growth is maintained in the membrane-in-gel patch and bacterial release is tunable by the thickness of PEI/PSS bilayer.

5.3.4 The impact of the LbL coating on free bacteria

When bacteria are released from a membrane-in-gel patch cultured in broth, 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 (Scheme 5.7). 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 5.5) and their CFUs (Figure S5.9) 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)₅ALG and (ALG/CHI)₁ALG(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 S5.9a-c and e). However, when patches were coated with either (ALG/CHI)₁ALG/PEI or (ALG/CHI)₁ALG(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 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.

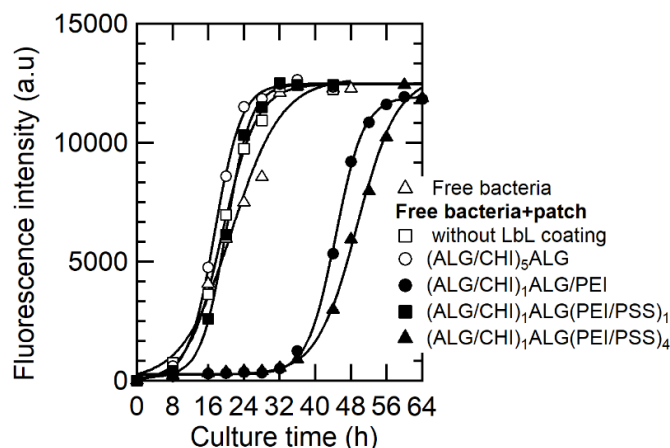


Figure 5.5. Integrated metabolic activity of free bacteria cultured in the broth in the presence of patches. Membrane-in-gel patches coated with/without LbL multilayers were added in free bacterial suspensions, with added alamarBlue™. The fluorescence intensity of the supernatant at 585 nm (excitation wavelength 470 nm) was recorded vs the culture time; lines are drawn to guide the eye.

5.3.5 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 5.3a), or patches coated with $(\text{PEI/PSS})_n\text{PEI}$ ($n>0$) (Figure 5.4a), 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 $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_4$ 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 5.6a 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 by breaking the gel using a tweezer 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 5.6b: 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.

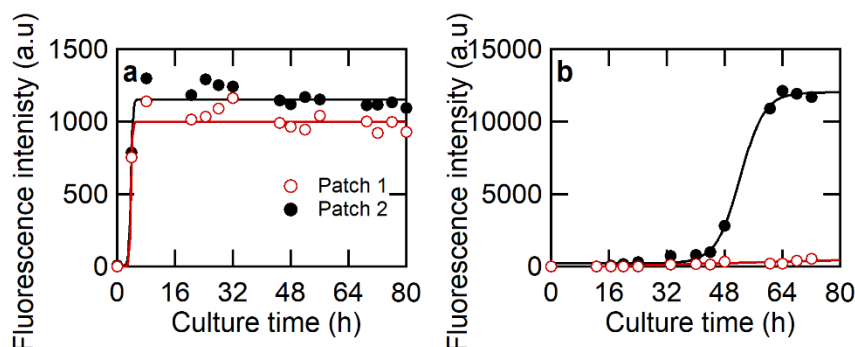


Figure 5.6. The state of bacteria in membrane-in-gel patches coated with $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_4$. Two membrane-in-gel patches were prepared and coated with $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_4$, named patch 1 and patch 2. The patches were placed in a broth with alamarBlue™ 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.

5.3.6 Discussion

The previous results demonstrate the possibility to encapsulate bacteria in a membrane-based patch surrounded by a shell made of LbL multilayers. The bacteria exhibit metabolic activity which, depending on the number of bilayers and nature of the outer layer, either stops after some time, or increases exponentially most of the time due to bacteria escaped from the patch and growing in the broth. However, for two intermediate samples $(\text{ALG/CHI})_1\text{ALG/PEI}$ and $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_2$, an exponential increase of metabolic activity occurs before viable CFUs are detected in the supernatant. By including PEI in the LbL multilayer, the coating exhibits bactericidal activity, which therefore contact-kills escaping bacteria, or kills possibly alive escaped bacteria. 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.

PEI/PSS multilayers are thus an efficient way to not only tune the metabolic activity of encapsulated cells, but also modulate bacterial escape. Comparing all samples with a topical application in mind, the patches coated with $(\text{ALG/CHI})_1\text{ALG}(\text{PEI/PSS})_2$ show the most interesting prospects for the

treatment of skin dysbiosis. *S. epidermidis* has 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.

5.4 Conclusions

A bacteria-containing membrane-in-gel patch, built by embedding bacteria in the modified pores of a track-etched PC membrane, followed by embedding the PC 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.

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. 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.

Supporting information

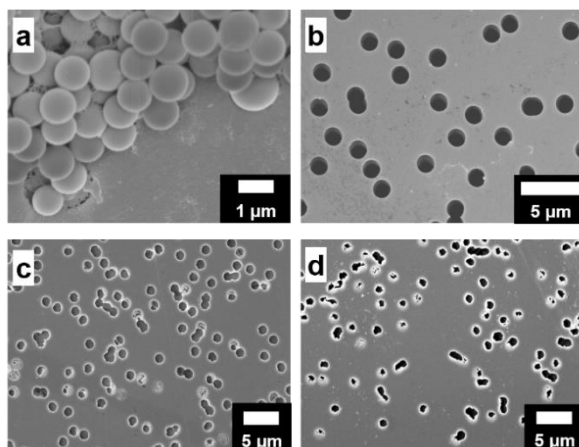


Figure S5.1. SEM images of (a) microbeads and (b-d) 1.6 μm PC membrane: (b) blank PC membrane; (c) the top surface and (d) the bottom surface of (ALG/CHI)₄-modified PC membrane. The membrane was coated with a 20 nm-thick layer of gold in a Cressington 108 carbon/A sputter, and was observed on a JSM-7600F, JEOL Ltd scanning electron microscope at 5 kV.

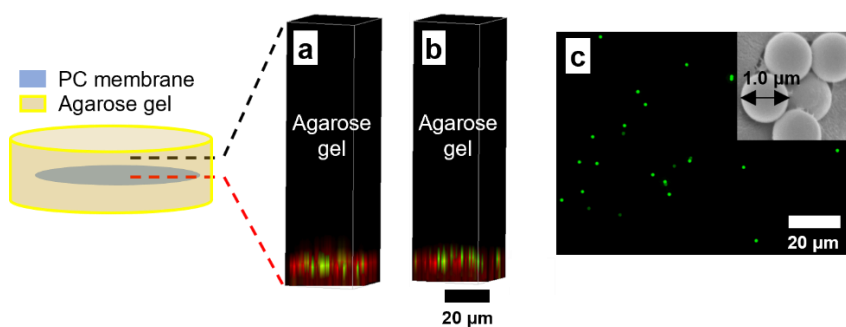
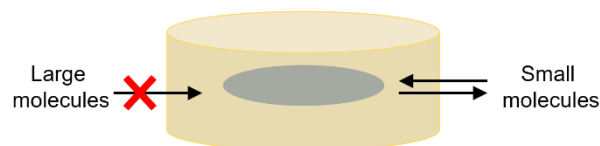
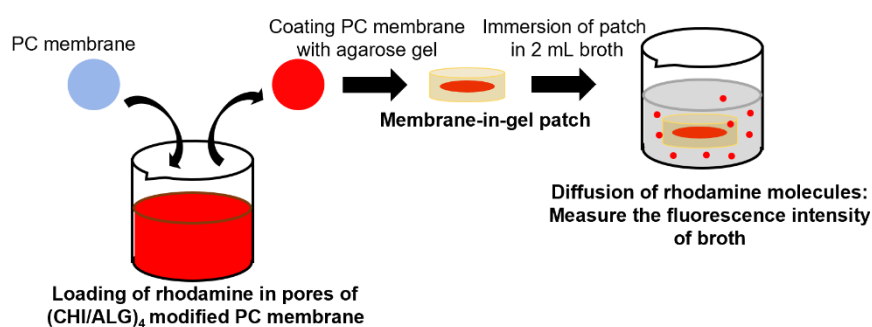


Figure S5.2. 3D-reconstructed CLSM images of membrane-in-gel patches loaded with fluorescent microbeads (a) before and (b) after 12 h immersion in a 150 mM NaCl solution. Green fluorescent microbeads were loaded in 1.6 μm (CHI/ALG)₂(PAH-Rh/ALG)₂-modified membrane (red fluorescence); the membrane was then coated with agarose to prepare a membrane-in-gel patch, which was immersed in 150 mM NaCl aqueous solution for 12 h and observed by CLSM with Z-stack scanning. (c) Epifluorescence microscopy image (inset SEM image) of microbeads used for loading.

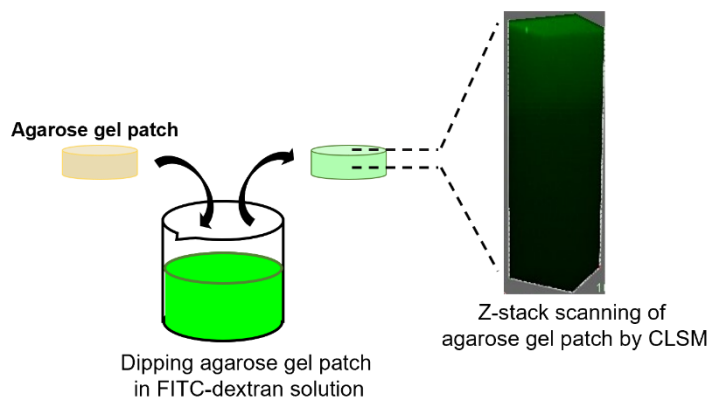
a. Diffusion of molecules in patch



b. Outward diffusion of rhodamine B in the membrane-in-gel patch



c. Inward diffusion of FITC-dextran in agarose gel patches



Scheme S5.1. Scheme illustrating (a) the diffusion test to check the selective penetration of a membrane-in-gel patch; (b) outward diffusion of rhodamine B from a membrane-in-gel patch; (c) inward diffusion of FITC-dextran in a agarose gel patch.

Diffusion of rhodamine B loaded in the membrane-in-gel patch.

As shown in Scheme S5.1b, a circular shaped (CHI/ALG)₄-modified membrane with a diameter of 12 mm was dipped in a rhodamine B solution (0.1 mg/mL) for loading. After 12 h, the membrane was taken out from the rhodamine B solution and the extra solution on the top and bottom surfaces of the membrane was swept away. Then, the membrane was coated with agarose gel, leading to a membrane-in-gel patch with a diameter of 16 mm. The membrane-in-gel patch was immersed in 2 mL broth and was cultured at 37 °C, 120 rpm. The variation of the fluorescence intensity of the supernatant vs culture time is shown in Figure S5.3.

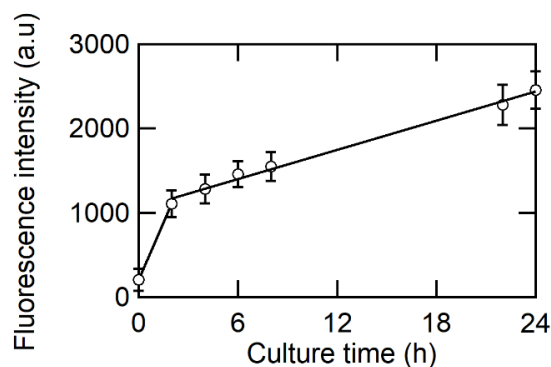


Figure S5.3. Outward diffusion of rhodamine B from a membrane-in-gel patch. The fluorescence intensity of the broth was measured at an excitation wavelength of 546 nm (bandwidth 9 nm) and an emission wavelength of 588 nm (bandwidth 20 nm). Pure broth was incubated in same conditions and used as a negative control. Each point is an average of three samples.

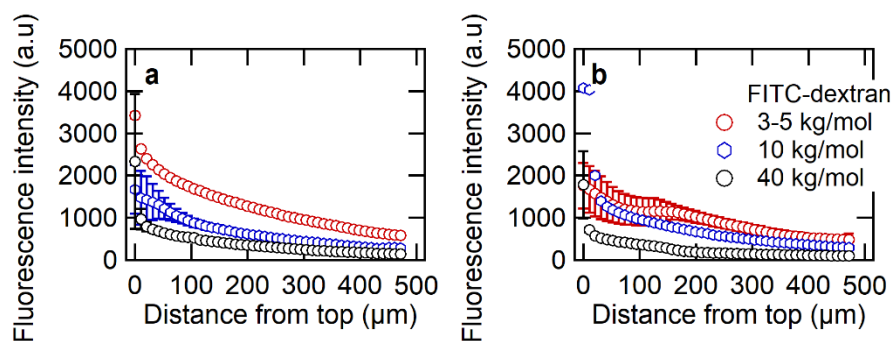
Diffusion of FITC-dextran to patch.

Figure S5.4. Inward diffusion of FITC-dextran in agarose gel patches. An agarose gel patch with the same diameter (16 mm) and thickness (1.3 mm) as the membrane-in-gel patches was prepared, without PC membrane inside. After dipping in 0.5 mg/mL aqueous solutions of FITC-dextran of different molar masses (3-5 kg/mol, 10 kg/mol and 40 kg/mol) for (a) 2 h and (b) 4 h, the agarose gel patch was observed from its top surface by CLSM to a depth of 480 μm with Z-stack scanning. The variation of the fluorescence intensity of FITC-dextran vs distance from the top surface of the patch is displayed in the graphs.

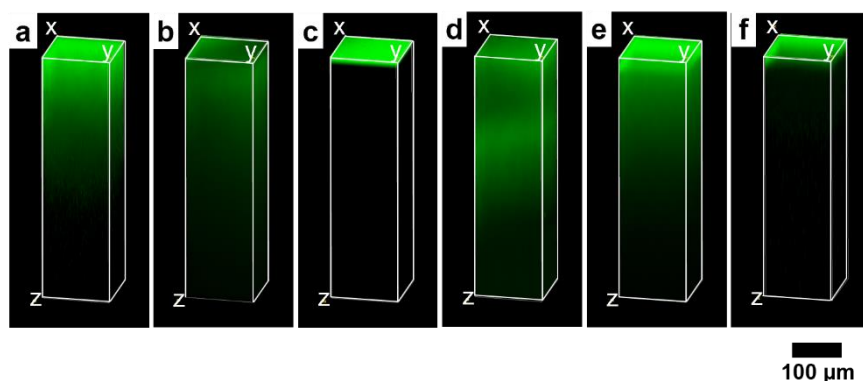


Figure S5.5. 3D-reconstructed CLSM images of agarose gel patches dipped in FITC-dextran solutions. An agarose gel patch of same thickness (1.3 mm) and diameter (16 mm) as the complete patches were dipped in 0.5 mg/mL aqueous solutions of FITC-dextran of different molar masses: (a, d) 3-5 kg/mol, (b, e) 10 kg/mol, and (c, f) 40 kg/mol. Two dipping times (a-c) 2 h and (d-f) 4 h were tested. Agarose gel patches were observed from the top surface by CLSM down to a depth of 480 μm with Z-stack scanning. The top surface of

the 3D-reconstructed images corresponds the top surface of the agarose gel patches.

Diffusion of PAH-Rh in agarose gel patch.

An agarose gel patch was prepared without the support of a PC membrane. The viscous agarose solution (40°C) was transferred between two glass slides to fabricate an agarose gel patch with the same thickness and diameter as membrane-in-gel patches (thickness 1.3 mm, diameter 16 mm). Then, the agarose gel patch was dipped in a PAH-Rh aqueous solution (0.5 mg/mL) to deposit a polycation layer on the gel surface; four dipping times (30 s, 60 s, 90 s and 120 s) were tested. After deposition, the agarose gel patch was dipped in liquid nitrogen to make a cross-sectional cut which was observed by epifluorescence microscopy (Figure S5.6) in order to determine the diffusion length of the polycation. Table S5.1 summarizes the average diffusion thickness of a single PAH-Rh layer in the agarose gel for different dipping times. The diffusion of PSS (127 kg/mol) in polyelectrolyte gels showed a coefficient of $4.1 \pm 0.1 \times 10^{-7} \text{ cm}^2/\text{s}$ with 100 mM NaCl.^[85] Diffusion coefficients of macromolecules in agarose gel showed a range of 10^{-6} to $10^{-7} \text{ cm}^2/\text{s}$, depending on the molecule size.^[63] The diffusion coefficient of PAH-Rh (15 kg/mol) can be estimated from our results for 30 s diffusion time, providing a rough range of $(1.3 \pm 0.1) \times 10^{-6} \text{ cm}^2/\text{s}$, within the same order of magnitude as previous research.

An agarose gel patch coated with (PAH-Rh/ALG)₅ multilayers was also prepared by alternately dipping the patch in PAH-Rh and ALG solution for 60 s. The cross-section of the agarose gel patch was observed with CLSM (Figure S5.7), demonstrating the deposition of LbL multilayers on the external skin of the gel.

Table S5.1. Average diffusion thickness of a PAH-Rh single layer in agarose gel.

Dipping time (s)	30	60	90	120
Average diffusion length (μm)	21±2	19± 3	22±9	26±9

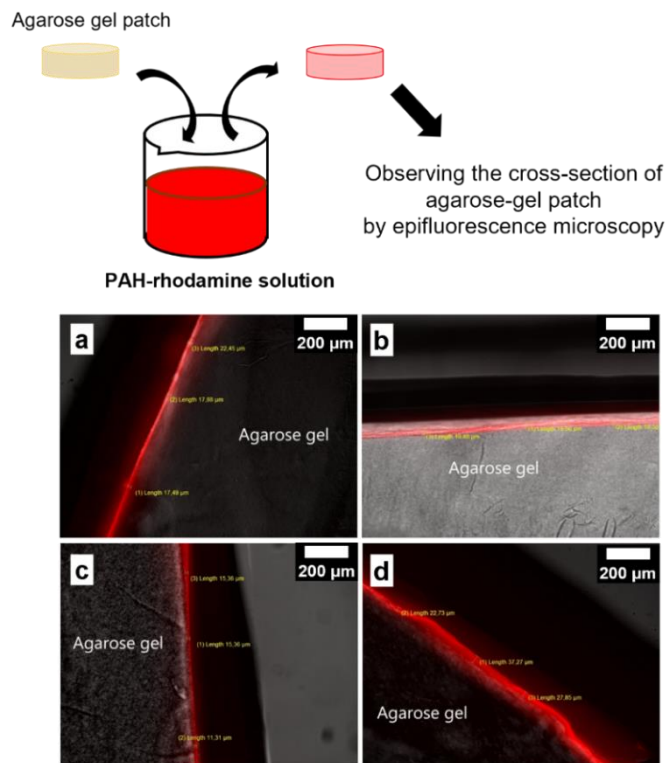


Figure S5.6. Epifluorescence microscopy images of a PAH-Rh single layer deposited on agarose gel patch with different dipping times of (a) 30 s, (b) 60 s, (c) 90 s and (d) 120 s.

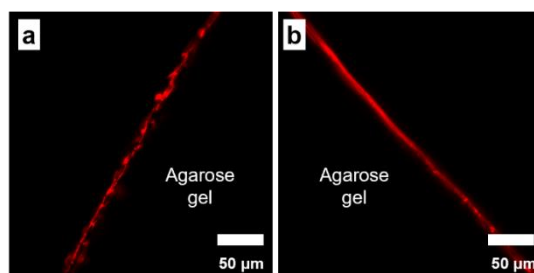
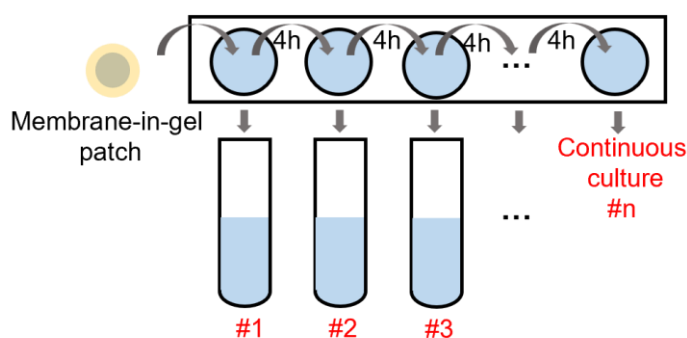


Figure S5.7. Diffusion of PAH-Rh in an agarose gel patch. After dipping in a PAH-Rh solution for 60s, the agarose gel patch was placed in liquid nitrogen to make cuts. The cross-section of the agarose gel patch was observed with CLSM to measure the diffusion depth of PAH-Rh in the gel. (a) CLSM Image of an agarose gel patch coated with $(\text{PAH/ALG})_4(\text{PAH-Rh/ALG})_1$. (b) Image of an agarose gel patch coated with $(\text{PAH-Rh/ALG})_5$.

Culture of patch in refreshed broth.

As shown in Scheme S5.2, a membrane-in-gel patch was placed in a well containing 2 mL broth to start culture (37 °C, 120 rpm). After 4 h, the membrane-in-gel patch was taken out from the well and triply rinsed with fresh broth. Then, the membrane-in-gel patch was transferred to a new broth-containing well to continue culture. The supernatant in well #1 was transferred to a vented culture tube and continuously cultured. This refreshing step was repeated every 4 h, obtaining supernatants #1, #2, #3, etc. Eventually, the membrane-in-gel patch was continuously cultured in the last well #n.



Scheme S5.2. Schematic illustration of the culture of membrane-in-gel patch in refreshed broth. Membrane-in-gel patch was placed in well #1 to start culture and was transferred to a new well with fresh broth every 4 h culture. The supernatant was collected in a vented culture tube. Eventually, the membrane-in-gel patch was leaved in well #6 and continuously cultured.

A positive control of free bacteria was cultured at the start time to check the state of bacteria. 10 μL of bacterial suspension ($\text{OD}_{540} = 0.042$) was added in 2 mL broth, corresponding to 1% of the number of bacteria used for membrane loading. Pure broth was cultured as a negative control, and a new negative control was used when the membrane-in-gel patch was transferred to a new well. AlamarBlueTM cell viability reagent (200 μL) was added in each broth. The fluorescence intensity of all supernatants was measured 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.

Since the membrane-in-gel patch was removed from broth after 4 h culture, the large increment of fluorescence intensity during culture of this supernatant demonstrates the presence of free bacteria in the supernatant. As shown in Figure S5.8a, the fluorescence intensity of supernatant #1 slightly increased at 4 h culture, showing the metabolic activity of bacteria in the patches. For the patch coated with (ALG/CHI)₅ALG, a significant increase was observed at 52 h culture, illustrating the presence of free bacteria in the supernatant. By contrast, the fluorescence intensity of supernatants #1 rarely changed after removing the membrane-in-gel patch coated with (ALG/CHI)₁ALG(PEI/PSS)_n (n=1-5). This demonstrates that PEI/PSS coating is more efficient in controlling bacterial escape. The fluorescence intensity of all supernatants at 4 h culture time increased from Figure S5.8a to f, showing that the activity of bacteria increased with the patch culture time. In Figure S5.8e, bacteria escape was observed on patches coated with (ALG/CHI)₁ALG(PEI/PSS)₁, 2, and 3 and the lag time increases with the PEI/PSS bilayer number, demonstrating that bacteria release depends on the thickness of PEI/PSS coating. The fluorescence intensity of patches coated with (ALG/CHI)₁ALG(PEI/PSS)₄ and (ALG/CHI)₁ALG(PEI/PSS)₅ did not show a large increase in all supernatants, demonstrating that (ALG/CHI)₁ALG(PEI/PSS)₄ multilayers have attained the critical thickness required to prevent bacteria release in refreshed broth.

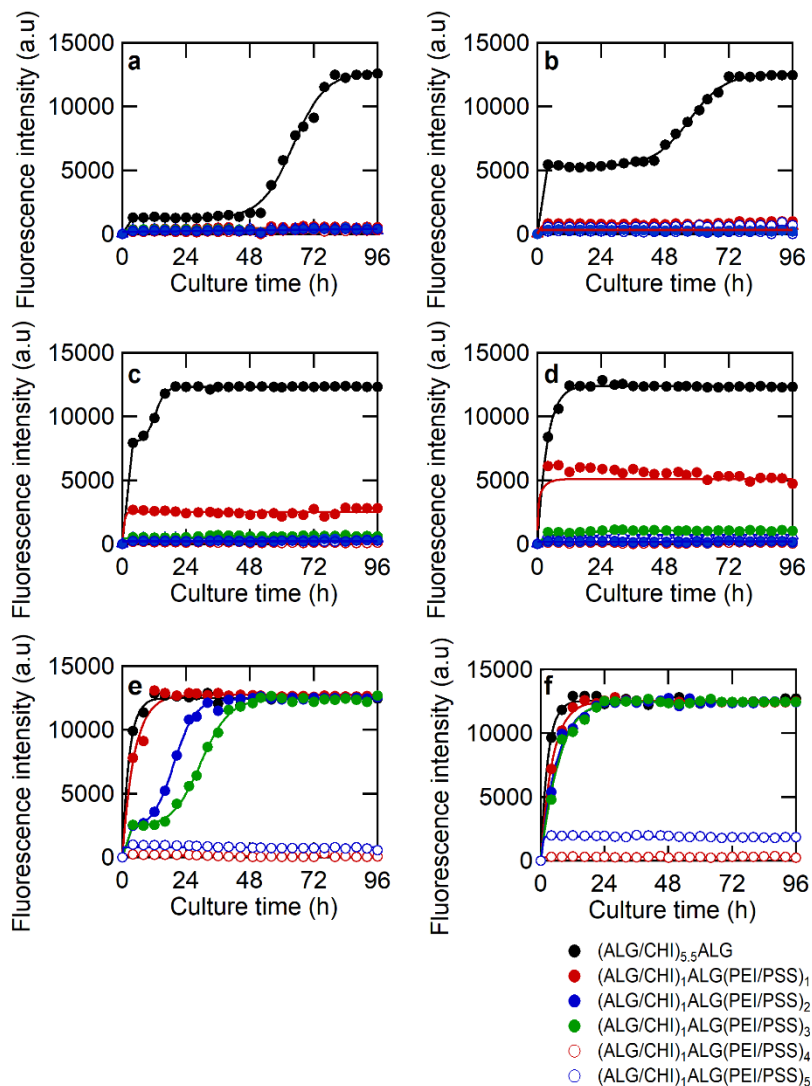


Figure S5.8. Metabolic activity of *S. epidermidis* encapsulated in membrane-in-gel patches. The LbL-modified membrane-in-gel patch was cultured at 37 °C in 2 mL broth with 200 μ L alamarBlue™ cell viability reagent. After every 4 h of culture, the bacteria-gel patch was transferred to a new broth/alamarBlue mixture and the previous mixture was collected. Lastly, the bacteria-gel patch was continuously cultured in a broth/alamarBlue mixture. The fluorescence intensity of all supernatants at 585 nm (excitation wavelength 470 nm) was measured; curves are drawn to guide the eye. (a) supernatant #1, (b) supernatant #2, (c) supernatant #3, (d) supernatant #4, (e) supernatant #5 and (f) supernatant #6 (continuous culture).

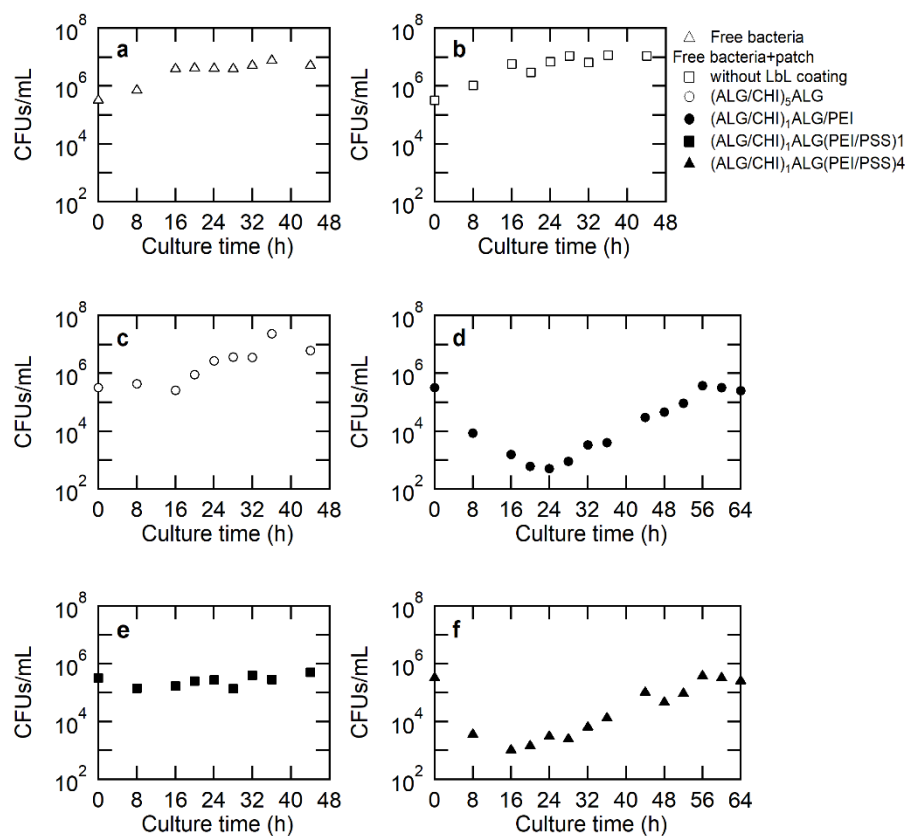


Figure S5.9. The effect of LbL multilayers deposited on the membrane-in-gel patch on the proliferation of free bacteria suspended in the broth. Fresh bacteria and membrane-in-gel patches coated with/without LbL multilayers were added to a broth. The number of CFUs per mL was measured using a single plate serial dilution spotting technique as described in the companion article.

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Chapter 6 Conclusions and prospects for future work

6.1 Conclusions

Commensal bacteria encapsulation offers to benefit from some factors secreted by bacteria while preventing their uncontrolled proliferation; it is increasingly viewed as essential for the development of bacterio-therapeutical applications. In this thesis, two bacteria encapsulation methods using LbL-modified PC membranes were studied, aiming at developing skin-applicable materials.

In the first method, *S. epidermidis* was entrapped in a mat made of LbL MTs. Previously, LbL tubes could only be liberated by dissolving the membrane template in organic solvents, which is not compatible for biological applications. To release LbL tubes in more benign conditions, a sacrificial compartment composed of PVCL/PMAA was used as an external compartment in the tubes.

In chapter 3, we focused on the growth and disaggregation of such H-bonded PVCL/PMAA LbL films, depending on pH, NaCl concentration, and PVCL molar mass. The PVCL/PMAA LbL assembly was performed at pH 2.0, and we showed that film growth is independent of NaCl concentration and PVCL molar mass. These results are compatible with previous studies and can be explained by the fact that PVCL/PMAA is a strong H-bonded system. The disaggregation of PVCL/PMAA films under pH triggers was then studied. The PVCL molar mass was found not to impact the pH_{crit} of PVCL/PMAA film. By contrast, the pH_{crit} of PVCL/PMAA films decreased with salt concentration, being 7.1, 6.6, and 6.1 for 0, 50, and 150 mM NaCl, respectively. The long-term stability of PVCL/PMAA film showed that film disaggregation is a time-dependent process and that the disaggregation kinetics can vary substantially around pH_{crit} . Due to its tunable stability, the PVCL/PMAA system shows great perspectives for the controllable release of various materials, especially bioactive molecules.

Using the PVCL/PMAA multilayers as a sacrificial compartment, we showed then the release of pH-stable LbL nano/micro-tubes. Floating of films was also proved, demonstrating that sacrificial compartments are a versatile way to

liberate various structures/materials. Interestingly, the release process is gentle (pH 6.5, 150 mM NaCl) and fully bio-compatible, which is promising for biological encapsulation. In addition, the LbL tube liberation proportion showed that only non-intersecting pores contributed to tube release, eliminating overlapped tubes from the collection process.

In chapter 4, loading of *S. epidermidis* in bicompartimentalized MTs and the metabolic activity of *S. epidermidis* in resulting MT mats were studied. The LbL assembly process was modified by tuning the decrusting solution. The use of either gentle (150 mM) or strong (3 M) saline solutions produced closed and open pores, respectively. This strategy allowed us to close the pores when and where needed, leading to an improvement in materials loading and limiting materials escape from MTs. Using microbeads as simplistic model particles, the loading efficiency was optimized, depending on the pore size of membrane, the microbead concentration, and the charge type of the innermost layer of MTs. A homogeneous microbead dispersion was observed in PC membranes of 1.6 μm pore diameter, showing the best trapping efficiency. The investigations on microbead concentration and the charge type of last layer showed that the fast trapping of microbeads was unfavorable for encapsulation efficiency. A 50x dilution factor of microbeads (3×10^6 microbeads/mL) prevented the quick blockade of pores, leading to more microbeads being trapped in MTs. It was also demonstrated that multilayers ended with a polyanionic ALG layer were conducive to an increased and more homogeneous microbead trapping in the pores. This counter-intuitive result can be explained by the fact that, when the pore coating is ended by a positively-charged polyelectrolyte, the interaction with negatively-charged particles is strong and results in aggregation and rapid pore blockade.

Following the optimized parameters, *S. epidermidis* was entrapped in (PVCL/PMAA)₅PVCL(ALG/CHI)₄ALG-modified MTs. An (ALG/CHI)₆ALG MT mat was obtained, which maintained the bacterial viability. However, a low metabolic activity of *S. epidermidis* in the mat was observed. A series of bacteria, including free bacteria, bacteria in PC membranes coated with/without (ALG/CHI)₉ALG, and bacteria in PC membranes before/after pH 6.5 treatment were checked, demonstrating high metabolic activities. However, bacteria in (ALG/CHI)₆ALG MT mats lost their activity, which may due to the antibacterial impact of CHI, the physical barrier of ALG/CHI compartment and/or the low encapsulation capability of MT mat.

Bacterial encapsulation in MTs then release of the loaded MTs to form a MT mat provides an elegant way to entrap *S. epidermidis* in a fibrillar structure in gentle aqueous and biocompatible conditions. Moreover, the size, length and

density of pores can be tuned to various cells or molecules. Multilayered MT mats can also be produced by gradually filtering different MTs in order to obtain multifunctional mats.

The strategy developed in this thesis to release the LbL tubes based on hydrogen bonded PVCL/PMAA compartment can be applied to release entrapped molecules or particles, simply by tuning the pH and ionic strength. However, the encapsulation process based on MT mats is complex to implement. Moreover, the encapsulation efficiency remains limited. Due to these drawbacks, we did not continue in the optimization of this approach and proposed a second encapsulation method.

In chapter 5, a new *S. epidermidis* encapsulation method was thus studied. Since bacteria in LbL-modified PC membranes can maintain their high activity, a soft and biocompatible agarose coating was cast on the PC membrane, leading to a membrane-in-gel patch. The agarose gel coating supplies a physical barrier that prevents the free diffusion of bacteria, but would allow the delivery of beneficial substances produced by *S. epidermidis*. In addition, the hydrous agarose gel coating provides a soft system that can be applied to skin. The bacterial activity in membrane-in-gel patches was tested in broth. Simultaneously, a single plate serial dilution spotting technique was used to monitor bacterial escape. This technique supplies a way to quantitatively test the bacterial release, especially for long incubation times, which can be generalized to evaluate multiple bacterial encapsulates.

We also demonstrated LbL assembly on agarose gel. Two types of polycations with antibacterial effect, PEI and CHI, were deposited on the membrane-in-gel patches. This densely-packed LbL coating provides an anti-escape barrier, whose properties can be modulated by playing with bilayer composition and the number of bilayers. ALG/CHI coatings only delay very moderately bacterial escape and subsequent growth, whereas the more bactericidal PSS/PEI coating can control bacterial release and prevent the growth of possibly alive escaped bacteria, depending on the bilayer number. Interestingly, the anti-escape ability of the coating is stronger when PEI is used as the outer layer. Using the second method, a bacterial hybrid material was obtained, which can be easily and conformally applied to skin, and prevents adventitious release of the bacteria while allowing to deliver secreted beneficial factors.

In this thesis, new bacteria encapsulation methods have been developed based on LbL assembly in porous PC membranes. Especially the second method, encapsulating bacteria in a membrane-in-gel patch, meets our objectives described in chapter 1 (Scheme 1.1): the metabolic activity of bacteria is maintained for at least 24h; the release of bacteria from the patch is prevented by depositing on the patch surface a coating containing bactericidal

compounds; the patch is permeable to small molecules, allowing nutrients to penetrate and beneficial secreted molecules to be released; the hydrated soft surface of the patch makes it suitable for skin applications

Moreover, the methodology developed to fabricate membrane-in-gel patches offers several advantages: all the steps are conducted in aqueous biocompatible conditions, which is gentle and less stressful for encapsulated cells; the characteristics of LbL assemblies, used to fabricate an anchoring layer to immobilize bacteria in membrane pores and to produce a functional coating preventing the release of bacteria from the patch, can be easily tuned by varying the nature of the polyelectrolyte used and the number of deposited layers; the use of agarose gel provides a hydrated microenvironment to protect bacteria and also a soft surface for skin application. Compared to generally methods developed for bacterial encapsulation, such as electrospinning or 3D printing, our approach is easy to operate without using sophisticated instruments. Compared to chemical/UV initiated bulk gel encapsulation, our process does not require chemically aggressive reagents that can be detrimental for cells. Moreover, 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. All these features make the membrane-in-gel patch an attractive encapsulating system not only for skin bacteria encapsulation but also for various biological active cells/molecules.

6.2 Prospects for future work

In this thesis, an erasable H-bonded LbL system was studied and used to release nano/micro-structures by changing pH. Besides this, ionic strength, PVCL molar mass and dipping times are also essential factors to regulate the release process. For example, a slow disaggregation of PVCL/PMAA multilayers at pH 6.0 can be used to achieve controllable release of target materials, especially biomolecules, in physiological conditions. In addition, inspired by observations on H-bonded PVCL/PMAA multilayers, other triggers such as light- or temperature could be developed for LbL multilayers for bio-applications.

Bacterial encapsulation was studied, demonstrating a membrane-based method to entrap bacteria, regulate bacterial activity, and control bacterial leakage. However, we are still far to reach the clinical application of this bacterial patch to regulate skin homeostasis. Indeed, a series of complementary studies are needed to comprehensively learn the efficiency of this encapsulation method and the activity of *S. epidermidis* in the patch. For example,

- The impact of patches on pathogen growth such as *S. aureus* should be tested on agar plates to evaluate the function of bacteria in patches.

Conclusions and prospects for future work

- The activity of bacterial patch in depleted media such as artificial sweat should also be tested.
- The leaking of bacteria from patches is another essential factor to be investigated since *S. epidermidis* is known as a pathogen when it goes into the bloodstream. Patches can be placed on a model of human skin, such as porcine ear skin, to evaluate bacterial penetration. To do this, the thickness and composition of antibacterial multilayers coated on patches can be tuned to get the best efficiency.

Then, *in vivo* characterization of patches should be carried out.

- The bioactivity of *S. epidermidis* in patch should be tested on an animal model.
- Further *in vivo* studies on skin dysbiosis treatment such as atopic dermatitis, psoriasis, acnes etc. should then be considered for clinical applications.

Due to the benign preparation conditions and the ease of regulation of the characteristics of patches, including pore diameter, patch diameter, and thickness of agarose gel, this versatile technology should be easily applied to entrap diverse cells for skin application, such as *B. subtilis*, *S. hominis*, *L. rhamnosus*, etc. The bactericidal LbL multilayers deposited on the patch can be easily modulated in both composition and thickness, bringing various possibilities for the control of cell release and surface modification. Therefore, our encapsulation method should be of interest for the development of a range of applications, depending on the type of entrapped cells. For example, *L. plantarum*, a bacterium that benefits the skin in tissue repairing, can be entrapped in this patch for wound healing. By doing this, not only entrapped *L. plantarum* but also bactericidal coatings, could protect wounds from pathogen inflammation. Prospects are thus numerous for the technology we have developed.

Dissemination

Publications

Xu, W.; Ferain, E.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Hydrogen-bonded multilayers for the release of polyelectrolyte nanotubes in biocompatible conditions. *ACS Appl. Polym. Mater.* 2019, 1 (9), 2407-2416.

Xu, W.; Malek Mohammadi Nouri, P.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Encapsulation of commensal skin bacteria within membrane-in-gel patches. *Adv. Mater. Interfaces*, accepted.

Oral communications

Xu, W.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Commensal bacteria encapsulation in porous membrane for topical applications. IMCN PhD students' day, Louvain-la-Neuve, virtual, May 21, **2021**.

Xu, W.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Membrane-based encapsulation methods for commensal skin bacteria. Belgian Polymer Group meeting, virtual, May 27-28, **2021**.

Xu, W.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Encapsulation of bacteria in membrane-based patches with antibacterial surface. Materials Research Society Spring Meeting, virtual, April 17-23, **2021**.

Xu, W.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Erasable hydrogen-bonded multilayers for the biocompatible release of templated nanostructures. 83rd Prague Meeting on Macromolecules-Polymer in Medicine, Prague, Czechia, June 23-27, **2019**.

Poster communication

Xu, W.; Ferain, E.; Demoustier-Champagne, S.; Glinel, K.; Jonas, A. M. Hydrogen-bonded multilayers for the release of polyelectrolyte nanotubes in biocompatible conditions. IMCN PhD students' day, Louvain-la-Neuve, Belgium, May 29, **2019**.