

École polytechnique de Louvain

Development of a bacterial encapsulation method for the fabrication of bacterial patches for skin medical treatment

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Abstract

Staphylococcus epidermidis (*S. epidermidis*) is one of the commensal bacterium present in human skin microbiota. This Gram-positive bacteria coexists with many different types of microorganisms on human skin and it plays an essential role in defense against pathogenic bacteria. However, *S. epidermidis* can become pathogenic if it enters the blood stream. That is why in this study, development of a bacterial patch is investigated to prevent release of bacteria, while maintaining the bacterial activity to treat skin disorders.

The encapsulation method involved loading *S. epidermidis* in pores of a track-etched polycarbonate membrane modified with (chitosan(CHI)/alginate(ALG))₄, followed by agarose gel coating. Finally, films produced by Layer-by-Layer technique (LbL) and incorporating antibacterial polycations were deposited on the surface of the agarose gel to prevent release of encapsulated bacteria. Three types of antibacterial polycations were tested to produce films, namely poly(*L*-lysine) (PLL), poly(ethylenimine) (PEI), and CHI. Bacterial patches coated with PLL and PEI-based LbL films were characterized through methods of refreshed broth and continuous culture, respectively, in order to measure bacterial activity and bacteria release from the bacterial patch. The results were compared with CHI-based films in each case.

PLL showed similar efficiency to CHI regarding the bacterial patch. However, increasing the (PLL/hyaluronic acid (HA)) bilayer number could further delay the release of bacteria. The bacterial patch coated with (ALG/CHI)_{1.5} (PLL/HA)₁₆ showed the best efficiency, among other bacterial patches, by delaying the release of bacteria for up to 4 hours, while preserving the metabolic activity of bacteria.

PEI demonstrated much stronger antibacterial effect compared to CHI. Increasing (PEI / poly(styrene sulfonate)(PSS)) bilayer number could delay the release of bacteria. However, it also reduced the bacterial activity inside the bacterial patch. Additionally, multilayers ended with PEI showed much stronger bactericidal effects, leading to more delay in release of bacteria, while decreasing the bacterial activity inside the bacterial patch. Bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)_{1.5} showed the best efficiency as it showed the highest bacterial activity inside the bacterial patch, while inhibiting the release of bacteria completely for at least 52 hours.

In this study, a novel encapsulation method for *S. epidermidis* was successfully developed.

ABSTRACT

This method can thus provide a new way to treat different types of skin disorders. However, further studies have to be conducted to investigate other types of antibacterial multilayers in this system. Furthermore, *in vitro* and *in vivo* testing are also required for checking the efficiency of the bacterial patch on pathogenic skin bacteria.

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List of abbreviations and symbols

ALG	Alginate
Alkynyl-PSBMA	Alkynylpoly(sulfobetaine methacrylate)
A. vinosum	Allochromatium vinosum
Azide-PSBMA	Azide-poly(sulfobetaine methacrylate)
C. acnes	Cutibacterium acnes
CFU	Colony forming unit
CHI	Chitosan
CNT	Carbon nanotubes
CD	Cyclodextrin
DENCOOH	Carboxyl-terminated polyether dendrimer
E. coli	Escherichia coli
ECM	Extracellular matrix
FITC	Fluorescein isothiocyanate
HA	Hyaluronic acid
LbL	Layer-by-Layer
MIC	Minimum inhibitory concentration
OD540	Optical density at wavelength of 540 nm
PAA	Polyacrylic acid
PAAm	Polyacrylamide
PAH	Poly(allylamine hydrochloride)

LIST OF ABBREVIATIONS AND SYMBOLS

PAH-Rho	Polyallylamine hydrochloride-rhodamine
PAMAM	Poly(amidoamine) dendrimer
PAni	Polyaniline
PC	Polycarbonate
PDDA	Poly(dimethyldiallylammonium chloride)
PDL	Poly(D-lysine)
PEG	Poly(ethylene glycol)
PEI	Poly(ethylenimine)
PGA	Poly(glycolic acid)
PLL	Poly(<i>L</i> -lysine)
PMAA	Poly(methacrylic acid)
PPFPA-b-PS	Poly(pentaurophenylacrylate)-b-poly(styrene)
P. aeruginosa	<i>Pseudomonas aeruginosa</i>
PSF-N₃	Azido-functionalized polysulfone
PSS	Poly(styrene sulfonate)
PVP	Poly(4-vinylpyridine)
PVPON	Poly(vinylpyrrolidone)
QACs	Quaternary ammonium compounds
S. aureus	<i>Staphylococcus aureus</i>
S. epidermidis	<i>Staphylococcus epidermidis</i>
SEM	Scanning electron microscopy
UV	Ultraviolet

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Chapter 1

State of the art

This study aims to devise a novel encapsulation method of *Staphylococcus epidermidis* (*S. epidermidis*) to take advantage of its beneficial properties against skin disorders. In this chapter, we will start by discussing the most important component, the bacteria. Next, we will discuss the literature on different components of the encapsulation method, including polycarbonate membrane, Layer-by-Layer (LbL) assembly, antibacterial materials, antibacterial LbL films, agarose gel, and bacterial encapsulation.

1.1 Commensal bacteria

Commensal bacteria that reside on human body, including skin, gut, and oral cavities, play a vital role in human homeostasis. Mucosal surfaces in human body, especially the gastrointestinal tract have the most densely packed commensal bacteria habitat. Around 10^{14} commensal bacteria live in human intestine. The intestinal surface is constantly exposed to various pathogenic and commensal bacteria and the imbalance between these bacteria can cause inflammatory bowel disease. [1]

Several studies have shown that commensal bacteria regulate immunophysiological functions, promoting resistance to pathogenic bacteria and benefiting the host. For instance, gut bacteria help with digestion and absorption of food, help to repair tissue injury, and even fight with pathogenic bacteria and external threats. In exchange, these commensal bacteria benefit from the favorable microenvironment in human gut. [1]

Researchers take advantage of the natural properties and functions of these bacteria to combat different diseases. For instance, commensal gut bacteria show anti-inflammatory properties and many studies have investigated strategies using these bacteria for treating diseases, such as colorectal cancer, [2, 3], and ulcers. [4] Additionally, fecal microbiota transplantation has been widely employed to treat infectious and inflammatory disorders. In this method, distal gut bacteria from a healthy person is transplanted to the patient in order to re-establish the microbiota balance after dysbiosis. Fecal microbiota transplantation has

shown great promise in combatting *Clostridium difficile* infection and inflammatory bowel disease. [5, 6]

Gastrointestinal tract is not the only commensal bacteria habitat in human body. Skin is sterile before birth and is exposed to thousands of microorganisms afterwards. Human skin microbiota includes bacteria, virus, fungi and protozoa. It contains more than 1000 species of bacteria and the number of genes encoded in these bacteria are 150 times more than those in human genome. The maintenance of such homeostatic conditions is of utter importance, as an imbalance, due to external stimuli like temperature, moisture, or pathogenic microorganisms, could lead to many different types of inflammatory diseases and even cancer. *S. epidermidis* and *Cutibacterium acnes* (*C. acnes*) are two important commensal bacteria on human skin, which play essential roles in skin homeostasis. [7]

1.1.1 *Staphylococcus epidermidis*

S. epidermidis is a Gram-positive bacterium that exists as a commensal bacterium on human skin. [8] It is an aero-anaerobic facultative bacterium and its favorable growth temperature is between 30° C and 37° C. [9] It is the species which is most frequently isolated from human epithelia and it is quite resistant against harsh conditions, such as high salt concentration or osmotic pressure. [8] *S. epidermidis* cells are spherical and 0.5 μm to 1.5 μm in diameter. [10] Gram-positive bacteria, such as *S. epidermidis* have a thick peptidoglycan layer surrounding the cell wall, whereas the peptidoglycan layer in Gram-negative bacteria, such as *Escherichia coli* (*E. coli*) is much thinner and is surrounded by an outer membrane. The typical schematic illustration of the cell membranes of Gram-positive and Gram-negative bacteria is shown in Fig. 1.1. [11] Bacterial membranes have a negative charge which is associated with lipopolysaccharides in Gram-negative and to teichoic acid in Gram-positive bacteria. [12]

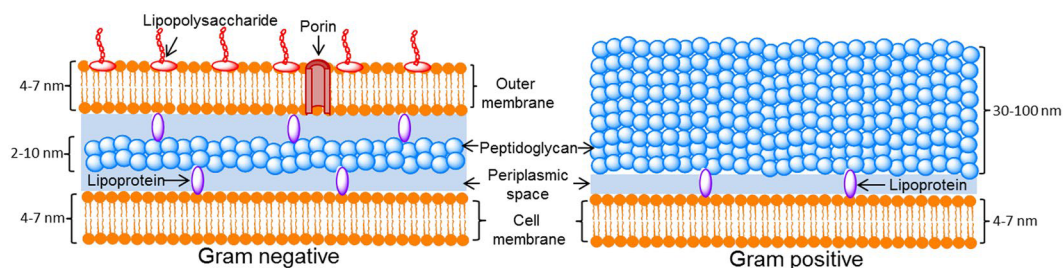


Figure 1.1: Schematic illustration of the cell membranes in Gram-positive and Gram-negative bacteria. [11]

S. epidermidis is abundant in human skin microbiota, and it can inhibit the growth of *C. acnes*, which is responsible for acne vulgaris. A certain balance is always kept between these two types of bacteria in a healthy skin. Scanning electron microscopy (SEM) images of the two bacteria are shown in Fig. 1.2. However, when the skin microbiota balance is disrupted, called skin dysbiosis, due to external factors, such as moisture or temperature, these bacteria can become pathogenic, and diseases like acne vulgaris or atopic dermatitis can occur. [9]

Wang et al. tested the inhibitory effect of microorganisms present on human finger tips on *C. acnes*. They noticed inhibition zones forming around the colonies of a certain type of bacterium, later determined to be *S. epidermidis*. [13] *S. epidermidis* can ferment the glycerol present on human skin to produce short chain fatty acids, such as acetic, butyric, lactic and succinic acids. Succinic acid has an inhibitory effect on biofilm formation of *C. acnes*. Therefore, *S. epidermidis* can effectively prevent acne vulgaris. [9] Furthermore, Cogen et al. showed that phenol-soluble modulins secreted by *S. epidermidis* can induce selective antimicrobial action against pathogenic bacteria, such as *Staphylococcus aureus* (*S. aureus*). [14] The effect of *S. epidermidis* in skin defense mechanism, inhibiting the growth of *A. Streptococcus* and *S. aureus* has also been shown by Lai et al. by *in vivo* testing on mice. [15] *S. epidermidis* has also been used in a clinical trial by Nodake et al. as a skin care method. They showed that treatment using *S. epidermidis* can suppress skin water evaporation and increase skin lipid content, leading to higher skin moisture, in addition to keeping a low acidic condition on the skin. [16] Moreover, Nakatsuji et al. showed that a strain of *S. epidermidis* is also quite effective for protection against skin tumor in mice. [17]

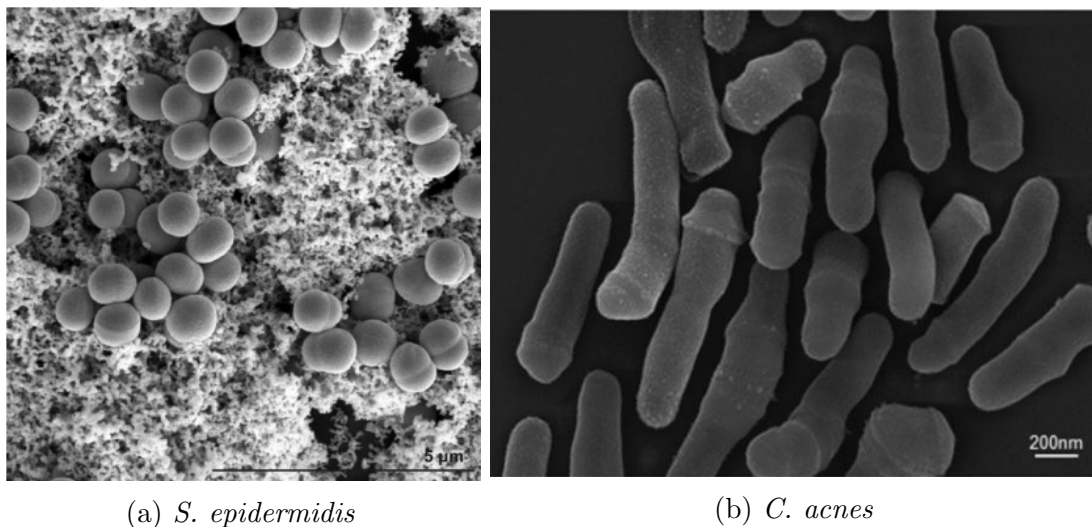


Figure 1.2: SEM images of two important bacteria in human skin microbiota, *S. epidermidis* and *C. acnes*. [18, 19]

However, *S. epidermidis* can cause infections if it enters the blood stream, and it is also one of the main sources of nosocomial infections, especially on medical devices. It can attach on the surfaces of devices and form biofilm aggregates. These biofilms are more resistant to antimicrobial agents like antibiotics and disinfectants, as well as host immune system mechanisms, such as antibodies and phagocytes compared to planktonic bacteria. Some biofilms also show resistance to mechanical breakage.[20, 21] Even though *S. epidermidis* is a common type of bacterium in human skin microbiota and has an essential role in skin

ecosystem, it can become an opportunistic pathogen quite easily. Therefore, a method should be applied to well control the growth of *S. epidermidis*, in order to maintain its benefits for treating skin disorders, while avoiding potential infection.

1.2 Polycarbonate membrane

Track-etched polycarbonate (PC) porous membranes are commonly used for bioanalysis. The membranes are produced by tracking polycarbonate sheets with ion beams from accelerators, followed by chemical etching to create well-defined pores along the sheet thickness. The pores can be in different shapes, including cylinders, cones, etc. A schematic illustration of a track-etched PC porous membrane can be seen in Fig. 1.3. What makes these types of membranes applicable in different applications is the fact that pore size and pore density can be accurately and independently tuned. The pore size can be tuned by controlling parameters, such as etchant composition, component concentration and temperature, and the pore density can be tuned by ion beam irradiation conditions. Pore size of PC membranes can be reduced even down to 10 nm, and the pore density range is from 1 to 10^{10} cm⁻². The thickness of PC membranes can vary from around 6 μ m to 50 μ m. Due to the accurate control over pore size and pore density, transport phenomena can be modelled easily, which helps with different applications, such as filtration, bioanalysis and, especially investigating cellular and bacterial activities. Additionally, track-etched PC membranes are chemically stable, non-staining, and have good mechanical properties. [22, 23] In this study, PC membranes are employed for immobilizing *S. epidermidis* inside the pores.

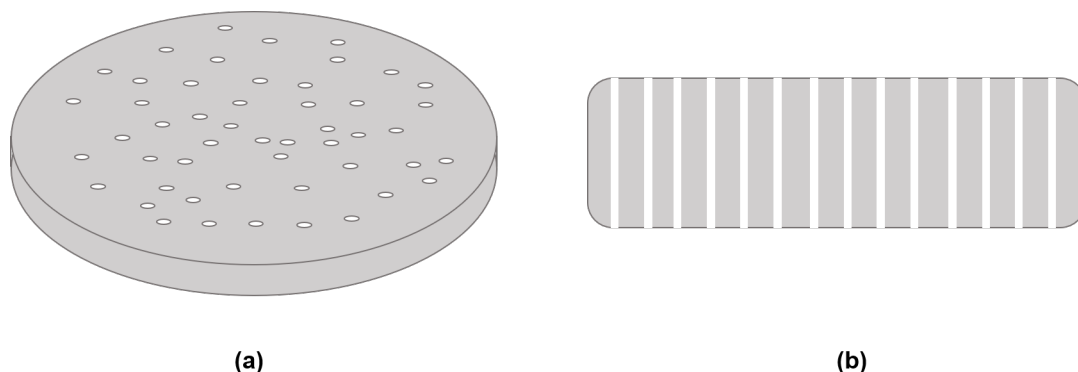


Figure 1.3: Schematic illustration of track-etched PC porous membrane from (a) above and (b) side view cross-section

1.3 Layer-by-Layer assembly

1.3.1 Overview

LbL assembly consists of alternately adsorbing different materials onto a substrate for film fabrication. Generally, in LbL assembly, the substrate is exposed to different materials, followed by a rinsing step to remove loosely bound materials, leading to the assembly of multilayers on the surface. This procedure can be conducted via different methods. Dipping method, spin-assisted method and spray-assisted method are among the conventional and most common methods. Composite films can be fabricated using LbL assembly in a sequential manner driven by different types of interactions. Traditionally, LbL assembly was mainly driven by the electrostatic interaction between oppositely charged polyelectrolytes. Nowadays, other interactions, such as hydrophobic interaction, hydrogen bonding, or even biologically specific interaction, in addition to covalent bonding are among the driving forces for assembling LbL multilayers. Due to the vast range of interactions used in this method, many types of materials can be used in LbL assembly. The material choice can range from different types of macromolecules, such as polypeptides, proteins and synthetic polymers, to inorganic materials and even biological species like cells. A general scheme of LbL assembly of oppositely charged polyelectrolytes using dipping method is shown in Fig. 1.4.

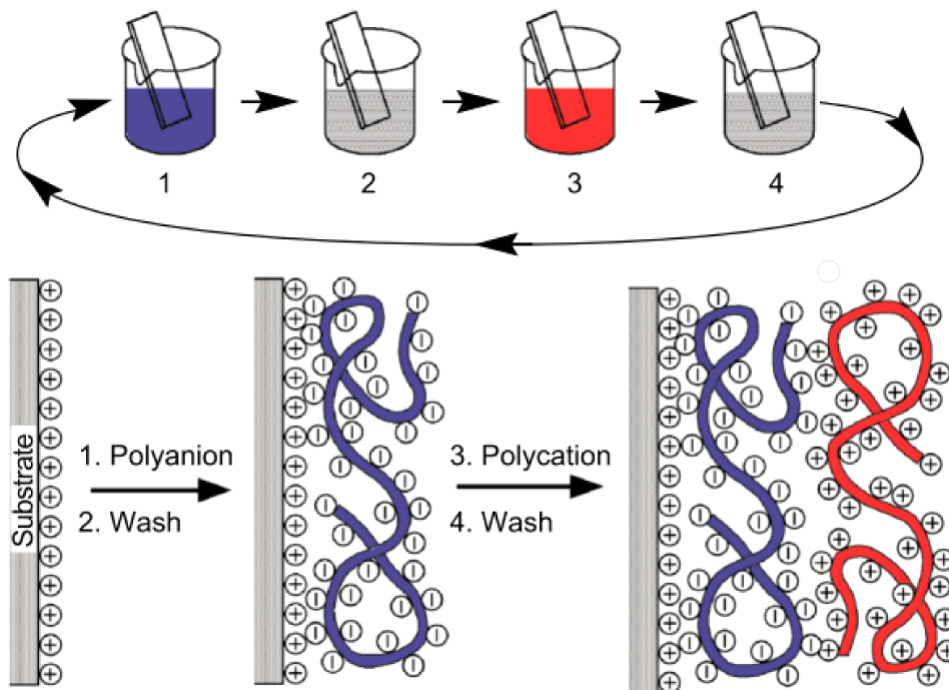


Figure 1.4: Schematic illustration of LbL assembly of oppositely charged polyelectrolytes via dipping method. [24]

LbL assembly is thus a very convenient method, used in many different applications. It has caught great attention due to the fact that it is a very simple, fast, and versatile method and it is also quite adjustable. The advantage of using LbL assembly instead of conventional methods for nanofilm fabrication lies in the precise control over film chemical composition and structure, and the fact that it is quite suitable for coating large areas of a non-flat surface. Additionally, LbL assembly is usually used with aqueous solutions, which makes the method more convenient and more compatible with biomedical applications. [25, 24, 26]

1.3.2 Types of interactions

One of the great points about LbL assembly, which makes it quite flexible, is the fact that different layers can be assembled together through interactions, including electrostatic interaction, hydrogen bonding, hydrophobic interaction, biologically specific recognition, and even covalent bonding. [27]

Electrostatic interaction

Electrostatic interaction is the most widely used driving force for assembling LbL constructs. Weak polyelectrolytes, including weak polycations and weak polyanions are deposited at a certain pH, such that the electrostatic interaction between them drives the assembly process. The choice of deposition pH is related to the pK_a of the weak polyelectrolytes. The protonation and deprotonation of these polyelectrolytes are reversible by changing the pH of solution. Simple weak polyanions and weak polycations become deprotonated when pH goes above their pK_a . Therefore, deposition pH should be above the pK_a of weak polyanion while below the pK_a of weak polycation to optimize the electrostatic interactions between weak polyanions and weak polycations. However, polyelectrolytes, such as proteins and complex polypeptides are composed of both acid and base groups, and the charge reversal pH is no more equal to the pK_a and instead, an isoelectric point is defined. In the case of strong polyelectrolytes, the charge density does not depend on pH and thus, pH does not play a role in LbL assembly using strong polyelectrolytes. [28, 29, 30] A schematic illustration of LbL assembly on 2D and 3D surfaces driven by electrostatic interaction is shown in Fig. 1.5. [31]

By alternately dipping the substrate in polycations and polyanions, accompanied by a rinsing step in between, well-controlled films can be manufactured by LbL assembly. However, other parameters such as temperature, solvent and ionic strength can also affect the physico-chemical properties of the polyelectrolytes, and consequently the final structure of the LbL films. Additionally, the morphology and mechanical properties of the LbL assembled films can also be varied by tuning the polyelectrolyte molar mass and charge density. [27]

Aside from polyelectrolytes, plenty of charged materials have also been employed for fabricating LbL assembled films. For example, negatively charged carbon nanotubes have been used to fabricate ultrathin and conductive membranes for energy applications. [32] In another study, a type of layer silicate clay, montmorillonite, was used to prepare montmorillonite-chitosan

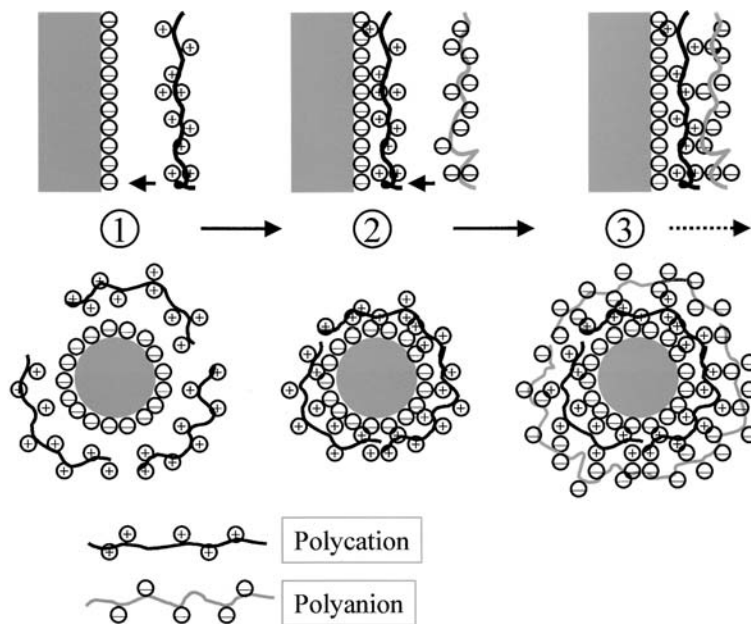


Figure 1.5: Schematic illustration of LbL assembly on 2D and 3D surfaces driven by electrostatic interaction. [31]

(CHI) hollow and hierarchical mesoporous spheres by LbL assembly for applications, such as targeted drug delivery and catalyst supports. [33] Alumina and carbon black particles have also been assembled in toluene by LbL assembly to prepare nanocomposite thin films. [34]

Even though electrostatic interaction is the most common driving force employed in LbL assembly, it limits the use of LbL to charged species. That is why other types of interactions have also been investigated for making LbL multilayers. [27]

Hydrogen bonding

Hydrogen bonding is also one of the most common driving forces employed in LbL assembly. Presence of hydrogen bonding donors and acceptors on the LbL building blocks is necessary for hydrogen bonding. [35] Zhang et al. showed the feasibility of creating multilayers out of poly(4-vinylpyridine) (PVP) and carboxyl-terminated polyether dendrimer (DENC₂COOH), as hydrogen bonding acceptor and donor, respectively. They determined the hydrogen bonding between these compounds using Fourier transform infrared spectroscopy. The structure of each compound and the resulting deposition cycle is shown in Fig. 1.6. [36]

There are three main advantages to using hydrogen bonding instead of electrostatic interaction for assembling LbL multilayers. First of all, stimuli-responsive films can be easily produced by employing hydrogen bonding LbL assembly at mild pH values. These films can respond to external stimuli, such as temperature and pH (in case of using at least one weak polyelectrolyte for hydrogen bonding) and are widely used for drug delivery and controlled

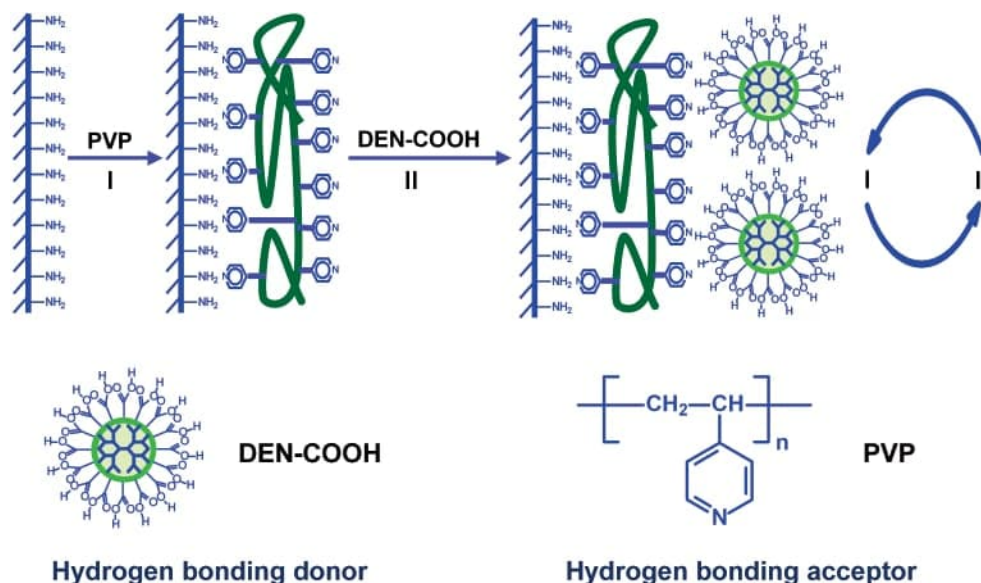


Figure 1.6: The deposition cycle of PVP and DENCOOH via LbL assembly using hydrogen bonding as the driving force. [36]

release applications. Studies have shown that when one weak polyelectrolyte is involved in the hydrogen bonding LbL assembly, the deposition pH plays an essential role in the film buildup. For instance, it was shown that in LbL assembly of polyaniline (PAni) with other polymers, such as poly(vinylpyrrolidone) (PVPON), polyacrylamide (PAAm), or poly(ethylene glycol) (PEG), a decrease in deposition pH of the second polymer solution can lead to both increase (PVPON, and PAAm) and decrease (PEG) of multilayer thickness. Variation of pH can even be used to disaggregate hydrogen bound multilayers, due to ionization of sites on polymer chains, blocking the hydrogen bonding mechanisms. Therefore, they can be used to fabricate stimuli-responsive films and drug delivery carriers. Aside from deposition pH, increasing the molar mass of PVPON in the same study, led to a much higher film thickness. [37, 38]

The second advantage is the possibility of converting the films into single or two-component hydrogels by well-controlled chemical crosslinking between polymer chains. This can extend the stability of multilayers for applications requiring a wider range of pH. [37] Sukhishvili and Granick assembled films made of polyacids and polybases, typically used for LbL assembly via electrostatic interactions. These polyacids and poly bases can form hydrogen bonds when they are uncharged. However, they are not very stable when exposed to external stimuli, such as temperature, pH or ionic strength. They showed that films made of (Poly(methacrylic acid)(PMAA)/PEG) were only stable up to pH 4.6, above which the ionization of polyacids caused the film to collapse. [39] The application of such films can be limited. That is why thermal, ultraviolet (UV)-, and photo-crosslinking methods have been used in literature to enhance stability of hydrogen bound LbL multilayers. [38]

The final advantage is that hydrogen bonds allow using polymers with low glass transition temperature, such as PEG, creating the opportunity to use such multilayers in new applications like proton-exchange membranes in fuel cells. [37]

Hydrophobic interaction

Hydrophobic interaction occurs when there are hydrophobic groups present in an aqueous medium. The hydrophobic groups tend to cluster together to decrease the area in contact with the aqueous medium, resulting from entropic effect. LbL assembly of tannic acid (TA) and gelatin driven by hydrophobic interaction was reported by Zhao et al. The hydrophobic groups of gelatin can form clusters to form hydrophobic pockets. Aromatic rings on TA can subsequently form hydrophobic interactions with the hydrophobic groups on gelatin backbone (Fig. 1.7). [40] Hydrophobic interaction can be combined with other interactions, such as electrostatic interaction [41] and hydrogen bonding [40, 42] to strengthen the LbL assembled films.

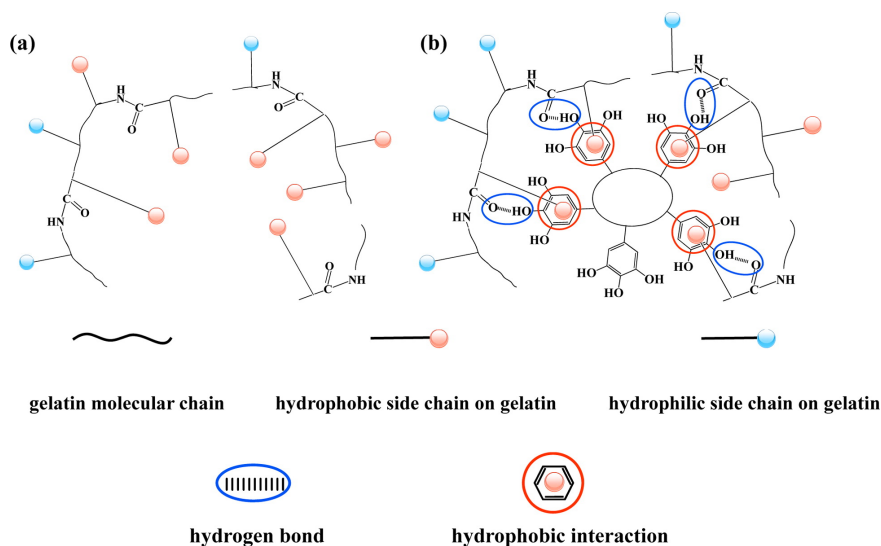


Figure 1.7: Hydrophobic interaction between TA and gelatin, reinforced by hydrogen bonds as a way to form LbL multilayers. [40]

Biologically specific interaction

Biologically specific interaction is composed of different types of interactions, such as hydrogen bonding, electrostatic interaction, hydrophobic interaction, and complexation. This type of interaction needs a quite accurate steric matching so that a high specificity can be obtained. Avidin-biotin, lectin-carbohydrate interactions, and DNA hybridization are more commonly used for biologically specific interaction in LbL assembly. [35] Fibronectin, which is one of the important proteins in extracellular matrix (ECM), has great biocompatibility and cell adhesion properties and is used for applications like tissue engineering. It is also known

for its interactions with ECM proteins. Nakahara et al. reported the LbL assembly of fibronectin with heparin, gelatin, and dextran sulfate using biologically specific interaction. They successfully deposited the nanofilms, and a schematic illustration of the nanofilms is shown in Fig. 1.8. [43]

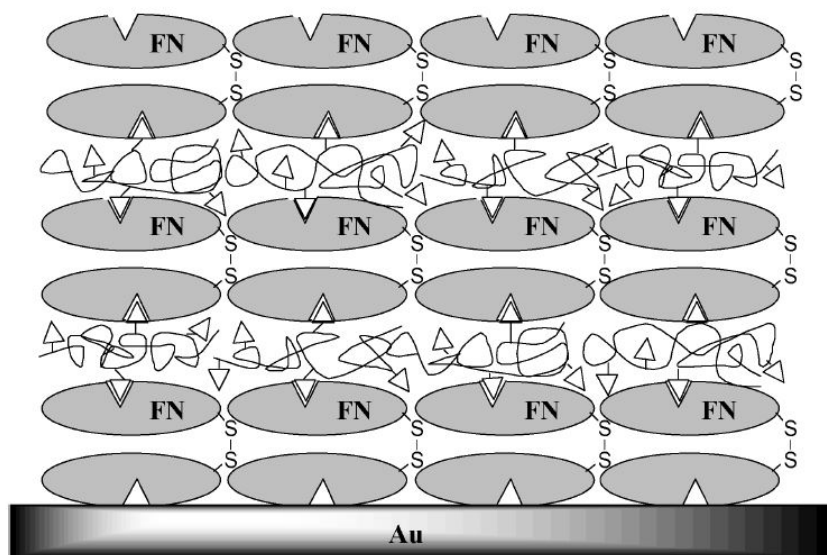


Figure 1.8: Schematic illustration of nanofilms based on fibronectin and gelatin/dextran sulfate/heparin using biologically specific interaction. [43]

Covalent bonding

Though the above-mentioned interactions can be used in LbL assembly to fabricate multilayers, the mechanical properties of multilayers are low. The stability of multilayers is an essential aspect that limits their application as the multilayers can be easily disaggregated under extreme conditions, such as strong saline solutions, low/high pH or temperature. Covalent bonds can meet the requirement of preparing LbL assembled multilayers with high stability, which is highly attractive in many applications, especially in the biomedical field. Additionally, a much more diverse range of materials is available for LbL assembly. [44] The building blocks for forming chemical bonds should have reactive moieties. There are many different chemical reactions which can be used for making these chemical bonds, such as Schiff base, click reactions, amine alkylation, etc. Heating or UV illumination might be used to start the reaction.

Aside from the building blocks and the type of chemistry used for assembly of the layers, the structural engineering strategy used for multilayer fabrication is also quite important as it can affect the stability, mechanical properties, and permeability of the film. In covalent bonding, diverse multilayer structures can be obtained by tuning the crosslinking degree and crosslinking spatial location. Stability of the films can also be tuned by employing stable

or labile covalent bonds. The multilayer properties can thus be controlled by changing the structural engineering strategy in order to fabricate multilayers for specific applications. The properties and the corresponding applications of fully and partially crosslinked multilayers are shown in Fig. 1.9. [44]

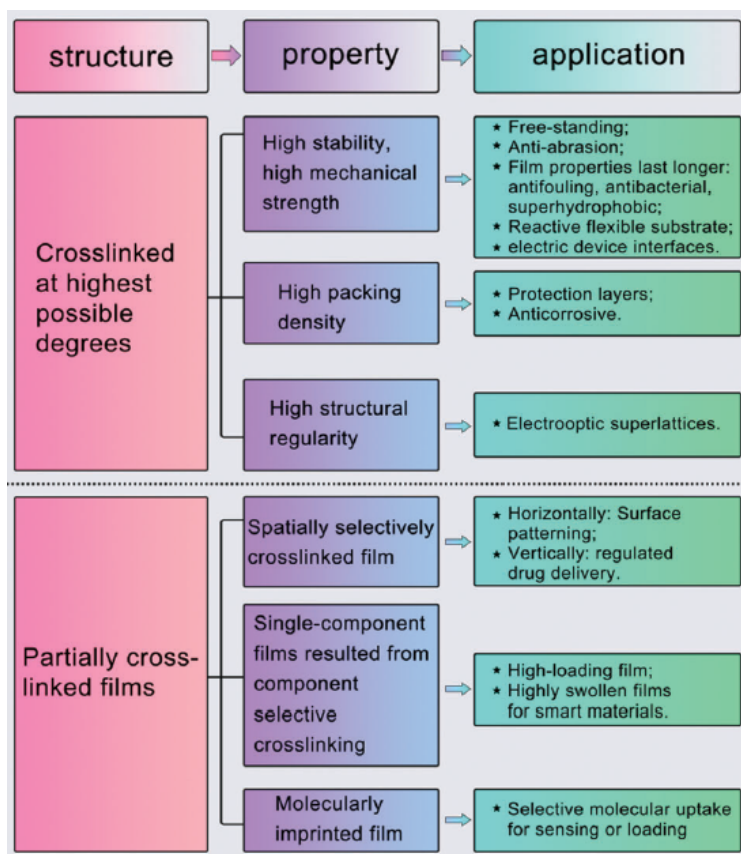


Figure 1.9: Main features and applications of fully and partially crosslinked multilayers. [44]

Generally, two different approaches are used for creating covalent bonds in LbL assembly. The covalent bonds can either be formed through direct chemical reactions on the substrate, where the covalent bonding is the driving force for film buildup, or it can be done through post-crosslinking of the pre-fabricated LbL multilayers produced from non-covalent forces.

Film buildup using covalent bonds

In this approach, covalent bonding is used at each fabrication step to build up LbL films. As a result, the choice of materials are no longer limited to charged or polar materials. Furthermore, small molecules can also be included in the LbL films, whereas the charges on small molecules are not usually sufficient for non-covalent LbL assembly. However, the labor,

time and energy needed in this approach is higher compared to using post-crosslinking as chemical reactions have to be conducted for buildup of every layer. These reactions might require long times or complex experiments based on their type.

Covalent LbL assembly has provided researchers with the tools to be able to tune the properties and the structures of nanofilms, while retaining the integrity and strength of the films for different applications, including nanofabrication and biomedicine. [44] Zhang et al. reported covalent LbL assembly of triazole on a copper substrate using copper(I)-catalyzed azide-alkyne cycloaddition click chemistry reaction. The copper substrate was alternately dipped in the click assembly solution, containing dodecylbenzenesulfonyl azide and 3-butyn-1-ol, and CuCl solution to form LbL films. They demonstrated that by depositing three layers, an anti-corrosion efficiency of 96.9% could be achieved in a 3% NaCl solution. [45] Huang et al. successfully prepared nanofilms made of active ester, poly(pentaurophenylacrylate)-b-poly(styrene) (PPFPA-b-PS), and amine rich polymer, poly(ethylenimine) (PEI) using covalent bonds. PPFPA-b-PS is a hydrophobic copolymer, while PEI is hydrophilic. Therefore, the resulted film showed switchable wettability by changing the outmost polymer layer. The switchable wettability also enabled the researchers to tune cell adsorption on the surface by changing the outmost layer, leading to higher cell adhesion on hydrophobic films ended with PPFPA-b-PS. Additionally, the outmost surface could be easily post-modified, obtaining a free standing film. [46] Xiang et al. alternately deposited two zwitterionic copolymers bearing alkynyl-poly(sulfobetaine methacrylate) (alkynyl-PSBMA) and azide-poly(sulfobetaine methacrylate) (azide-PSBMA) on an azido-functionalized polysulfone (PSF-N₃) membrane using click reaction. After modification, the membrane showed protein and platelet adsorption resistance, along with longer clotting times, creating blood-compatible membranes. Additionally, alkynyl-citric acid deposited on the outmost layer using click reaction induced anticoagulant properties to the membrane. [47]

Post-crosslinking using covalent bonds

Another approach for covalent LbL assembly is to fabricate LbL films using non-covalent interactions, mostly electrostatic interaction, and crosslink the films using covalent bonds afterwards. The crosslinking can be done via temperature, UV, or using bifunctional agents. One of the advantages of this method is the lower needed time, labor and energy, as build up of the films using non-covalent interactions can be done much easier, compared to films deposited using covalent bonds, and the crosslinking can be conducted in a one-step process. Moreover, the control and adjustability over crosslinking degree and film architecture in this approach is much higher. However, the material choice is limited to crosslinkable charged or polar materials.

An example would be the crosslinking of LbL assembled (poly(allylamine hydrochloride) (PAH)/polyacrylic acid (PAA)) films, where raising the temperature to around 200° C leads to amide formation from amine groups and carboxylic acid groups in PAH, and PAA, respectively. [44] Crosslinking can also be done by using crosslinking agents like genipin, which is a natural

crosslinker for materials, such as collagen, CHI and gelatin. The reaction can be done at room temperature and it can be accelerated at higher temperature. Silva et al. fabricated hollow tubes as blood vessel substitutes by depositing (CHI/alginate (ALG)) multilayers on a sacrificial template, followed by template leaching. Then, they crosslinked the tubes using genipin, leading to increase in mechanical properties and decrease in water uptake. [48] Another approach is using photochemistry to crosslink LbL films. In this method, a bifunctional molecule is infiltrated into pre-fabricated films using non-covalent LbL assembly. Then, crosslinking is done by UV irradiation. Zhang et al. fabricated (PAH/PAA) films using electrostatic LbL assembly and they were able to successfully enhance the stability of the films by infiltration of 4,4'-diazostilbene-2,2'-disulfonic acid disodium salt into the LbL films, followed by subsequent UV irradiation. The crosslinked films did not dissolve by ultrasonication in saturated sodium dodecyl sulfate solution for 10 minutes, in contrast to non-crosslinked LbL films. A scheme of the LbL assembled films and the crosslinking reaction can be seen in Fig. 1.10. [49]

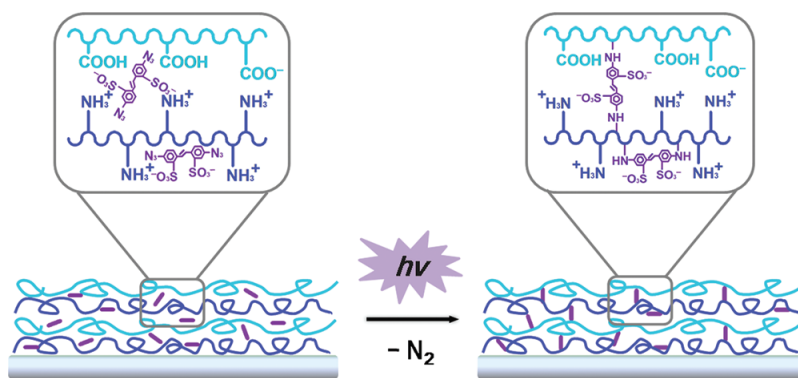


Figure 1.10: Schematic illustration of LbL assembled (PAH/PAA) film and the photocrosslinking reaction between 4,4'-diazostilbene-2,2'-disulfonic acid disodium salt and the polyelectrolytes [49]

1.3.3 LbL methods

LbL assembly is a simple technology, which allows the operator to deposit nano- or micro-films without much effort. LbL assembly methods include conventional and unconventional methods. In conventional methods, common technologies and driving forces are used to fabricate LbL multilayers. There are three main conventional methods for assembling LbL multilayers: 1. Dipping method 2. Spin-assisted method 3. Spray-assisted method. A schematic illustration of each method is shown in Fig. 1.11. [25]

Unconventional methods, however, take advantage of building blocks, which are not usually associated with LbL assembly. For instance, metal ions, inorganic-organic hybrids, and cellular multilayers have been used to fabricate LbL multilayers. Additionally, novel technologies

like inkjet deposition are also part of unconventional methods for LbL assembly. [24] In this section, we will only focus on conventional LbL assembly methods.

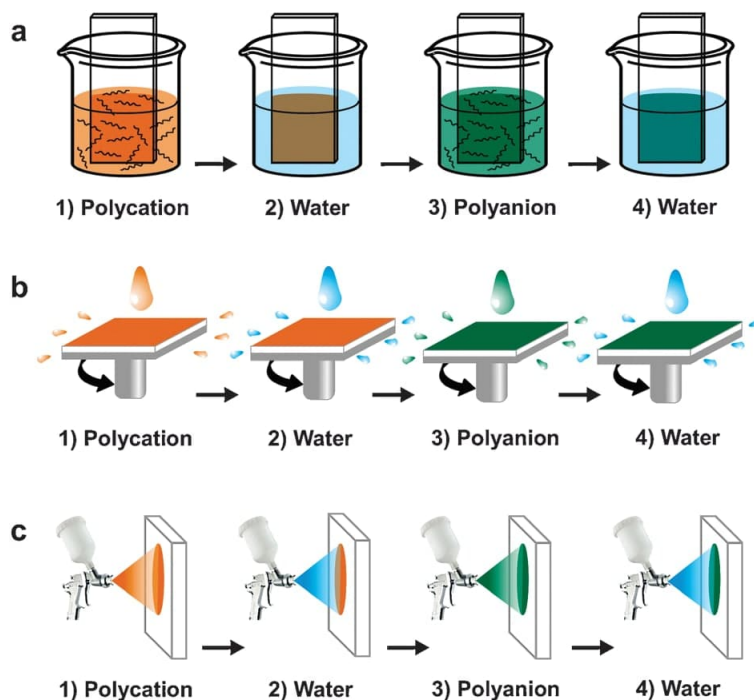


Figure 1.11: Schematic illustration of main conventional methods for LbL assembly; (a) Dipping method (b) Spin-assisted method (c) Spray-assisted method. [25]

Dipping method

The traditional dipping method consists of alternately dipping a substrate in polyelectrolyte solutions, or other types of building blocks used for LbL assembly, along with a rinsing step in between. When the substrate is initially dipped in polycation, or polyanion solution depending on the charge of the substrate, the polyelectrolyte diffuses to the substrate and is adsorbed to form an adsorbed layer on the surface. This causes a reversal of charge on the surface of the substrate. A rinsing step is required between adsorption steps to wash away any non-adsorbed polyelectrolyte chains, as well as to avoid contamination of the following polymer solution. The second polyelectrolyte with the opposite charge can then be deposited on the previous film by dipping the substrate in the solution, as shown in Fig. 1.11a. A dipping robot is usually used in order to fabricate LbL films using the dipping method. The dipping method is a very simple approach for LbL assembly. However, it is generally used to fabricate thin films due to the long dipping time needed for depositing one layer, which is around several minutes. For making dry films in the micrometer range, several hundred layers have to be deposited which makes the process quite time-consuming. [25] In this study, dipping method is used to build LbL assembled multilayers.

The major parameters controlled in the dipping method are temperature, ionic strength, and pH (only if weak polyelectrolytes are used), which greatly affect the morphology, film thickness and composition of the multilayers. This gives a quite accurate control over the properties of the LbL assembled films. [25]

Silicon tubes, a model of catheters, have been reported to be dipped in chlorhexidine-loaded micelles and PAA for deposition. After LbL assembly modification, both the inner and the outer surfaces of silicon tubes were modified with antimicrobial films. They also chemically crosslinked the films in order to improve film stability and tune release rate of encapsulated chlorhexidine. [50]

Spin-assisted method

In spin-assisted LbL assembly, each layer is deposited on a charged substrate by the help of a spin coater. Each solution is poured on the spin coater, followed by rotation of the plate at a fixed rate. This will deposit one layer of the polymer on the plate. Similar to the dipping method, this is followed by a washing step, before the next layer is deposited in a similar manner. The main advantage of the spin-assisted method is its ability to build up films in micrometer range as the deposition step for each layer and the subsequent rinsing step takes only a few seconds. Thus, several hundred layers can be deposited in a relatively short time. Furthermore, by using spin-assisted LbL assembly, non-polar or uncharged materials can also be used as the building blocks. Finally, the films obtained using this method are smoother and have much higher internal order compared to the traditional dipping method. The main parameter determining the film thickness in this method is the spinning rate. However, spin-assisted LbL faces more difficulties when coating uniform films on large or non-planar surfaces. [25]

Zhao et al. used spin coating to create anti-corrosion films in order to protect AZ31 magnesium alloys. They used (PVPON/PAA) as the building blocks. Spin coating was used instead of dipping method in this case due to the fact that spin coating was able to fabricate smooth, dense and defect-free films. On the other hand, the substrate coated using dipping method was prone to corrosion due to pin holes and rough surfaces. They demonstrated that the dense and defect-free films could show excellent corrosion protection and adhesion strength. [51]

Spray-assisted method

Spray-assisted LbL assembly uses nozzles to alternately spray polyelectrolyte solutions onto the designated vertical substrate. In each step, a pump is used to add pressure on the spray to keep a constant spraying rate. After the spraying stage, the excess solution is removed from the substrate by solution draining. The obtained films using this method are much smoother than the films fabricated using dipping method and the deposition time for the spray-assisted method is much lower, in the range of a few seconds. Parameters, such as the

distance between nozzle and substrate, solution flow rate, and spraying/drying time can all be controlled as a way to tune the film composition and structure. [25]

As an example, Heo et al. showed how spray-assisted LbL assembly could be used to deposit graphene oxide sheets on a polyethylene substrate. They tried to induce oxygen barrier properties by alternately depositing negatively charged graphene oxide and positively charged amine-functionlized graphene oxide, on the substrate. They concluded that the oxygen transmittance rate decreased significantly due to the presence of the densely packed graphene oxide layers. Additionally, the substrate was able to maintain its transparency, which made this coating suitable for food packaging applications. [52]

1.3.4 Linear vs exponential growth

Most of the polyelectrolytes employed in LbL assembly demonstrate a linear growth behaviour, which means that the thickness of the film increases linearly after each deposition step. Considering the thickness gained after each step, around several nanometers, it can be understood that the final thickness of films would not be very thick in linear growth. However, certain LbL multilayers, such as the ones involving poly(*L*-lysine) (PLL), as polycation and ALG, hyaluronic acid (HA), or poly(glycolic acid) (PGA), as polyanion show a different behaviour. These films grow in an exponential manner, meaning that deposition of even one layer of polyelectrolyte can sometimes increase the thickness up to several hundred nanometers. This is due to the fact that in these types of films, at least one of the components, such as PLL, can diffuse in and out of the whole thickness of the film and form complex films with the incoming new polyanion layer. Using exponential growth, one can rapidly deposit very thick films, which is not easy in linear LbL assembly. However, the exponential growth only occurs during the first several cycles. After a certain number of cycles, the ability of the polyelectrolyte to diffuse in and out of the film is inhibited due to the restructuring of the bottom layers. In the case of (PLL/HA) multilayers, Porcel et al. showed that the exponential-to-linear transition happens after 12 bilayers. [53] The diffusion of the polyelectrolyte in the film is also dependent on its molar mass as it determines its diffusion coefficient. Alkekhia and Shukla recently showed the diffusion of PLL in (PLL/HA) multilayers by incorporating a layer of fluorescent PLL in the film and observing the diffusion in the film thickness using confocal laser scanning microscopy. They used PLL with different molar masses (PLL³⁰: M_w 6.9 kDa, PLL⁹⁰: M_w 19 kDa, PLL⁴⁰⁰: M_w 85.1 kDa)

It can be seen in Fig. 1.12 that the thickness of the films obtained using PLL³⁰ is much lower than the rest. As for the PLL⁴⁰⁰, the fluorescent-PLL layer can be seen only on the top surface of the film, in contrast with lower molar mass PLL, where the fluorescence emission can be seen throughout the whole film. This demonstrates that PLL can diffuse through the whole film when its molar mass is low enough. [54]

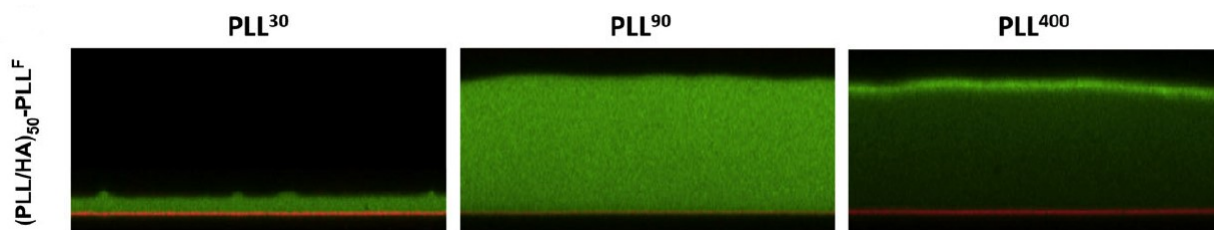


Figure 1.12: Confocal laser scanning images of the cross-section of $(\text{PLL}/\text{HA})_{50}\text{-PLL}^F$ multilayers using PLL with three different molar masses. [54]

1.3.5 Classes of materials

Biomacromolecules

Proteins, such as collagen and fibronectin are abundant in the human body, especially in the ECM, which surrounds the cells. Proteins are all made of amino acid units attached by peptide bonds and they can carry charges due to presence of amine or acids groups on the backbone. That is why they can be assembled as films driven by electrostatic interaction. These proteins can induce biocompatibility and cell recognition ability to the LbL film, which eventually can lead to cell adhesion, growth and differentiation. [27] Huang et al. used the combination of CHI and collagen for LbL assembly on polycaprolactone /cellulose acetate electrospun mats, as polycation and polyanion, respectively. They observed that deposition of collagen greatly enhances cell migration of normal human dermal fibroblasts *in vitro* and up-regulate the secretion of ECM *in vivo* to improve skin regeneration as a wound healing approach. [55]

Polypeptides also show high biocompatibility, biodegradability, and cell recognition sites. Examples such as PLL and poly(D-lysine) (PDL) are quite commonly used for LbL assembly due to their excellent biological properties. Zhou et al. designed a gene and drug co-delivery system using hollow mesoporous silica and LbL assembly of positively charged PLL and negatively charged gene, which released drugs and genes upon enzyme degradation of PLL. [56]

Polysaccharides are among the most important types of biomacromolecules used in LbL assembly. They consist of monosaccharides linked together by O-glycosidic bonds. Polysaccharides are very useful for LbL assembly as many of them have acid groups, and few have amine groups, on the backbone, which makes them suitable polyelectrolytes for LbL assembly. They also have great biocompatibility and biodegradability features. CHI, ALG, HA and dextran sulfate are some of the most important polysaccharides used in different domains. [27] Wang et al. used CHI and dextran sulfate in a dual drug delivery system for anti-cancer drugs. They used LbL assembly of dextran sulfate and CHI on a CHI nanoparticle to make a double-layer. The nanoparticle and the external CHI layer was loaded with two different anti-cancer drugs. Therefore, they were able to devise a pH-sensitive drug delivery system. The schematic illustration of the drug delivery system is shown in Fig. 1.13. [57]

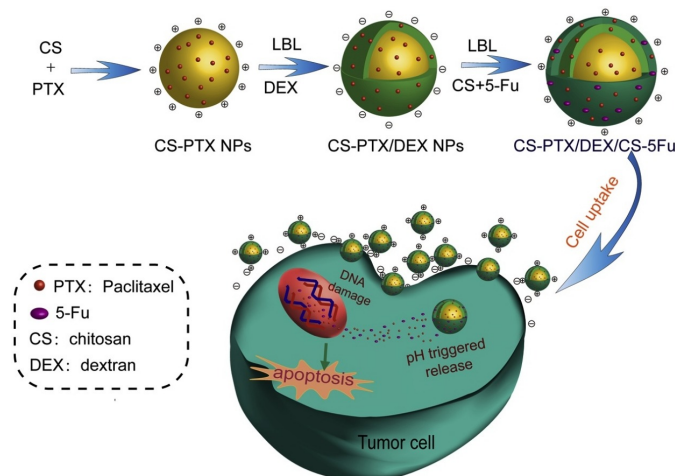


Figure 1.13: Schematic illustration of the dual drug delivery system for anti-cancer drugs using LbL assembly of dextran sulfate and CHI on CHI nanoparticles. [57]

Inorganic compounds

Aside from biomacromolecules, which are the most common type of materials used for LbL assembly, some inorganic compounds are also used as a way to induce exceptional mechanical, electrical, and surface properties. Carbon nanotubes (CNT), for instance, have been used a lot in research recently, mostly to induce electrical conductivity. Jia et al. used LbL assembly of CNT and CNT-based inorganics for creating a conductive high temperature proton exchange membrane. They alternately deposited a type of cationic polyurethane as polycation, and CHI and CNT-based inorganics as polyanions in order to develop such membranes. [32]

Different layers of silicate clays have also been used in LbL assembly. They can act as adsorbents, ion-exchange reagents or as diffusion barriers. Montmorillonite is one of the most abundant natural clays and it has a distinctive 2D sheet morphology. It has been used by Chen et al. to make hierarchially mesoporous and hollow spheres made of CHI and montmorillonite based on LbL assembly. [33] Furthermore, rectorite is also used as an antibacterial compound in LbL assembly. Chen et al. deposited CHI, as polycation, and rectorite, as polyanion, on a silk fibroin nanofibrous scaffold. Their results showed that deposition of rectorite actually increased the antibacterial properties of the scaffold against *S. aureus* and *E. coli*. [58]

1.4 Antibacterial materials

The application of antibacterial materials is proven to be one possible way to selectively kill bacteria for a wide range of applications, especially in the biomedical field. Among the diverse types of antibacterial materials, antibiotics are the most commonly used ones, ranging from household use to hospital care. Silver nanoparticles, and also antibacterial macromolecules,

including synthetic polymers, biopolymers, proteins, enzymes, etc. are also among widely used antibacterial materials.

Depending on the working principle, the antibacterial films can be classified into three types: 1) Bacteria-repelling films, 2) Leachable films, and 3) Contact-killing films, as shown in Fig. 1.14. [59]

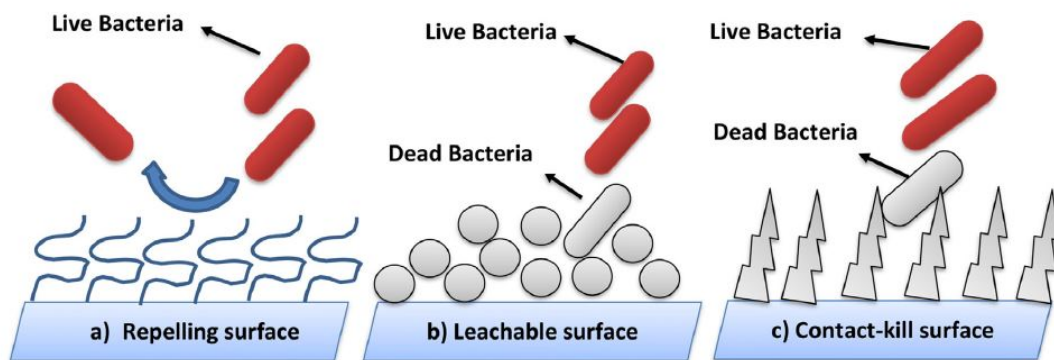


Figure 1.14: Schematic illustration of three different strategies for creating antibacterial films. [59]

1.4.1 Bacteria-repelling films

Bacteria-repelling films prevent the adhesion of bacteria to the film without killing them. Bacteria generally adhere to surfaces through electrostatic and hydrophobic interactions. [60] There are different approaches to inhibit the contamination and biofilm formation of bacteria on the surface. One of these approaches is to control the hydrophobicity and roughness of the films. [59] The effect of roughness and hydrophobicity on bacterial adhesion is complex and the correlation depends on the type and strain of bacteria, as well as the substrate. Furthermore, surface topography is also quite influential on bacterial adhesion. [61] Crick et al. used a rough and superhydrophobic thin film made of a silicone elastomer to reduce the adhesion of *E. coli* and methicillin-resistant *S. aureus* without chemical modification. [62] On the other hand, Tang et al. showed that higher roughness can lead to higher adhesion of *S. epidermidis* on fluoroalkylsilane-coated silicone because the surface irregularities can protect the bacteria against shear forces. The higher roughness also provides higher contact area between the substrate and the bacteria. However, the effect of roughness can only be seen above a certain threshold value. Regarding the effect of hydrophobicity, Tang et al. showed a reduction in *S. epidermidis* adhesion with increase in surface hydrophobicity. [63] In contrast, MacKintosh et al. showed a higher adhesion of *S. epidermidis* in hydrophobic layers. [64] The mechanical properties of the surface are also important for bacterial adhesion. Lichter et al. fabricated multilayers of PAH and PAA, and they changed the stiffness of the film over a range of 1 MPa to 100 MPa by changing the pH. They stated that layers with

higher Young's modulus showed much greater adhesion of *S. epidermidis*, such that a 100-fold increase in layers stiffness led to approximately 100-fold increase in bacteria colony density.[65]

Another way to hinder the attachment of bacteria is using bacteria-repelling coatings. Some types of coatings can repel bacteria through actuation or electrostatic repulsion. Other types prevent bacterial adhesion by resisting protein fouling, since bacterial adhesion occurs through a layer of adsorbed proteins. One of the most common polymers used for preventing bacterial adhesion is PEG. PEG brushes are known for their protein resistance, as well as their flexible chains, which can induce high steric repulsion against bacteria. As the molar mass of PEG chains increases, the antibacterial efficiency increases as well. However, PEG is not suitable for long-term use as it becomes oxidized in complex media. [60] PAA polymer brushes have also been fabricated to prevent bacterial adhesion. As the pH goes above the isoelectric point of PAA, 4.5, the PAA brushes become ionized and they stretch out in the media. Yadav et al. stated that even though the chains become hydrophilic at this point, they can repel bacteria due to a physical actuation effect and the electrostatic repulsion between the negatively charged bacteria membrane and PAA chains. This effect allows changing the antibacterial properties of the surface by just changing the pH. The authors showed that increasing the chain length or brush thickness dispersity can improve the antibacterial properties of the brush. A schematic image of the PAA brushes is shown in Fig. 1.15. [66]

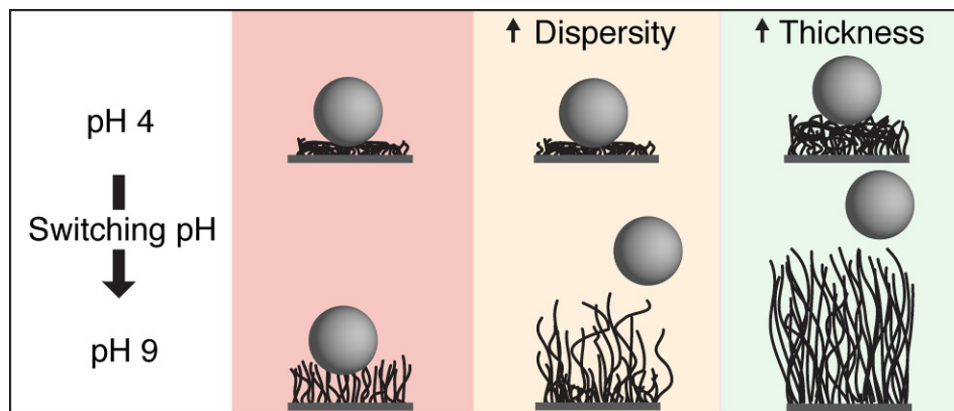


Figure 1.15: Effect of brush thickness and thickness dispersity on antibacterial efficiency of PAA polymer brushes. [66]

Even though the bacteria-repelling films can prevent surface contamination and subsequent biofilm formation, they still do not kill the microorganisms. Therefore, the bacteria are present in the environment and can contaminate other targets. Other approaches using bactericidal compounds can be used instead to kill the bacteria, either using release-based or contact-killing strategies. [59]

1.4.2 Leachable films

The second type of antibacterial films are based on release of antibacterial agents in the surrounding environment. The most used antibacterial agents in this approach are nanoparticles like silver or zinc oxide, antibiotic drugs, and nitric oxide. [59] Meng et al. deposited LbL assembled multilayers of (PAA/poly(dimethyldiallylammonium chloride) (PDDA)) on a silk substrate to create a 3-dimensional matrix for subsequent *in-situ* synthesis of silver nanoparticles. They demonstrated that the release of Ag^+ could last up to 120 hours, showing long antimicrobial activity against *S. aureus*. [67]

Antibiotics are still the main choice for antimicrobial purposes, especially in nosocomial infections. In order to fabricate antibacterial films for wound dressings, implants, etc., the antibiotic drug is usually incorporated in the coating to allow controlled release of the drug in the corresponding environment. [59] Zhuk et al. investigated the LbL assembly of TA and the cationic antibiotic, gentamicin, on a silicon substrate in order to create a self-defensive film. The film showed no release of gentamicin at pH of 7.4 for months. However, when pH was decreased to 4.5, either by adding acid in the medium or due to acidification of environment by bacteria, such as *S. epidermidis*, gentamicin was released from the film because the TA ionization degree decreased and the bonds between TA and the antibiotic were weakened. The release rate could be controlled by changing the (TA/antibiotic) film deposition parameters or using different types of antibiotics with stronger or weaker bonds to TA. [68] Even though antibiotics and silver nanoparticles are quite effective against different types of bacteria, they have quite big drawbacks. Silver nanoparticles, for instance, are known to be toxic to mammalian cells, causing DNA damage and apoptosis in human cells. These effects limit the use of silver in biomedical applications. [69, 70] Moreover, antibiotic resistance has become one of the current major problems worldwide. Emergence of multidrug resistance in Gram-positive and -negative bacteria has caused a serious problem in treating microbial infections. That is why using alternative methods for combating bacteria is being investigated. [71]

1.4.3 Contact-killing films

The last antibacterial strategy is employing contact-killing films. These types of films can directly kill bacteria upon contact. Contact-killing films generally do not induce resistance in bacteria, except in the case of immobilized antibiotics. This makes them quite favorable compared to other methods. Quaternary ammonium compounds (QACs) are among the most common contact-killing polymers. They consist of four alkyl groups attached covalently to a nitrogen atom, thus carrying a positive charge. (R_4N^+) Three major mechanisms have been proposed for the antibacterial properties of QACs. The first one is the **polymeric spacer effect**, which states that the antibacterial group, here QACs, situated at the end of the polymer chains or on lateral chains can penetrate the cell wall of Gram-positive bacteria and damage the inner cell membrane, leading to bacteria death. Thus, considering this mechanism, the molar mass of the attached polymer chains have to be high enough to be

able to reach the inner membrane of bacteria. As some studies have shown this mechanism not to be valid in all cases, **Ion-Exchange mechanism** has been suggested in literature. This mechanism involves the exchange of divalent cations, existing on bacteria membrane, with the ions of QACs. This can result in destabilisation and subsequent death of bacteria. In this mechanism, the chain length does not play an important role, rather surface charge density is an essential parameter. There exists a surface charge density threshold for optimum antibacterial effect. This threshold depends on the type of bacteria, their metabolic state, and the QACs structure on the surface. The third mechanism of QACs, namely **Phospholipid sponge effect**, has been proposed recently and states that the cationic surface of the material can pull anionic phospholipids out of bacterial membrane towards itself to create holes in the membrane, leading to bacteria death [59, 72]

Another group of antibacterial agents used for contact-killing is N-chloramine compounds. Cyclic and acyclic organic N-chloramines are durable and they show great antibacterial activity against a wide range of bacteria. Free N-chloramines initially chlorinate the protein matrix around bacterial membrane. Then, they can penetrate into the bacteria and oxidize main cell constituents and also denature proteins, leading to bacteria death. [59]

Aside from these common antibacterial agents, many other types of polymers have been studied as antibacterial agents with the ability to kill bacteria upon contact. For instance, antibacterial polycations are commonly used for biomedical applications. These materials only show antimicrobial properties in the pH range in which they carry a positive charge. Hereby, a number of important antibacterial polycations are explained in more detail.

1.4.4 Chitosan

Chitosan (CHI) is a widely used polysaccharide, synthesized by chitin deacetylation. Chitin itself is a biopolymer obtained from different sources, such as crustacea, fungi and insects. CHI is used in various biomedical applications, including wound healing [73, 74], drug delivery [73, 75], tissue engineering [76], and antibacterial films. [77, 78] The chemical structure of CHI can be seen in Fig. 1.16. [79] Due to the presence of amine groups in CHI, it can be ionized in the pH range below its pK_a , which is 6.3-6.5, leading to a positive charge on the polysaccharide. This positive charge is the basis for the antibacterial activity of CHI. There exists different mechanisms for the bactericidal properties of CHI and the dominance of a mechanism is determined by the type of bacteria. In the case of Gram-positive bacteria, the bactericidal property of CHI is associated with the electrostatic interaction between the positively charged CHI and polyanionic teichoic acids on the bacteria surface. Teichoic acids are responsible for vital cell functions, such as protection against external stress, enzyme activity control, etc. Therefore, the electrostatic interaction between CHI and teichoic acids can disrupt the vital cell functions and denature the cell membrane leading to cell death. In the case of CHI samples with low molar mass, penetration of oligo-CHI into the inner membrane results in distorting DNA synthesis and bacteria death. As for the Gram-negative bacteria, both chelation-based and electrostatic mechanisms have been proposed. [80]

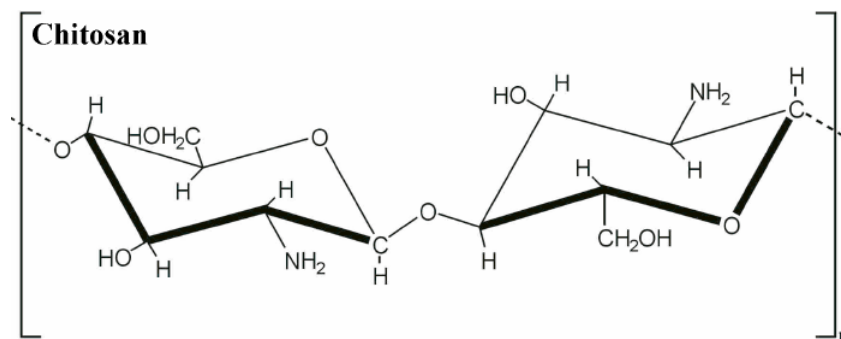
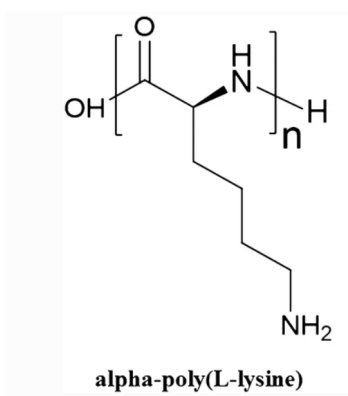


Figure 1.16: Chemical structure of fully deacetylated CHI. [79]

In either case, electrostatic interaction between CHI and the negatively charged bacteria plays an essential role in antibacterial features of CHI. [80] Therefore, increasing the deacetylation degree of chitin, using CHI at low pH, or even using quaternized CHI can enhance its antibacterial properties. Jiang et al. showed that by using quaternized carboxymethyl CHI intercalated in organic rectorite and ALG, LbL films show very good antibacterial activity against both *E. coli* and *S. aureus*. [81]

1.4.5 Poly(*L*-lysine)

α -poly(*L*-lysine) (PLL) is a synthetic polypeptide, which carries a high positive charge density below its isoelectric point, 10.6. [82] As can be seen in Fig. 1.17, the positive charge is due to the presence of amine groups in PLL structure. [83] PLL has been widely used in different applications, such as drug delivery [84, 85] gene delivery [86], cell tissue culture [87], etc. All these applications take advantage of the positive charge in PLL structure, leading to electrostatic interactions between PLL and cells, DNA/RNA or polyanionic macromolecules used in drug delivery. Moreover, the positive charge of PLL makes it a suitable candidate for antibacterial applications.

Figure 1.17: Chemical structure of α -poly(*L*-lysine) (PLL). [83]

Alkekhia and Shukla assembled (PLL/HA)₅₀ LbL films to investigate the effect of PLL molar mass on its antibacterial activity against both Gram-negative and Gram-positive bacteria. *S. aureus* bacteria coverage on the (PLL/HA)₅₀ coated glass substrates are represented in Fig. 1.18. Films deposited from three molar masses of PLL were very effective against *S. aureus*. They hypothesized that the antibacterial activity of (PLL/HA) films are determined by both contact-killing and the release of PLL into solution, and that the release of PLL is less when using higher molar mass because of the hindered diffusion of PLL in the film. [54] Therefore, in this study, we used PLL with a high molar mass in order to limit the dominant antibacterial mechanism to contact-killing.

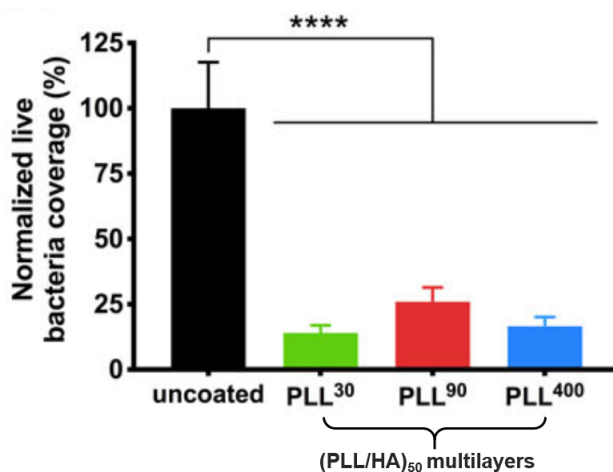


Figure 1.18: *S. aureus* normalized coverage on glass substrates coated with (PLL/HA)₅₀ films, ended with HA, using PLL with different molar masses. Three different molar masses of PLL were used; PLL³⁰: M_w 6.9 kDa, PLL⁹⁰: M_w 19 kDa, PLL⁴⁰⁰: M_w 85.1 kDa [54]

1.4.6 Poly(ethylenimine)

Poly(ethylenimine) (PEI) is a synthetic, basic, aliphatic polymer carrying a positive charge below its isoelectric point of around 11 due to the presence of amine groups. PEI exists in linear, and branched structure. The linear and one possible branched structure of PEI are shown in Fig. 1.19. [88] The linear PEI only contains secondary amines, whereas branched PEI has primary, secondary, and tertiary amines in its structure. PEI is widely used for gene delivery, similar to PLL, due to its complex forming ability with DNA, and also as a cell attachment factor for weakly anchoring cells. However, the disadvantage of PEI is that the highly cationic nature of PEI can also cause cell wall disruption and cell death. [89, 88, 90, 91] Vancha et al. showed that PEI coating of dishes could strongly enhance attachment of PC-12 and HEK-293 cells compared to other coatings, like collagen. [92]

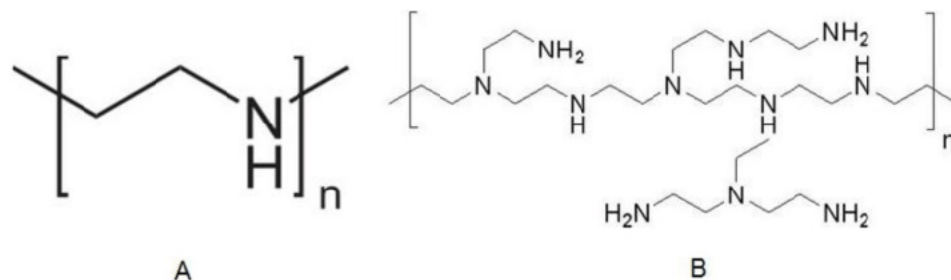


Figure 1.19: Chemical structure of (A) linear, and (B) branched PEI. [88]

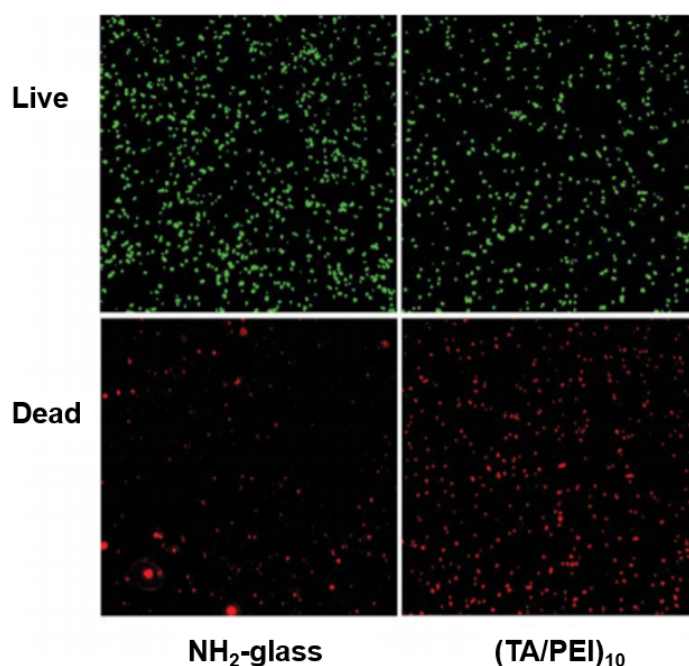


Figure 1.20: Live/Dead imaging of *S. aureus* bacteria in contact with amino-modified (NH_2 -) glass coated with and without $(\text{TA/PEI})_{10}$ films. The films were deposited by LbL assembly driven by covalent bonding between TA and PEI. [93]

PEI is also a great candidate for bactericidal coatings as the high positive charge density on the polymer can disrupt bacteria cell walls and induce permeabilization, like other polycations. Azevedo et al. reported the antibacterial activity of PEI and nanoparticles made of cross-linked PEI against both Gram-negative and Gram-positive bacteria, including *S. epidermidis*, concluding that they can inhibit bacterial growth and reduce biofilm metabolic activity of both types of bacteria, with a higher effect against Gram-positive ones. They also mentioned that incorporating quaternary ammonium moieties in PEI-based nanoparticles could lead to a higher antibacterial effect against *S. aureus* and *E. coli*. [89] However, He

and Chan reported a higher antibacterial efficiency of LbL films made of covalently bound (TA/PEI)₁₀ films, deposited on amino-modified (NH₂-) glass, against Gram-negative *E. coli* compared to Gram-positive *S. aureus*. The difference can be due to the different systems involved in the two studies. The Live/Dead imaging results can be seen in Fig. 1.20, where only around 50% of *S. aureus* bacteria were found dead. [93]

1.5 Antibacterial LbL films

LbL assembly is a powerful tool to fabricate antibacterial films and has been extensively employed for this application in the literature. The versatility of LbL assembly allows access to a wide range of materials, assembly methods and film architectures, which is very useful for creating antibacterial films. Antibacterial LbL films consist of bacteria-repelling films, leachable films, and contact-killing films, as explained in section 1.4. [94]

Bacteria-repelling LbL films mainly prevent adhesion of bacteria by changing the surface wettability, stiffness or charge. Tang et al. modified the surface of a polysulfone microfiltration membrane by LbL assembly of (PAH/PAA) multilayers as a way to prevent bacterial adhesion. The bacterial removal efficiency reached around 99% by LbL coating, which was attributed to the hydrated and swollen multilayers, hindering the direct contact of bacteria with polysulfone membranes. [95]

Leachable LbL films are based on incorporation of bactericidal agents, such as metal ions, antibiotics and antimicrobial peptides in the LbL films, followed by their release in the surrounding environment. Cado et al. created self-defensive leachable LbL films by depositing HA, functionalized with cateslytin, an antimicrobial peptide, and CHI on a planar surface. Hyaluronidase, secreted by pathogens like *S. aureus* enzymatically degraded the films, leading to release of cateslytin and complete inhibition of *S. aureus* growth. [96] Silver ions, antibiotics and nitric oxide have also been used extensively for this purpose. [67, 68, 94]

Contact-killing LbL films are fabricated by immobilizing bactericidal components, such as polycations, antibiotics, and antimicrobial peptides on LbL films. As a result, bacteria are killed upon contact. Lichter and Rubner used (PAH/poly(styrene sulfonate) (PSS)) multiayers to create contact-killing LbL films showing pH-responsive antibacterial activity against *S. epidermidis* and *E. coli*. [65] Nisin, a low molar mass antimicrobial peptide, has also been covalently bound in LbL films made up of synthetic oxidized poly(3,4-dihydroxyphenylalanine) and PAH deposited on stainless steel, showing antibacterial effect against *Bacillus subtilis* by contact-killing. [97]

The antibacterial mechanisms can also be used simultaneously in order to increase their effect. For instance, Zhan et al. fabricated LbL films by using HA and poly(amidoamine) dendrimer (PAMAM), combining the anti-adhesion properties of the films, due to improved hydrophilicity, and contact-killing properties of the positively charged PAMAM against *E.*

coli. [98] Negatively-charged heparin, an anti-adhesive agent, and positively charged CHI have also been used for this purpose, showing anti-adhesive and bactericidal activity against *E. coli*. [99]

1.6 Agarose gel

Agarose is a polysaccharide that constitutes one of the two components of agar. Agarose is a linear biomacromolecule with a molar mass of 120 kDa and is used widely for gel electrophoresis to separate large molar mass macromolecules, notably DNA molecules and proteins. Its structure is shown in Fig. 1.21. [100] The reason for using agarose gel is mainly its high gel strength compared to other types of hydrogels, like agar. Agarose dissolves in water only above 85 ° C. The sol-gel transition occurs by decreasing the temperature to the transition point. This point can vary based on agarose type, and concentration, and the transition is reversible by raising the temperature above 85° C again. [101] When agarose solution is cooled down, its structure goes from random coils in solution to double helices at first, and then to bundles of double helices when the gel is formed. [102] Agarose gel average pore size is from 100 nm to 300 nm. The pore size depends on the concentration of agarose and also the cooling rate. [103]

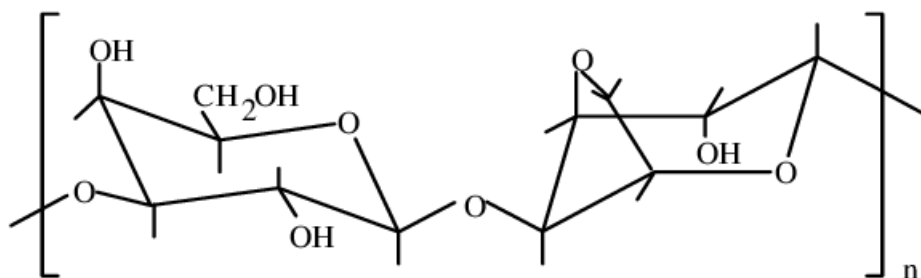


Figure 1.21: Chemical structure of agarose. [104]

Agarose has been widely used to encapsulate different types of cells. Jutila et al. successfully encapsulated chondrocytes in agarose gel to model the stiffness of healthy and unhealthy pericellular matrix, which is the environment of chondrocytes in articular cartilage, and to investigate the effect of gel stiffness on cellular response. [105] Kumachev et al. encapsulated mouse embryonic stem cells in agarose microgels with different elastic moduli to study the effect of microenvironment mechanical properties on cell fate. [106] Agarose has also been used to encapsulate bacteria. Eun et al. encapsulated *E. coli* in agarose microparticles to isolate, screen and test the effect of antibiotics against bacteria using very low amounts of reagents, compared to conventional methods of agar plates. [107] Bacterial encapsulation in agarose was also reported by Tuson et al. to measure the Young's modulus of live bacteria. [108]

1.7 Bacterial encapsulation

1.7.1 Overview

Bacterial encapsulation involves the entrapment of bacteria in a matrix. The encapsulation of bacteria is used for different purposes. Encapsulation is an effective way to protect the activity and function of bacteria from environmental stresses, including pH, temperature, salt ions, and moisture. Encapsulation has also shown to control the physico-chemical properties of the bacteria environment by tuning the properties of the matrix, such as porosity, or chemical stability. [109] That is why bacterial encapsulation has been widely studied recently in various applications, such as probiotics delivery in food industry [110, 111, 112], microbial fuel cells [113], environmental remediation [114, 115], diagnostics [116, 117, 118], etc.

One of the important parameters in bacterial encapsulation is the cell viability after encapsulation. Therefore, the encapsulation condition should be tuned to retain the most number of bacteria. The matrix should also be biocompatible with the entrapped bacteria. Additionally, the porosity of the matrix has to be chosen, such that the molecules necessary for bacteria growth, such as nutrients, oxygen, water, waste, and bacterial metabolites could pass through the matrix freely, while the bacteria are kept entrapped.

Bacteria are commonly encapsulated in hydrogels, such as ALG, agarose, and CHI or proteins like collagen and gelatin, as well as mesoporous silica. The choice of matrix material depends on the properties of bacteria, the application and the encapsulation method. [109] A wide range of methods have been studied to encapsulate bacteria in microbeads, microfibers, etc. Extrusion, electrospinning, LbL assembly, and microfluidics are among the most important methods. [111, 110, 117]

1.7.2 Extrusion

Extrusion technique is a very simple and cheap method to encapsulate bacteria in microbeads without using organic solvents, providing biocompatibility and flexibility. This method involves preparing a hydrocolloid solution composed of the matrix component and bacteria, and extruding the solution through a nozzle. A droplet is formed, and then falls into a hardening solution, which can solidify the droplet through ionic, chemical, photo- or thermal crosslinking, thus obtaining the microbead encapsulated with bacteria. The diameter of droplet depends on a series of parameters including the properties of the hydrocolloid solution (surface tension, viscosity, concentration), and process conditions (diameter of needle, liquid flow rate, distance between nozzle and gelling solution/collector). The size of the droplet can be reduced by adding pressure at high flow rate, or using droplet breaking systems, such as co-axial air flow, vibrational encapsulator, and jet-cutter. [111]

Among the widely used extrusion matrices, ALG is the most studied one for encapsulation of diverse bacteria because of its biocompatibility and easy operation steps. Castro-Rosas

et al. encapsulated *Lactobacillus paracasei*, in an ALG gel matrix using extrusion method for probiotics delivery in food products. They achieved an encapsulation efficiency of 87.6% and the encapsulated bacteria showed strong resistance to pH conditions, similar to stomach, and bile salts. They also showed higher viability during storage compared to free bacteria. [119] Li et al. encapsulated a genetically engineered strain of *E. coli* (*E. coli*-GFP) in ALG-based microbeads in order to take advantage of the *E. coli*-GFP towards pathogenic *Pseudomonas aeruginosa* (*P. aeruginosa*), while protecting the bacteria from external threats, such as pH, toxic compounds, etc. The encapsulation was done by electrostatic extrusion method and the divalent calcium ions in CaCl_2 act as ionic crosslinking agents, obtaining stable and robust microbeads with good permeability. The using of high voltage on extrusion allowed them to tune the size of microbeads between 100 and 300 μm by changing the electric potential, while keeping a high bacterial viability. They also tried photo-crosslinking methods afterwards to increase the stability of microbeads. A schematic illustration of the encapsulation process and the respond of *E. coli*-GFP to *P. aeruginosa* autoinducers is shown in Fig. 1.22. [120]

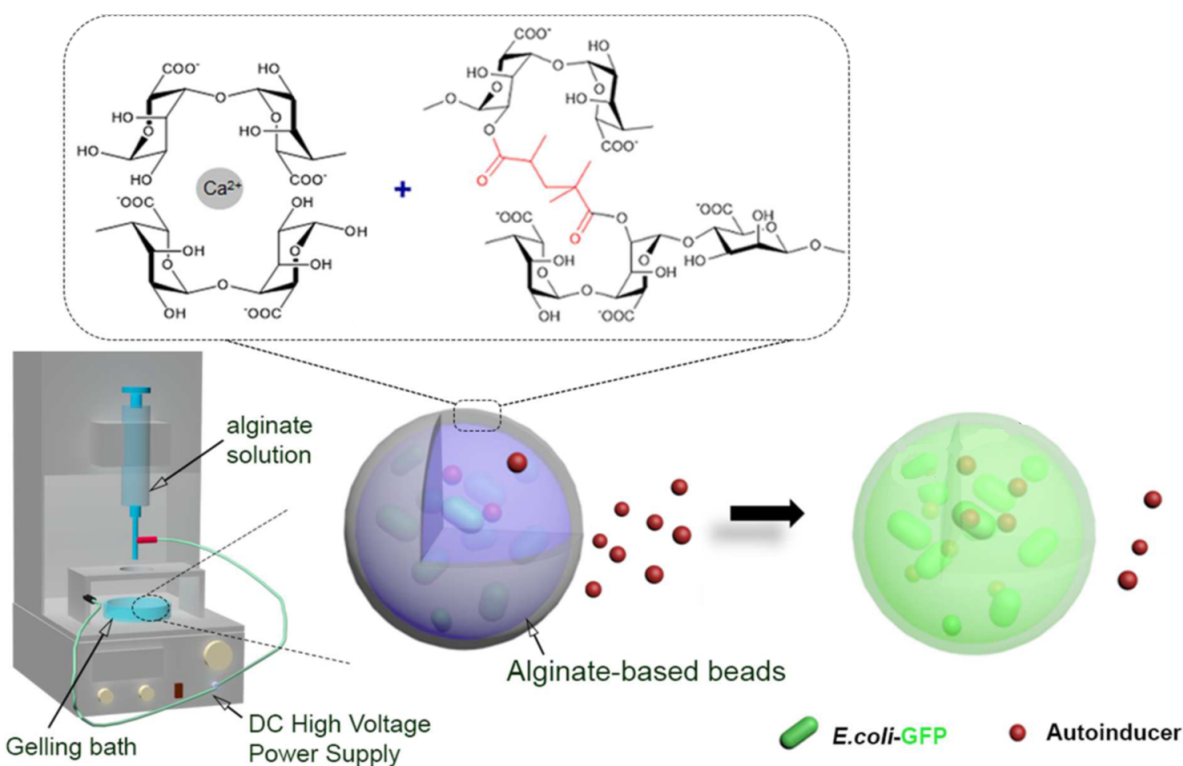


Figure 1.22: Schematic illustration of the electrostatic extrusion method, the encapsulation of *E. coli*-GFP in ALG-based microbeads, and chemical structure of photo- and ionic crosslinked ALG. *E. coli*-GFP can respond to autoinducers secreted from *P. aeruginosa* by expression of green fluorescence protein [120]

1.7.3 Electrospinning

Electrospinning is a cost-effective method to fabricate fibers in the range of 100 nm to 1 μm with a high surface-to-volume ratio. Principally, a high voltage source is applied on a polymer solution to let electrostatic force overcome surface tension. Under the effect of electrostatic repulsion force and Coulombic force, the natural hemispherical droplet at the tip of the capillary is distorted into a conical shape. This typical droplet is called Taylor cone, where fibers/particles are continuously produced. The solvent is thus evaporated and nano- or microfibers will be deposited on a collector. Fibrous scaffolds with high porosity and excellent mechanical properties can be obtained using this method. That is why electrospinning has been widely used in biomedical engineering applications. [121, 122]

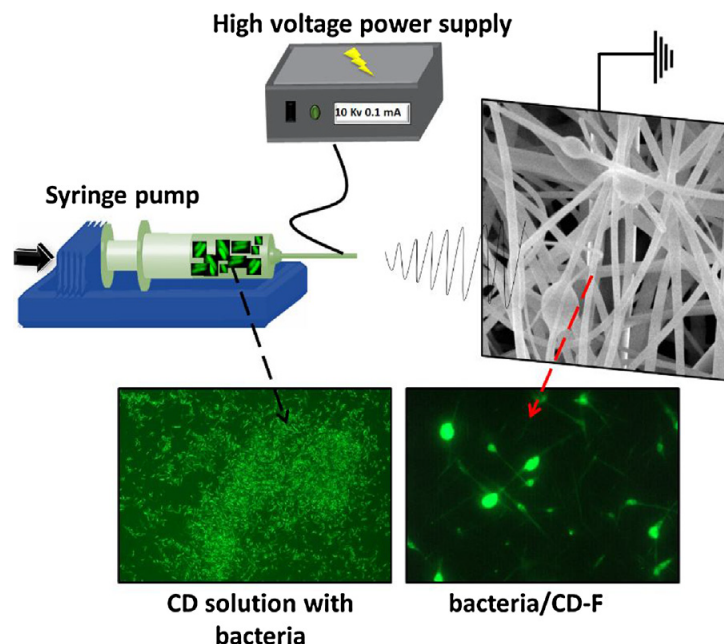


Figure 1.23: Schematic illustration of the encapsulation process of *Lysinibacillus sp.* NOSK bacteria in electrospun cyclodextrin (CD) fibers, along with fluorescence microscopy images of bacteria before and after encapsulation. (F: fiber) [115]

Bacterial encapsulation in electrospun fibers has been widely studied in the recent years. For this purpose, bacteria are dispersed in a polymer solution and during the electrospinning, bacteria are drawn into the charged jet and they become entrapped in the fabricated fibers. The hydrodynamic stress can damage the bacteria in the process. To prevent this effect, the viscosity of the polymer solution can be tuned to reduce the stress applied on the bacteria. Bacteria can also be encapsulated by core-shell electrospinning, where two polymer solutions are used; one for the shell and one for the core. In this approach, the bacteria can be dispersed in the core polymer solution, resulting in a bacteria-loaded core-shell fiber or even bacteria-loaded microtubes. [109]

San et al. encapsulated *Lysinibacillus* sp. NOSK bacteria in electrospun cyclodextrin (CD) fibers for bioremediation of heavy metals and reactive dye from wastewater. A schematic illustration of the encapsulation process can be seen in Fig. 1.23. They dispersed the bacteria in an aqueous solution of CD for bacterial encapsulation procedure. In addition to protection from environmental stress, CD also acted as the carbon source for bacteria. Despite the electrostatic field and osmotic changes, a significant number of viable bacteria maintained their functions. The encapsulated bacteria remained viable for 7 days at 4° C, and they were able to remove heavy metals and reactive dye from the water to a certain degree. [115]

1.7.4 LbL assembly

LbL assembly provides a versatile and simple method for encapsulating different kinds of microorganisms. As explained in section 1.3, LbL assembly allows great control over the properties and thickness of the multilayers. Thus, the cell surface properties, such as hydrophobicity and surface charge can be easily modulated. Transfer of oxygen, nutrients and bacterial metabolites can also be tuned, since the porosity of the multilayers can be controlled by changing the composition of multilayers or deposition cycle. In bacterial encapsulation, electrostatic interaction is the most common driving force for assembly of the multilayers, and the encapsulation process can be done in direct or indirect manner. [123, 124]

Direct bacterial encapsulation

In direct encapsulation, the polyelectrolytes are deposited directly on either single cells or cell aggregates. Thus, the polyelectrolytes, or other types of LbL building blocks such as particles, will be directly deposited on bacteria, which has been shown to bring functions into biohybrids. [124]

Franz et al. used different combinations of polyelectrolytes, such as PSS, PAA, and PGA as polyanions, and PAH and PDDA as polycations to directly encapsulate *Allochromatium vinosum* (*A. vinosum*) using LbL assembly. They concluded that the surface charge did not have an effect on the uptake of sulfide and elemental sulfur by bacteria. However, by increasing the number of layers, the multilayers could act as a physical barrier between bacteria and environment. The permeability of the multilayers could also be tuned by changing the polyelectrolyte combination. [123] Jonas et al. directly encapsulated *S. epidermidis* bacteria in (CHI/ALG)_n, and also (PAH/PSS)_n multilayers by LbL assembly. Multilayers deposition proved to delay the growth of bacteria, showing promising results in taking advantage of their metabolic activity for skin disease treatment. [125]

Indirect bacterial encapsulation

Indirect encapsulation of bacteria involves entrapping bacteria initially in a hydrogel or other templates, followed by LbL assembly of polyelectrolyte multilayers on top. Indirect encapsulation can be categorized in two types of bulk or hollow capsules. In bulk capsules, the core

is kept after the LbL assembly, whereas the core will be removed as a sacrificial component in hollow capsules. In case of bulk capsules using hydrogels, LbL coating provides stability to the hydrogel and allows a better control over diffusion properties, which is commonly used for hydrogel functionalization or drug delivery purposes.

Flemke et al. investigated the encapsulation of *E. coli* in porous calcium carbonate microparticles, followed by LbL assembly of (PAH/PSS) multilayers on top. They subsequently dissolved the calcium carbonate core, obtaining bacteria-loaded hollow capsules. The schematic illustration of the process is shown in Fig. 1.24. Using this method, cell viability of 40% was achieved. Additionally, the metabolic activity of encapsulated bacteria with LbL coating was delayed and the longest lag-phase was 36 hours. [126]

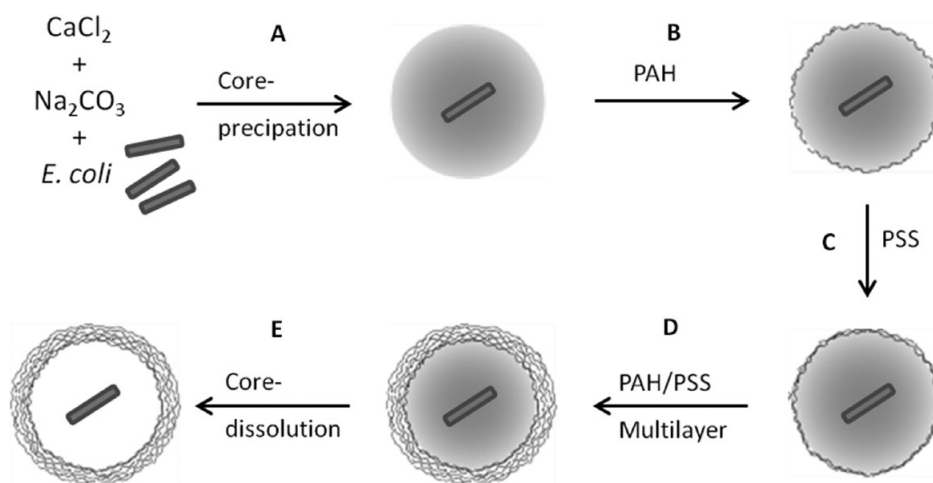


Figure 1.24: Schematic illustration of *E. coli* encapsulation in hollow microspheres using porous calcium carbonate microparticles as sacrificial component and LbL assembly of (PAH/PSS) multilayers [126]

1.7.5 Microfluidics

Microfluidic devices consist of channels in the range of ten to hundred micrometers and they are widely used for analysis and testing. Microfluidics have been employed in the field of bacterial encapsulation because it allows high throughput encapsulation using very small amounts of reagents. The flowing of fluid in the microenvironment of channels follows a laminar orientation that enables the accurate control of resulted particles. Additionally, multiple laboratory operations can be conducted in a short time using this technology. Bacterial encapsulation in microfluidics is conducted by entrapping bacteria in droplets, followed by crosslinking. Three of the most important configurations for droplet generation are shown in Fig. 1.25. In these systems, two immiscible liquids are used as dispersed and continuous phase. The bacteria is dispersed in a polymer solution as the dispersed phase and droplets are formed due to pressure or shear stress of the continuous phase. The continuous phase

can thus act as droplet carriers. In this system, channel geometry, solution viscosities and flow rate, and other parameters have to be taken into account to obtain uniformly-sized microbeads. [116, 117]

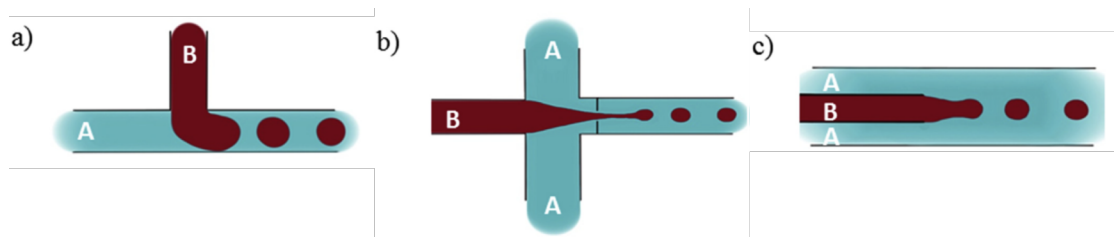


Figure 1.25: Schematic illustration three different microfluidics configurations for droplet generation; (a) T-junction, (b) flow-focusing, (c) co-flow (A: continuous phase, B: dispersed phase) [117]

Duarte et al. encapsulated single cells of *E. coli* in agarose gel using flow-focusing configuration for high-throughput screening. After incubation, bacteria formed microcolonies inside the microbeads, leading to amplified signal, making it suitable for screening. [118] Similar approaches have been used for investigating coexistence, interactions and signaling of *Streptomyces venezuelae*, *S. aureus*, and *E. coli* by encapsulating two of these bacteria in microfluidics double emulsions of water-oil-water. [127] Bacterial encapsulation by microfluidics has also been widely used for testing the effect of different antibacterial agents on bacteria in a very efficient way. [116, 128]

Chapter 2

Objectives

In this work, a novel approach is investigated for encapsulation of *S. epidermidis* to avoid its pathogenic consequences, while making use of its advantages for skin disease treatment. In a previous study, Jonas et al. tried to encapsulate *S. epidermidis* by depositing multilayers of (CHI/ALG)_n, and also (PAH/PSS)_n on each bacterial cell. Even though this approach was successful in delaying the growth of bacteria, the method was too demanding. [125] In another approach, Glinel et al. tried encapsulating *S. epidermidis* in a nanofibrillar patch. Briefly, they modified the pores of a PC membrane by depositing (PAH/PSS)₉ multilayers, resulting in the formation of nanotubes in the pores. Next, nanotubes were released by dissolving PC membrane, and the nanofibrillar patch was obtained by filtering a suspension of (PAH/PSS)₉ nanotubes and bacteria through a membrane. Finally, by depositing multilayers of (CHI/ALG)_n as additional antibacterial barriers on top of the patch, they were able to delay the release of bacteria from the patch by around 10 hours, while keeping their metabolic activity. [129]

Both methods mentioned above are quite complex and time-consuming. Moreover, they cannot prevent the release of bacteria, which can lead to pathogenicity if *S. epidermidis* enters the blood stream. That is why another approach is investigated in this study as a way to manufacture bacterial patches. Basically, in this approach, (CHI/ALG)₄ multilayers are deposited in the pores of a track-etched PC membrane to make their surface adhesive towards bacterial cells. Bacteria are then directly immobilized inside the modified pores. The resulting membrane is subsequently coated with agarose gel. Agarose acts as a physical barrier against the immediate release of bacteria from the pores of PC membrane. Additionally, the gel will be more friendly for skin application, and it also provides a wet environment, which is essential for bacterial activity. Afterwards, the patch is coated by LbL films incorporating antibacterial polycations to prevent the release of bacteria by contact-killing. PLL, PEI, and CHI have been investigated in this study as antibacterial polymers. The proposed method aims to prevent the release of bacteria, while maintaining their metabolic activity. A schematic illustration of the bacterial patch is shown in Fig. 2.1.

In this study bacterial patch is prepared, and the release and metabolic activity of bacteria is

investigated. The first objective is determining the successful fabrication of the bacterial patch. This involves the successful modification of PC membrane pores, encapsulation of bacteria in the pores of the modified membrane, and LbL assembly around the membrane-in-gel patch. The second objective of the study was to optimize the antibacterial barrier deposited around the bacterial patch by changing the LbL films composition, number of bilayers, and the outer polyelectrolyte. That is why the antibacterial effect of LbL films incorporating PLL, PEI, and chitosan on bacterial patch is studied and the effect of (PLL/HA) and (PEI/PSS) bilayer number, and the outer polyelectrolyte in (PEI/PSS) multilayers on antibacterial efficiency is tested.

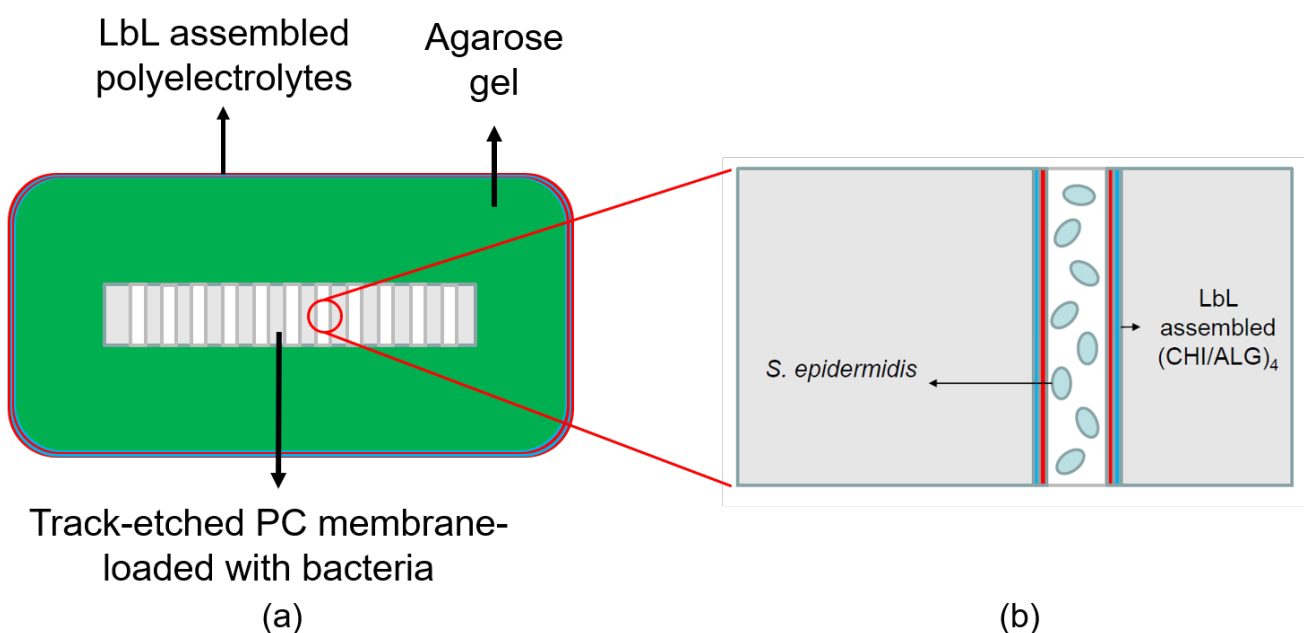


Figure 2.1: Schematic illustration of the cross-section view of the bacterial patch developed in this study; a) general structure of bacterial patch b) magnified image of the PC membrane showing modification of pore with LbL assembled (CHI/ALG)₄ and the encapsulation of bacteria inside the pores.

Chapter 3

Materials and Methods

3.1 Materials

Staphylococcus epidermidis (*S. epidermidis*, ATCC 12228) was purchased from ATCC and was kept in cryo-frozen state at -80° C. Track-etched porous polycarbonate (PC) membrane with a pore size of 1.6 μm , pore density of 10^7 cm^{-2} and thickness of 24 μm was supplied by it4ip, Belgium.

Protasan ultra pure chitosan (CHI, M_w 50-150 kDa, deacetylation degree $< 10\%$) was purchased from NovaMatrix, poly(*L*-lysine) (PLL, M_w 66 kDa) was purchased from Alameda Polymers and hyaluronic acid (HA, M_w 151-300 kDa) was obtained from Lifecore Biomedical. Sodium alginate (ALG), poly(ethylenimine) (PEI, M_w 750 kDa), sodium chloride (NaCl) and agarose were all purchased from Sigma-Aldrich. Poly(styrene sulfonate) (PSS, M_w 70 kDa) was purchased from Acros Organics. Poly(allylamine hydrochloride)-rhodamine (PAH-Rho, M_w 15 kDa, monomer to dye molar ratio of 392) was bought from Surflay Nanotec GmbH. Difco nutrient broth and agar were purchased from BD biosciences and alamarblue was purchased from Invitrogen.

Rhodamine B isothiocyanate, mixed isomers and fluorescein isothiocyanate (FITC)-dextran (M_w 3-5 kDa, 10 kDa, 40 kDa) were purchased from Sigma-Aldrich. Silica nanoparticles (1 μm diameter) were obtained from NanoCym.

Polyelectrolyte solutions, including CHI, ALG, PLL, HA, PEI, PSS and PAH-Rho solutions were prepared at 1 mg/mL, with NaCl concentration of 0.15 M. Rhodamine B and FITC-dextran solutions were prepared at 0.1 mg/mL and 0.5 mg/mL, respectively. Nutrient broth and agar were prepared at 8 g/L, and 23 g/L, respectively. pH of all polyelectrolyte and NaCl solutions were set to 6.0, except in the case of PC membrane pore modification, where pH was set to 4.0 instead. All solutions were prepared by using Milli-Q water with a resistivity of $18.2 \text{ M}\Omega\cdot\text{cm}$ obtained from a Merck Millipore system.

3.2 Free bacteria culture

All solutions and instruments in contact with bacteria were previously autoclaved using Tuttnauer autoclave or disinfected with 70% ethanol, purchased from VWR chemicals, and UV-light. Additionally, all operations were conducted under Airstream class-2 biological biosafety cabinet purchased from Esco.

Cryo-preserved *S. epidermidis* was firstly taken out of -80° C freezer and precultured overnight in 8 mL broth in a vented culture tube at 37° C and 180 rpm agitation using an orbital stirring. Then, the bacteria were transferred to 200 mL of broth in a bioreactor. The initial optical density (OD540) of the bacteria culture and pure broth was measured using Tecan Infinite M Nano+ microplate reader, and the bacteria were cultured in the bioreactor at 37° C and 120 rpm agitation. The OD540 of bacteria culture was checked and the culture was stopped when OD540 reached a value between 0.45 and 0.5, where the bacteria growth becomes exponential.

Next, the bacteria culture was centrifuged at 4000 rpm, 4° C using Eppendorf centrifuge 5804 R to remove the supernatant. Then, bacteria were twice rinsed with 0.15 M NaCl solution to remove the trace of broth. Finally, concentrated bacteria were re-dispersed in 0.15 M NaCl solution and stored at 4° C.

3.3 Bacteria staining

In order to detect *S. epidermidis* bacteria with confocal laser scanning microscope, the bacteria were stained by mixing 1 mL of bacterial suspension with 1.5 μ L of SYTO 9 green-fluorescent nucleic acid stain (excitation and emission wavelength at 480 and 500 nm, respectively), purchased from Invitrogen. The mixture was then stored for 20 minutes under biological safety cabinet before it was loaded in the PC membrane.

3.4 Preparation of bacterial patch

The preparation of bacterial patch involves many steps, all of which were done under a class-2 biological safety cabinet. All solutions were autoclaved using autoclave, except PLL, CHI, and ALG solutions, which were sterilized by filtering through 0.8 microns Pall life sciences Supor membrane.

3.4.1 Modification of the membrane pores

The pores of PC membrane were modified with (CHI/ALG)₄ multilayers at pH 4.0. To do so, PC membrane sheet was first cut into a 6 cm $\times$ 6 cm piece. For the first bilayer deposition, the membrane was dipped in CHI, then ALG solution for 20 minutes, each step followed by two rinsing steps in 0.15 M NaCl solution for 2 minutes. After the first bilayer, the dipping

time was reduced to 10 minutes. The bottom side of the membrane was decrusted rigorously using a cotton pad in 0.15 M NaCl solution after deposition of every two bilayers. When final decrusting of the bottom side was finished, the membrane was floated on a 3 M NaCl solution for 1 minute, top side down, followed by a quick decrusting of the top side using a cotton pad in 3 M NaCl solution. Finally, the membrane was rinsed three times in deionized water, and it was dried in the end.

Modified PC membrane was cut into a circle with a diameter of 12 mm using a puncher. Then, membrane was disinfected by dipping in 70% ethanol for 2 minutes, followed by three rinsing steps in deionized water for 30 seconds. The membrane was then put under germicidal UV lamp (254 nm) for final disinfection and dried overnight in safety cabinet in order to remove traces of ethanol.

3.4.2 Bacteria loading

Bacteria was diluted with 0.15 M NaCl solution until OD₅₄₀ of around 0.042. Circle PC membrane was put in a Swinney filter unit to load bacteria into the pores of the modified membrane. 1 mL of bacteria culture was diluted as a factor of 50 with 0.15 M NaCl solution and 10 mL of diluted bacterial suspension was filtered through the Swinney filter unit in 2 minutes using a syringe, as shown in Fig. 3.1. The loading step is repeated until finishing all diluted bacteria suspension. Then, PC membrane was taken out from Swinney filter unit and both surfaces of membrane were triple decrusted with cotton swab in 0.15 M NaCl solution to remove bacteria deposited on the top surface of the membrane.

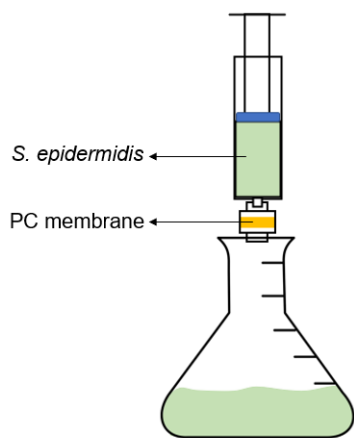


Figure 3.1: Schematic illustration of bacteria loading process.

3.4.3 Agarose gel preparation

4% agarose solution was autoclaved and the temperature was then gradually decreased to around 42° C, where a viscous semi-gel was obtained. Then, the bacteria-loaded PC membrane was dipped in 4% agarose solution for a few seconds. Next, it was rapidly sandwiched between two glass slides separated with a stack of four cover slips, as shown in Fig. 3.2. Then, agarose gel was solidified at room temperature and a bacterial membrane in gel patch with a diameter of 16 mm was obtained. Finally, the bacterial patch was stored in 0.15 M NaCl solution to keep it from drying.

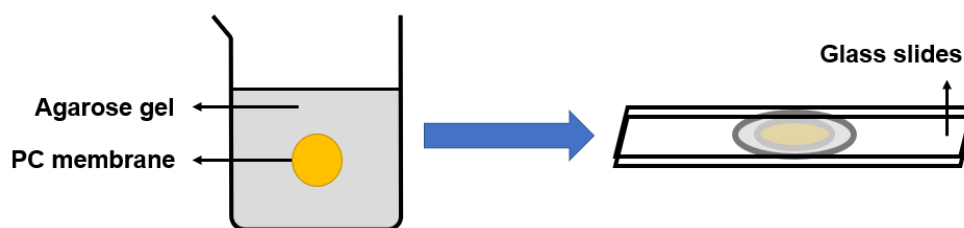


Figure 3.2: Schematic illustration of membrane embedding in agarose gel.

3.4.4 Surface modification of the patch

LbL assembled multilayers were deposited on bacterial patch at pH 6.0, as shown in Fig. 3.3. Bacterial patch was immersed in polyelectrolyte solutions for 1 min to do layer deposition, followed by twice rinsing in 0.15 M NaCl solution. PLL solution was sonicated for 10 minutes before LbL deposition.

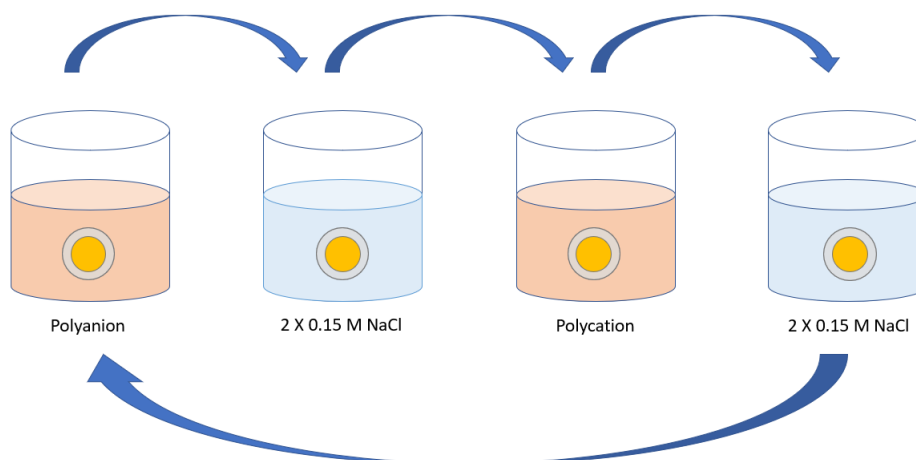


Figure 3.3: Schematic illustration of surface modification of patch using LbL assembly.

3.5 Culture and study of bacterial patch

3.5.1 Bacterial patch culture in refreshed broth

The patch was placed in 2 mL of broth for incubation. 2 mL of pure broth and 1% free bacteria were also prepared as negative and positive controls, respectively. 200 μ L of alamarblue was added in all tubes in order to quantify bacteria metabolic activity. All samples were cultured in well plates in incubator at 37° C and 120 rpm agitation.

Every two hours, patch was removed from broth, and rinsed three times using fresh broth. The supernatant was transferred to a vented tube for further culturing, and the patch was put in fresh broth with alamarblue to continue culture, and to eventually test the growth of bacteria released from the bacterial patch. This procedure was done four times and finally, the bacterial patch was continuously cultured in well plates, leading to four groups of supernatants corresponding to 2, 4, 6, and 8 hours of culture and supernatant from continuous culture, as shown in Fig. 3.4. The fluorescence intensity of all supernatants was measured at an emission wavelength of 585 nm, an excitation wavelength of 470 nm, and temperature of 37° C. For each measurement, 200 μ L of solution was transferred into 96-black well plates and they were poured back in the main samples after measurement to keep the volume of the broth constant. 15 μ L of deionized water was added to supernatant after each 4 hours of culture in order to compensate for evaporation of water during long culture.

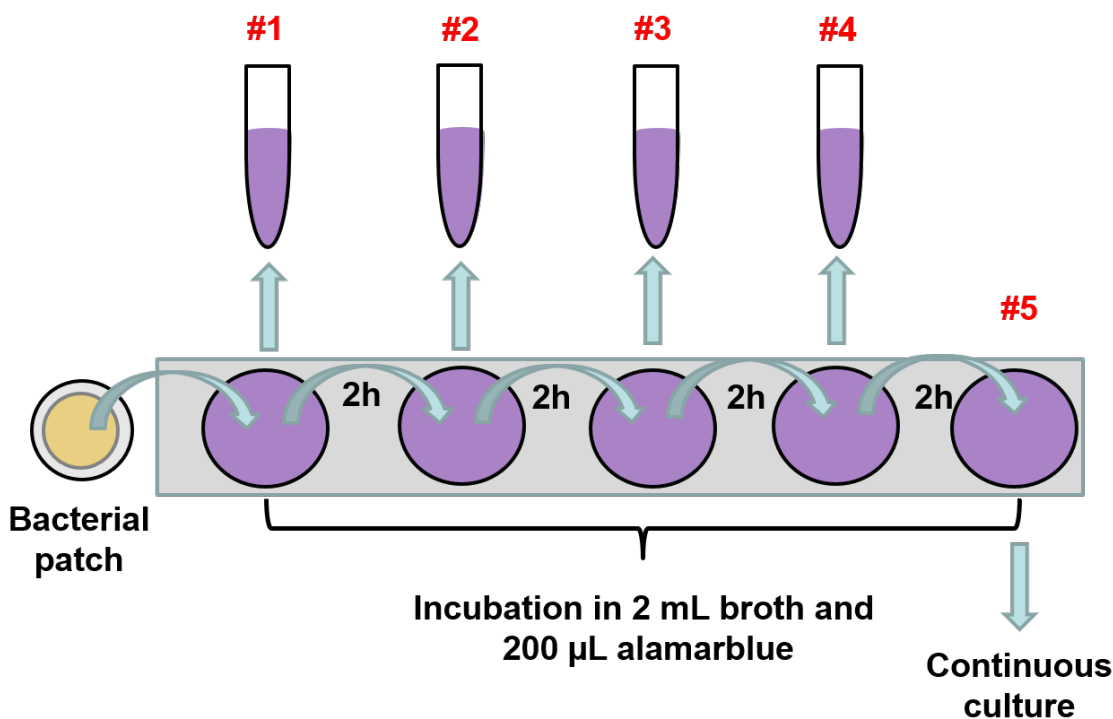


Figure 3.4: Schematic illustration of bacterial patch culture in refreshed broth.

3.5.2 Bacterial patch continuous culture

The bacterial patch was placed in 2 ml of broth and continuously cultured. Control samples were prepared as in the previous experiment, and 200 μL of alamarblue was added in all broth. The fluorescence intensity of supernatant was measured using microplate reader as described before, and the release of bacteria from bacterial patch was measured by single-plate serial dilution spotting. [130] After every four hours culture, 20 μL of supernatant was serially diluted by a factor of 10 with 0.15 M NaCl solution and five serial dilutions, as well as 20 μL of undiluted supernatant were inoculated on an agar plate. After incubation for two days at 37° C, the number of bacterial colonies on agar plate were counted to determine the colony forming units (CFUs/mL) in supernatant. A scheme of the CFUs on agar plate inoculated with serial dilutions and undiluted supernatant after two days is shown in Fig. 3.5.

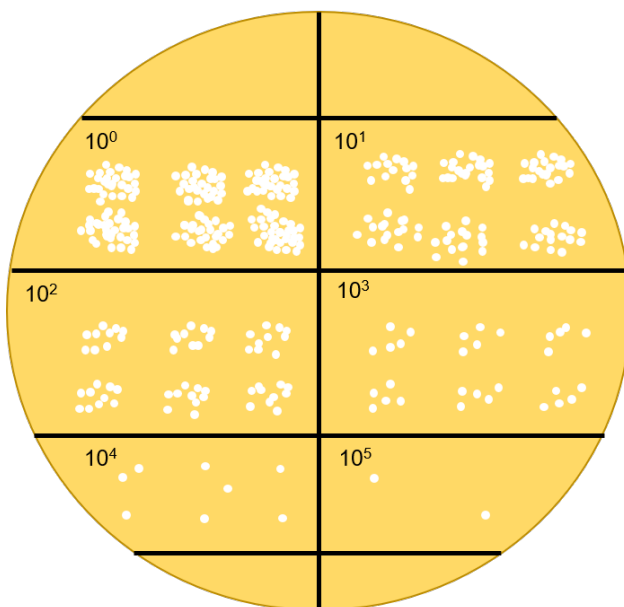


Figure 3.5: Schematic illustration of CFUs on agar plate inoculated with 20 μL of undiluted supernatant, and five serial dilutions as a factor of 10. The numbers indicate the dilution factor.

3.6 Minimum Inhibitory Concentration (MIC) of PLL, PEI and CHI

PLL, PEI, and CHI solutions were disinfected by filtering through 0.8 μm membranes and a series of PLL, PEI, and CHI solutions were prepared by two-fold serial dilution using broth. Bacteria were diluted with broth until a concentration of 10^6 bacteria/mL. Then, 1 mL of polyelectrolyte solution was mixed with 1 mL of diluted bacteria suspension, with the concentration of polymer ranging from 128 $\mu\text{g/mL}$ to 0.5 $\mu\text{g/mL}$. Pure broth and free

bacteria sample were also prepared as controls. All samples were cultured in incubator at 37° C and 180 rpm agitation. The turbidity of all samples were checked by the naked eye after one day culture. In solutions where bacteria were able to grow, the solution became turbid, whereas in solutions where the growth of bacteria was inhibited due to presence of polyelectrolyte, the solution stayed transparent. MIC was chosen, among the serial dilutions, as the minimum concentration of polyelectrolyte solution, which inhibited the growth of bacteria, resulting in a transparent solution. Thus, the solutions with lower concentrations than MIC were turbid, as the concentration of polyelectrolyte solution was not enough to completely inhibit the bacterial growth.

3.7 Diffusion tests

3.7.1 PAH-rhodamine diffusion test in agarose gel

To observe the diffusion of polyelectrolytes into agarose gel and to make sure that the polyelectrolytes used to prepare the antibacterial LbL coatings do not diffuse through the agarose gel to kill the bacteria entrapped into the membrane, PAH-Rho (M_w 15 kDa) was taken as a model macromolecule. After preparing an agarose gel disk, without embedded PC membrane, with thickness of 3 mm and diameter of 16 mm, the disk was dipped into PAH-Rho solution for 30, 60, 90, and 120 seconds, followed by three rinsing steps in 0.15 M NaCl solution to deposit one layer of PAH-Rho on top of the agarose gel disk. Then, the agarose gel disk was kept in water and covered with aluminum foil to prevent fluorescence loss. The agarose gel disk was broken in liquid nitrogen and the cross-section was observed using Olympus IX71 epifluorescence microscope to determine the diffusion thickness of the fluorescent polyelectrolyte inside the gel.

To observe the effect of bilayer number on diffusion of PAH-Rho, two different sets of multilayer structures were deposited on top of agarose gel disks. The first set (set 1) was (CHI/ALG)₄(PAH-Rho/ALG)₁ and the second set (set 2) was (PAH-Rho/ALG)₅. The LbL assembly was done by dipping the agarose gel disk in the corresponding polyelectrolyte solutions for 1 minute, followed by two rinsing steps in 0.15 M NaCl solution for 15 seconds. The agarose gel disk was then broken in liquid nitrogen and the cross-section was observed using epifluorescence microscope and Zeiss LSM 710 confocal laser scanning microscope.

3.7.2 Rhodamine B diffusion test from agarose gel to media

The diffusion of fluorescent molecule rhodamine B out of patch was investigated as a model for diffusion of bacterial metabolites out of the patch. Firstly, a circle with diameter of 30 mm was made out of (CHI/ALG)₄ modified PC membrane and it was dipped in rhodamine B solution overnight. The membrane was cut into a circle with diameter of 12 mm the next day and was coated with agarose gel, as explained in section 3.4.3. Then, the agarose gel disk embedded with PC membrane was cultured in 2 mL of broth at 37° C and 100 rpm agitation, along with pure broth as control. The fluorescence emission of supernatant was

measured after every 2 hours of culture at an excitation wavelength of 546 nm and emission wavelength of 588 nm.

3.7.3 FITC-dextran diffusion test from media to agarose gel

To investigate the effect of molecule size on diffusion into agarose, dextran labelled with FITC with different molar mass of 3-5 kDa, 10 kDa, and 40 kDa were used. An agarose gel disk with a thickness of 3 mm and a diameter of 16 mm was prepared without embedding PC membrane inside. The agarose gel disk was dipped in FITC-dextran solutions with different molar mass for 2 and 4 hours at room temperature.

The diffusion of FITC-dextran from media into agarose gel disk was characterized by taking Z-stack images along the thickness of agarose gel disk using confocal laser scanning microscope and measuring the fluorescence intensity at each point.

Chapter 4

Results and Discussion

4.1 Successful fabrication of bacterial patch

The bacterial patch was prepared by first modifying the pores of PC membrane with $(\text{CHI}/\text{ALG})_4$ multilayers, followed by loading *S. epidermidis* in the membrane. In the next step, bacteria-loaded PC membrane was coated with agarose gel with a thickness of around 1.2 mm. A digital image of the bacterial patch can be seen in Fig. 4.1. To determine successful modification of membrane and bacteria loading inside modified pores, $(\text{CHI}/\text{ALG})_4$ films, tagged with fluorescent rhodamine, and SYTO 9 stained *S. epidermidis* were used. The characterization was then conducted on confocal laser scanning microscope using Z-stack scanning mode. Orthogonal images of $(\text{CHI}/\text{ALG})_4$ modified PC membrane with and without encapsulated bacteria are shown in Fig. 4.2. The red color corresponds to $(\text{CHI}/\text{ALG})_4$ multilayers deposited in pores of the PC membrane, while green color corresponds to *S. epidermidis*. By looking at the XZ and YZ images, it is clear that $(\text{CHI}/\text{ALG})_4$ multilayers are successfully deposited along the thickness of the membrane. *S. epidermidis* is observed along the thickness of PC membrane, as is shown in Fig. 4.2c and d, showing the successful encapsulation of bacteria in modified PC membrane. These results were obtained by Wanlin Xu.



Figure 4.1: Digital image of bacterial patch

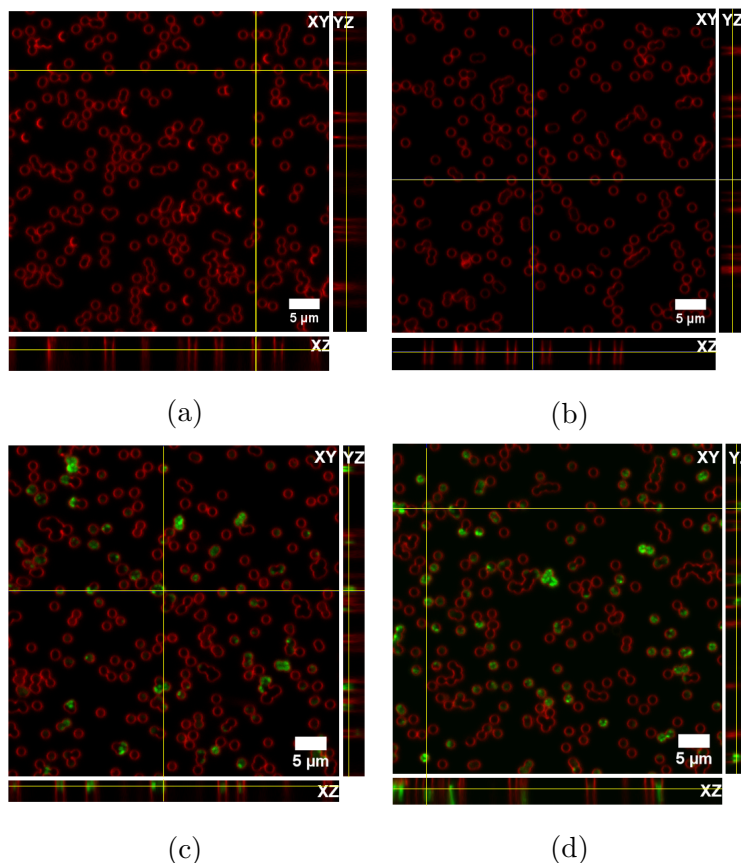


Figure 4.2: Orthogonal images of $(\text{CHI/ALG})_4$ modified PC membranes (a,b) without encapsulated *S. epidermidis*, and (c,d) with encapsulated *S. epidermidis*. The images were obtained using Z-stack imaging of confocal laser scanning microscope. Images (a,c) were taken from the top and (b,d) were taken from the bottom surface. The top side of XZ images in (a,c) corresponds to top side and in (b,d) corresponds to bottom side of PC membrane.

4.2 Characterization of LbL assembled polyelectrolytes

In order to observe the successful assembly of polyelectrolytes on the surface of agarose gel, fluorescent PAH-Rho, that can be detected using epifluorescence or confocal microscopes, was used as a model for polyelectrolytes. Another goal of this experiment was to check whether the polyelectrolytes, deposited on top of the agarose gel disks, can diffuse through the agarose gel disk or not. Since the polycations have contact-killing properties, if they diffuse and reach the PC membrane inside the gel, they can kill the encapsulated bacteria. So, the surface of the agarose gel, modified with PAH-Rho, was characterized.

Firstly, a single layer of PAH-Rho was deposited on top of an agarose gel disk, without embedded PC membrane. The dipping time in PAH-Rho solution was set to 30, 60, 90, and 120 s, followed by three rinsing steps in 0.15 M NaCl solution. Then, the agarose gel disk

CHAPTER 4. RESULTS AND DISCUSSION

was dipped in liquid nitrogen, and then broken to obtain a well-defined cross-section. Next, the cross-section was observed using epifluorescence microscope to determine the diffusion length of PAH-Rho inside agarose gel. Epifluorescence microscopy images of agarose gel disks can be seen in Fig. 4.3. The presence of red fluorescence on agarose gel disk demonstrates that polyelectrolyte can be deposited on agarose gel disks. The diffusion length of PAH-Rho in agarose gel disk was measured in different sections, and the average diffusion length is summarized in Table 4.1.

Table 4.1: Average diffusion length of a single layer of PAH-Rho in agarose gel disk based on six measurements per sample

Dipping time (s)	30	60	90	120
Average diffusion length (μm)	21.1 ± 2.3	18.9 ± 2.8	22.3 ± 9.3	26.2 ± 9.1

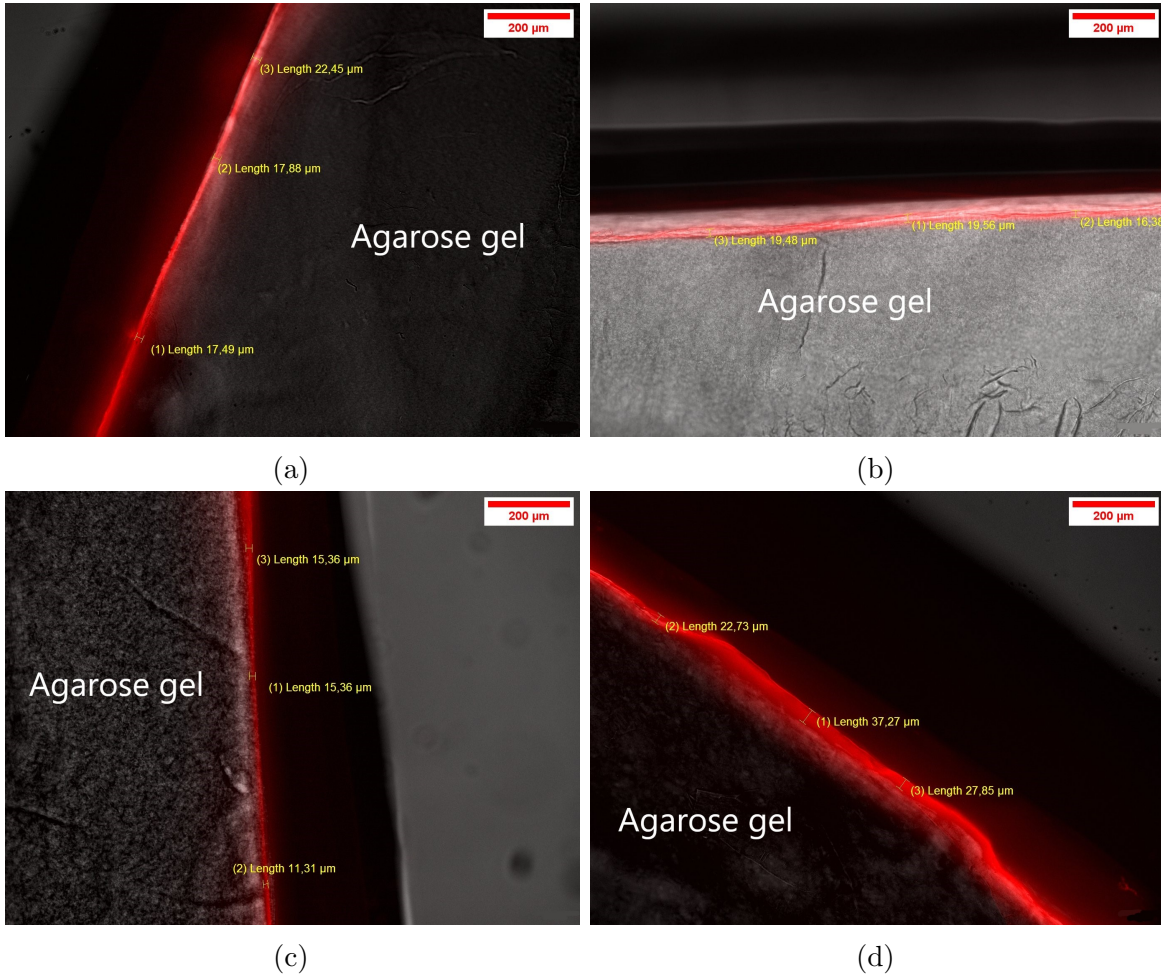


Figure 4.3: Epifluorescence images of single layer of PAH-Rho deposited on agarose gel disk with different dipping times of (a) 30 s (b) 60 s (c) 90 s (d) 120 s.

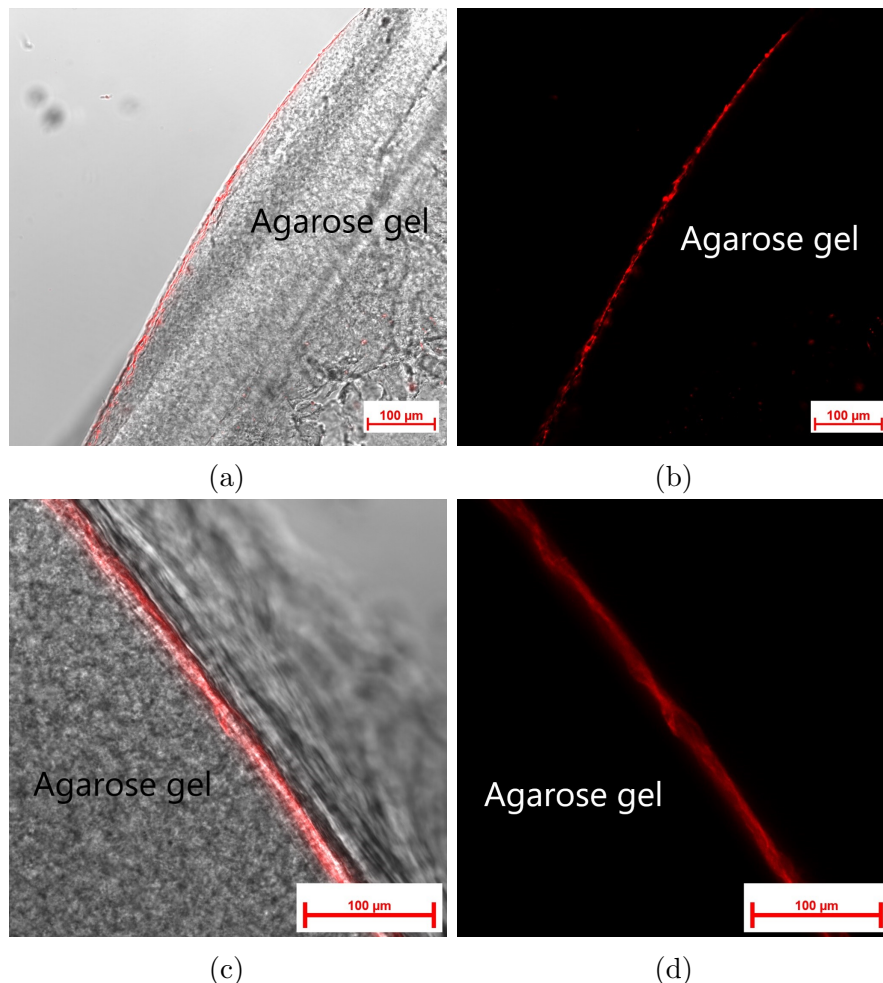


Figure 4.4: Confocal laser scanning microscopy images of agarose gel disk coated with (a,b) $(\text{CHI/ALG})_4(\text{PAH-Rho/ALG})_1$ multilayers and (c,d) $(\text{PAH-Rho/ALG})_5$ multilayers. (a,c) show the combined channel and (b,d) show the red channel.

According to the data, there is no general increase in the diffusion length of a single layer PAH-Rho when increasing the dipping time. First of all, agarose gel porosity distribution might be different from one agarose gel disk to another, and also vary from one part of the agarose gel disk to another. The pore size of agarose gel is determined by the concentration and cooling rate of the solution. Pernodet et al. mentioned a power law correlation between the concentration and pore size of agarose gel and reported the average pore size of 4% agarose gel to be 243 ± 5 nm, measured by atomic force microscopy. [131] However, the pore size might be different when the agarose gel is deposited on a membrane, rather than a flat surface. PAH-Rho (M_w 15 kDa) diameter of gyration is around 23.8 nm, which is much smaller than the average agarose gel pore size. [132] Additionally, the agarose gel disk is not homogeneous, containing very small cracks or other defects, leading to varied diffusion length in different sections of agarose gel disk. Another reason could be the sample

preparation for microscopy. After breaking the agarose gel disk in liquid nitrogen, a thin slide of agarose gel disk was cut and put on glass slide to observe the cross-section. During this process, the agarose gel was probably deformed, which can cause errors on PAH-Rho diffusion length measurement. However, the highest diffusion length of PAH-Rho is much smaller in comparison with the distance between the agarose gel disk surface and PC membrane, which is around 600 μm . The diffusion of polyelectrolytes is related to molecule size. The polycations used in this thesis are PLL and PEI, which have a molar mass of 66 kDa and 60 kDa, respectively. Their molar mass is much larger than PAH-Rho, 15 kDa. Therefore, their diffusion length is expected to be smaller than PAH-Rho. Thus, we can conclude that a single layer of polyelectrolyte deposition can not contact-kill bacteria encapsulated in PC membrane.

The diffusion length of polyelectrolyte multilayers was also studied and two sets of agarose gel disks deposited with $(\text{CHI/ALG})_4(\text{PAH-Rho/ALG})_1$ multilayers and $(\text{PAH-Rho/ALG})_5$ multilayers were prepared. Then, agarose gel disks were broken in liquid nitrogen and the cross-section was observed on confocal laser scanning microscope. PAH-Rho was deposited as the last layer on set 1 agarose gel disk, and five bilayers of PAH-Rho were deposited on set 2 agarose disk. As shown in Fig. 4.4, the diffusion length of one layer PAH-Rho is almost the same as five bilayers PAH-Rho. Furthermore, black and red intermixed patterns are observed on set 1 agarose gel disk, showing a heterogeneous fluorescent pattern, whereas the fluorescence emission is much more homogeneous and also has more intensity in set 2 where PAH-Rho exists in every bilayer. Alkehia et al. also showed that on PLL/HA multilayers, the newly absorbed polyelectrolyte can diffuse throughout the whole LbL multilayers, and the higher molar mass polyelectrolyte has a low diffusion capability, showing molar mass dependent diffusion. [54]

4.3 Characterization of molecule diffusion through patch

The diffusion of different molar mass molecules through patch was studied as a model to make sure that the bacterial metabolites can diffuse out of the bacterial patch and reach the medium. Rhodamine B was used as a model of small molecules. Rhodamine B was loaded into the $(\text{CHI/ALG})_4$ modified PC membrane and coated with agarose gel to get the patch. The release of rhodamine B from patch was characterized by measuring the fluorescence intensity of the supernatant over 24 hours. Three patches were fabricated and the fluorescence intensity is shown in Fig. 4.5. A sudden rise in fluorescence intensity can be seen in the first two hours in all patches, followed by a slower increase with an almost constant slope, which is in accord with typical diffusion behaviour. Patch 1 and patch 2 show very similar diffusion behaviour. In contrast, patch 3 shows a reduced fluorescence intensity. The agarose gel coating of patch 3 was broken before we started testing the diffusion of rhodamine B. Thus, we removed the original agarose gel and re-coated PC membrane with agarose gel, leading to the loss of rhodamine B. In the first few hours, the large difference between the concentration of rhodamine B in the patch and the medium, in addition to the presence of loosely bound rhodamine B in the PC membrane can lead to a large flux of molecules

diffusing out of the PC membrane. However, with the reduction of concentration gap, the diffusion becomes stable and follows a constant slope until the concentration of rhodamine B in supernatant becomes equal to the patch (not shown here).

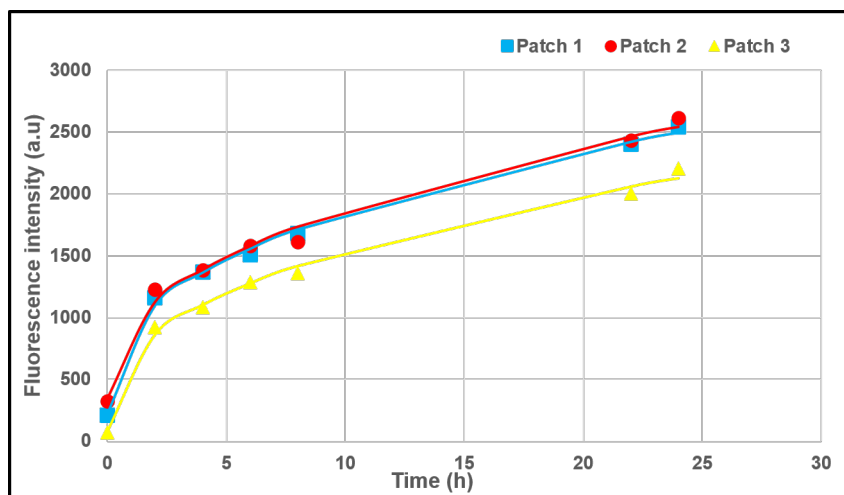


Figure 4.5: Fluorescence intensity measurements over time indicating rhodamine B release from $(\text{CHI}/\text{ALG})_4$ modified PC membrane coated with agarose gel.

The diffusion of large molecules through agarose gel disk without embedded PC membrane was tested by using FITC-dextran, and the effect of molecule size on diffusion was studied. A $100\mu\text{m} \times 100\mu\text{m}$ section near the middle of the agarose gel disk was observed on confocal laser scanning microscope using Z-scanning and the 3D-rebuilt images are shown in Fig. 4.6. It is worth noting that the images only show the fluorescence intensity until the depth of ca. $450\mu\text{m}$ into the agarose gel disk. This distance is a little less than the half-thickness of the agarose gel disk. Additionally, the fluorescence intensity of FITC-dextran obtained from confocal laser scanning microscope was plotted against the thickness of agarose gel disk. An average fluorescence intensity was calculated in every layer by taking all the points along a line going from top middle to bottom middle of the square section and the plots are shown in Fig. 4.7

The top side of 3D rebuilt image pertains to the surface of the agarose gel disk, which shows very high fluorescence emission in some cases. This is due to the fact that it was sometimes difficult to exactly identify the topmost surface of the patch. Therefore, the high fluorescent emission may result from the attached FITC-dextran solution on agarose gel disk surface. Despite the effect of attached FITC-dextran, the fluorescence intensity decreases along the thickness of each agarose gel disk, showing lower concentration of FITC-dextran in the middle of agarose gel disk. As the molar mass of FITC-dextran increases from 3-5 kDa to 40 kDa, the fluorescence intensity at the same thickness reduces, showing a molar mass-dependent behavior. During the same time interval, macromolecules with lower molar mass were able to diffuse more into the depth of agarose gel disk due to the limitation of agarose gel pore size. This effect is also clearly observed in Fig. 4.7.

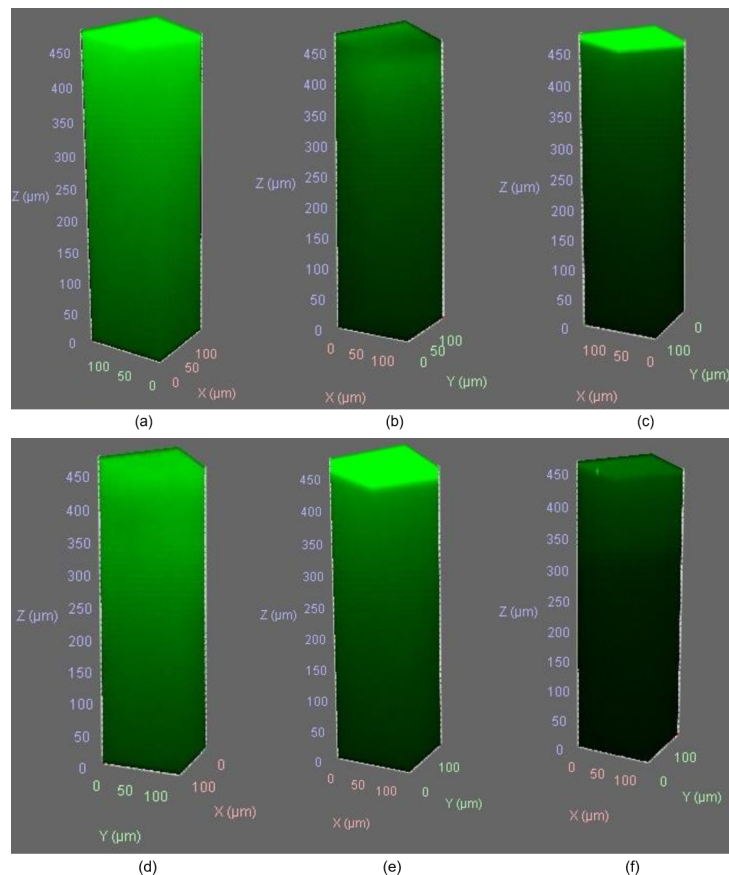


Figure 4.6: Confocal laser scanning microscopy 3D images taken in Z-stack scanning mode, obtained from agarose gel disks dipped in FITC-dextran solution with molar mass (a,d) 3-5 kDa (b,e) 10 kDa (c,f) 40 kDa for (a,b,c) 2 hours, and (d,e,f) 4 hours. Highest point in Z-scale corresponds to the surface of the gel.

Comparing the agarose gel disks dipped for 2 hours and 4 hours in FITC-dextran, no obvious increase in diffusion thickness or fluorescence intensity is observed. Comparing the plots shown in Fig. 4.7 does not show a clear effect of dipping time on the fluorescence intensity either. This can be associated with the fact that diffusion occurs very fast in the first hours of dipping. Therefore, after 2 hours, the concentration of FITC-dextran in agarose gel disk might have reached saturation and thus, keeping the sample in solution longer did not have a strong effect on the concentration.

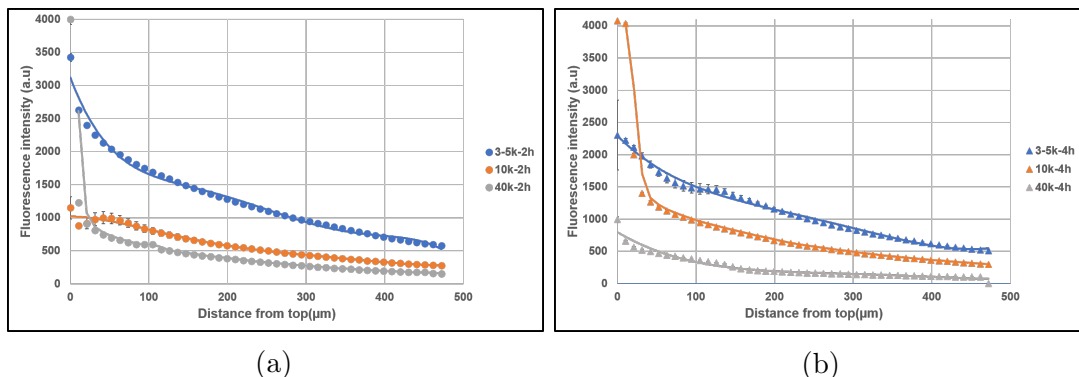


Figure 4.7: Fluorescence intensity profile of agarose gel disks dipped in FITC-dextran solutions with different molar mass of 3-5 kDa, 10 kDa, and 40 kDa for (a) 2 hours (b) 4 hours.

4.4 Characterization of bacterial patch

The bacterial patch was prepared by first loading bacteria in the $(\text{CHI}/\text{ALG})_4$ modified PC membrane, followed by agarose gel coating. The agarose gel coating was used to act as a physical barrier against the diffusion and immediate release of bacteria. Additionally, agarose gel coating is more skin-friendly for topical application and provides a wet environment for bacterial activity. To prevent the release of bacteria, multilayers containing antibacterial components, PLL and PEI were deposited on bacterial patch, and the antibacterial effect of multilayers incorporating PLL and PEI was compared with CHI, the most widely applied bactericidal polymer. Next, the metabolic activity of bacteria in bacterial patch and their release are discussed.

4.4.1 Bacterial patch coated with (PLL/HA) multilayers

The antibacterial effect of PLL and CHI against *S. epidermidis* was tested and the MIC of PLL and CHI are both 4 $\mu\text{g}/\text{mL}$. Antibacterial properties of Lysozyme was also tested against *S. epidermidis* for comparison. However, lysozyme showed no antibacterial effect below the concentration of 128 $\mu\text{g}/\text{mL}$, even though lysozyme is a widely used antibacterial agent. [133, 134]

After deposition of LbL films incorporating antibacterial polycations, bacterial patches were cultured in broth with alamarblue. As bacteria grow, the surrounding environment becomes more reducing. As a result, the resazurin in alamarblue is converted from a non-fluorescent oxidized state with a color of blue to a fluorescent reduced state with a color of red, called resorufin. So, the fluorescence intensity of alamarblue can indicate the metabolic activity of bacteria, and it can be characterized by measuring the fluorescence intensity of supernatant over time. [135] Every two hours, the bacterial patch was rinsed with broth and transferred to fresh broth to continue the culture, and the former supernatant was also cultured in incubator. Using this procedure, the fluorescence emission of supernatants

corresponding to the bacterial patch at different time intervals could be measured. In the end, four groups of supernatants corresponding to 2, 4, 6, and 8 hours culture were obtained, denoted as groups A to D, respectively. Lastly, bacterial patch was put in a new broth and continuously cultured, denoted as group E. The fluorescence intensity of group A-E supernatant was measured with time. The name of the groups and the corresponding bacterial patch culture time is shown in Table 4.2.

Table 4.2: Samples obtained in bacterial patch culture in refreshed broth

Bacterial patch culture time (h)	2	4	6	8	Continuous
Name of the group	A	B	C	D	E

Effect of multilayer composition

Multilayers with CHI and PLL polyelectrolytes were studied to compare the effect of multilayer composition on bacterial patch. Two different multilayer structures were used, namely $(\text{ALG}/\text{CHI})_{5.5}$ and $(\text{ALG}/\text{CHI})_{1.5}(\text{PLL}/\text{HA})_4$, both ended with a polyanionic layer. The numbers 1.5 and 5.5 mean that the multilayers deposition ends with ALG. $(\text{ALG}/\text{CHI})_{1.5}$ was used as primer multilayers for (PLL/HA) deposition to prevent contact-killing of bacteria when dipping the bacterial patch in PLL solution.

The fluorescence intensity of supernatants in groups A to E are shown in Fig. 4.8. The fluorescence emission values indicated on the plots are already subtracted from those of pure broth in the same conditions, and free bacteria was cultured at same condition as a positive control.

In all cases, a slight increase in fluorescence intensity was observed in the first two hours. This slight increase in groups A-D was plotted cumulatively against the total culture time of the bacterial patch corresponding to each group, as mentioned in Table 4.2. The plot is shown in Fig. 4.9.

The slight increase of fluorescence intensity in the first 2 hours culture is associated with the metabolic activity of bacteria in the bacterial patch. After 2 hours culture, bacterial patch was removed and put in next group to continue culture. Therefore, the fluorescence intensity at 2 hours rises from group A to group D. Furthermore, a higher fluorescence intensity is seen in the case of bacterial patch coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PLL}/\text{HA})_4$ multilayers. After the removal of bacterial patch, a plateau is observed in group A to group C. However, after around 30 to 40 hours, an exponential increase in fluorescence intensity is observed, which is associated with growth of leaked bacteria in supernatant, since bacterial patch was already removed at 2 hours culture. The lag time decreases from group A to group C, which shows increase of bacteria release with culture time. In group D exponential growth occurs at 2 hours culture, which also proves the higher number of bacteria release from bacterial patch. The fluorescence intensity change of group A to group D supernatant demonstrated

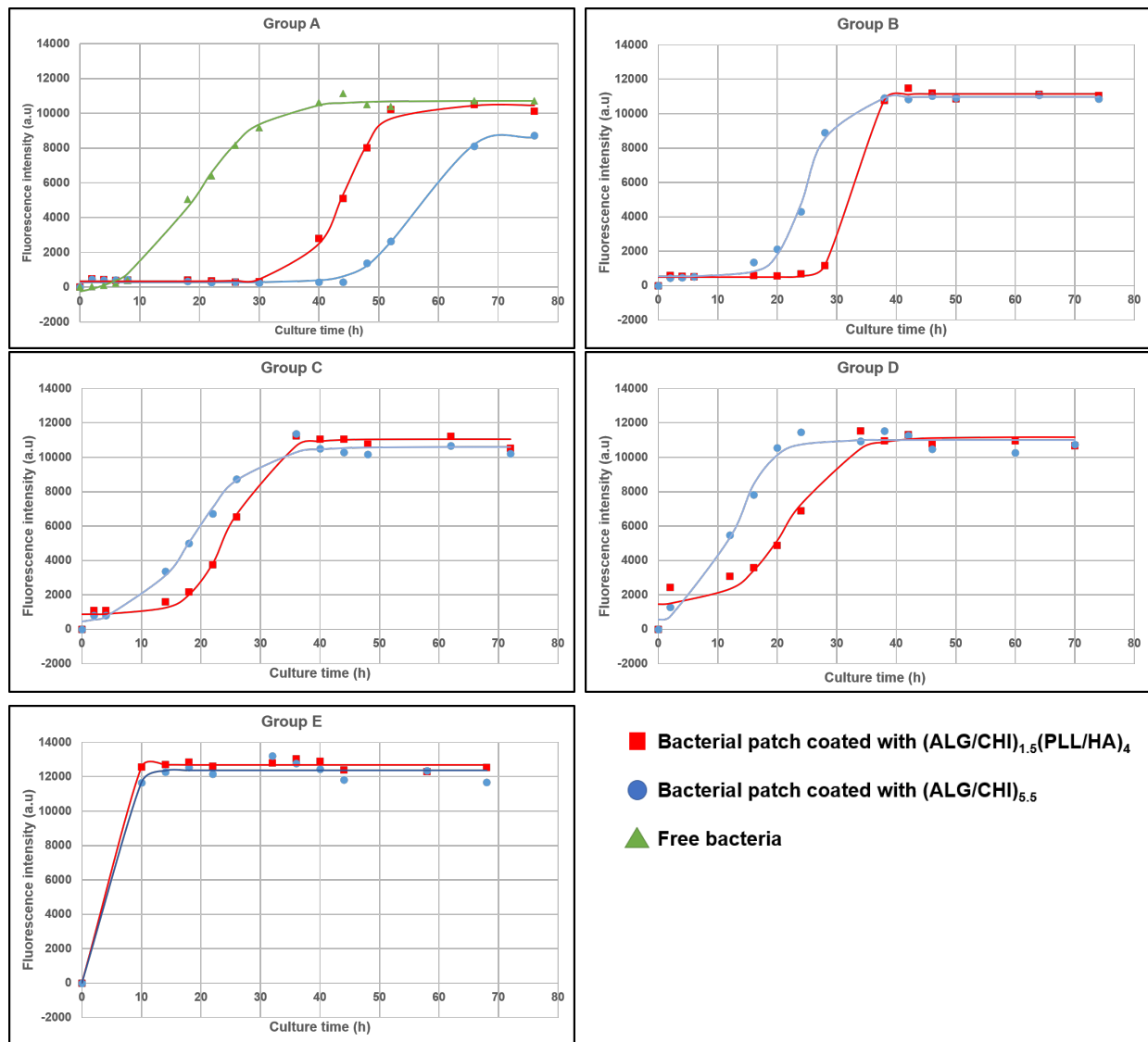


Figure 4.8: Fluorescence intensity of supernatants of bacterial patches with different multilayer compositions corresponding to 2, 4, 6, 8 hours culture, and continuous culture, denoted as groups A-E, respectively.

that bacteria are released from bacterial patch, and the two multilayers $(\text{ALG/CHI})_{5.5}$ and $(\text{ALG/CHI})_{1.5}(\text{PLL/HA})_4$ show similar effect on bacterial patch. In contrast with (ALG/CHI) multilayers which show linear growth, (PLL/HA) multilayers show exponential growth, where the polyelectrolytes can diffuse all through the multilayers. This behaviour is maintained up to 12 bilayers, after which a transition to linear growth occurs. [53] It is also worth noting that LbL assembly of these multilayers on agarose surface might also affect this transition. The deposition of 4 bilayers of (PLL/HA) might be less sufficient to show the antibacterial impact. Therefore, more bilayers of (PLL/HA) were deposited on bacterial patch and the

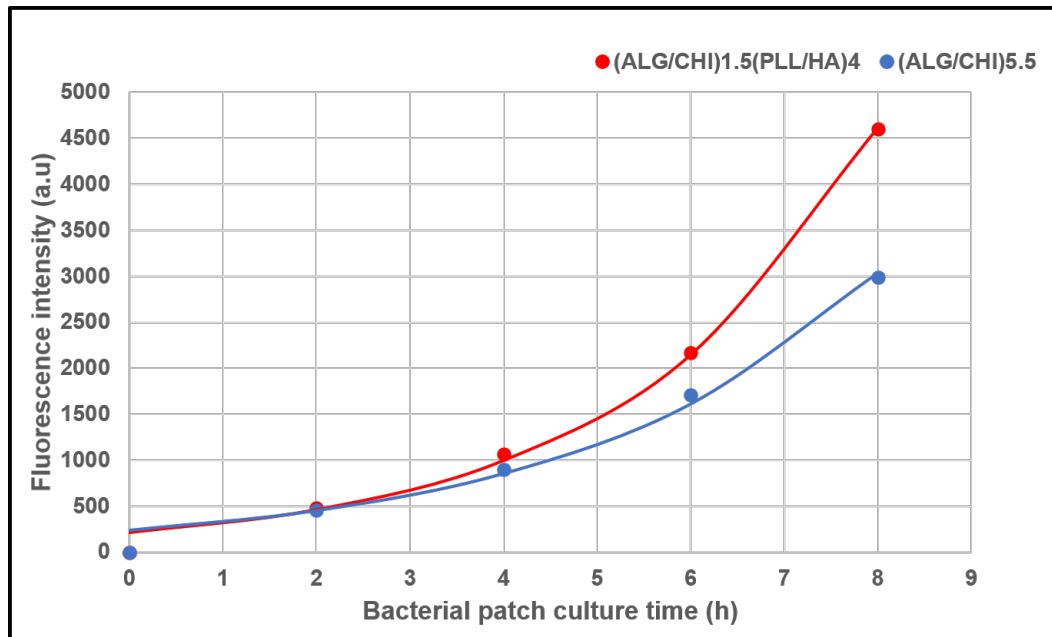


Figure 4.9: Cumulative fluorescence intensity at initial 2 hours culture for groups A-D plotted against the total culture time of bacterial patch, corresponding to each group.

effect of (PLL/HA) bilayer number was studied.

Effect of (PLL/HA) bilayer number

Bacterial patches coated with $(\text{ALG/CHI})_{1.5}(\text{PLL/HA})_n$ multilayers were fabricated, $n=12, 16, 20$. The bilayer numbers were selected to ensure obtaining a linear growth of multilayers. The fluorescence emission measurements were conducted as before. The results can be seen in Fig. 4.10. In group A, the fluorescence intensity of bacterial patches coated with $(\text{ALG/CHI})_{1.5}(\text{PLL/HA})_{12,20}$ multilayers starts to increase after around 50 hours. In groups B to group E, these bacterial patches show an exponential increase in fluorescence intensity until ca. 10000-11000. However, in the case of the bacterial patch coated with $(\text{ALG/CHI})_{1.5}(\text{PLL/HA})_{16}$ multilayers, fluorescence intensity does not increase in groups A and B and the exponential increase is only seen in group C to group E. Additionally, in groups B and C, it can be seen that the delay before exponential increase of fluorescence intensity is increased when 20 bilayers of (PLL/HA) are deposited, compared to 12 bilayers. However, this does not appear in the next groups. This delay also decreases generally for all bacterial patches from group A to group E. Finally, the plateau value before the exponential rise increases slightly from group A to group D for all bacterial patches.

The bacterial patches coated with $(\text{ALG/CHI})_{1.5}(\text{PLL/HA})_{12,20}$ multilayers show an increase in bacterial activity in group A, which can be associated with bacteria release at 2 hours. However, when 16 bilayers of (PLL/HA) are used, bacteria release is hindered for

at least 4 hours. Thus, bacterial patch coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PLL}/\text{HA})_{16}$ multilayers shows better performance, compared to other bacterial patches. We hypothesize that when 12 bilayers are used, as it is close to exponential-linear transition, the multilayer structure might not be very stable at this stage. Furthermore, in the case of 20 bilayers, the high thickness of multilayers can lead to the collapse of multilayers, especially on agarose gel surface. That is

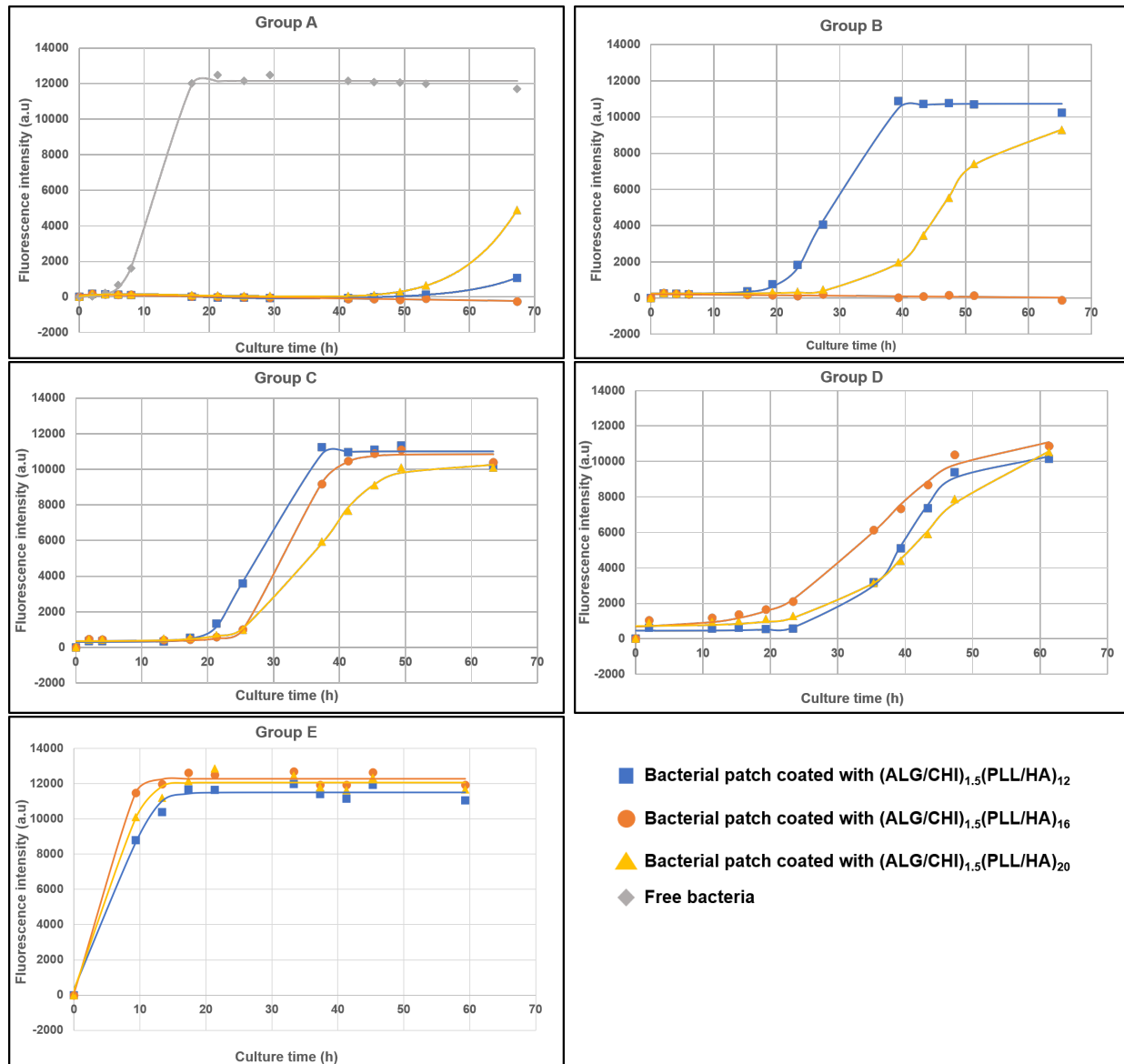


Figure 4.10: Fluorescence intensity of supernatants of bacterial patches coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PLL}/\text{HA})_{12,16,20}$ corresponding to 2, 4, 6, 8 hours culture, and continuous culture, denoted as groups A-E, respectively.

why selecting an intermediate value for (PLL/HA) bilayer number can lead to better results. Furthermore, by comparing the results in group B and group C, it is evident that the lag time

before exponential growth of bacteria is higher when using 20 bilayers of (PLL/HA), showing lower number of released bacteria. Thus, a higher (PLL/HA) bilayer number, can decrease the number of released bacteria from the patch. This effect is not seen distinctly in groups D and E because at this time point, a high number of bacteria is released from all bacterial patches. The higher number of released bacteria over time is also the reason why the lag time decreases generally from group A to group E. Finally, the plateau value, which shows the bacterial activity inside the patch, increases slightly from group A to group D because activity of bacteria inside the patch increases over time. So, each time the patch is cultured in fresh broth, a higher activity of bacteria inside the patch leads to a higher plateau value.

In conclusion, the bacterial patch coated with (ALG/CHI)_{1.5}(PLL/HA)₂₀ multilayers shows smaller release of bacteria, compared to using 12 bilayers. Nevertheless, it still releases bacteria at 2 hours. However, bacterial patch coated with (ALG/CHI)_{1.5}(PLL/HA)₁₆ multilayers prevents release of bacteria for 4 hours and shows the best properties among these bacterial patches. Moreover, the bacteria entrapped inside these bacterial patches are still active. This demonstrates that the encapsulation process in this experiment does not affect the bacterial activity.

4.4.2 Bacterial patch coated with (PEI/PSS) multilayers

PEI has a much stronger antibacterial effect against *S. epidermidis* with MIC of 1 µg/ml compared to CHI and PLL. Additionally, (PEI/PSS) films are denser than (PLL/HA) films, which makes (PEI/PSS) films more effective as a physical barrier to limit bacteria release. Bacterial patch coated with (PEI/PSS) multilayers were prepared, and the effect of (PEI/PSS) bilayer number and the outer layer polyelectrolyte were studied. In this section, bacterial patches were continuously cultured in broth. The fluorescence intensity of supernatant and the release of bacteria were tested.

Effect of multilayer composition

The results concerning the metabolic activity and release of bacteria from bacterial patches coated with (ALG/CHI)_{5.5} and (ALG/CHI)_{1.5} (PEI/PSS)_{1,4} multilayers are shown in Fig. 4.11. In bacterial patch coated with (ALG/CHI)_{5.5} multilayers, the fluorescence intensity is increased exponentially to ca. 12600. When bacterial patch is coated with (ALG/CHI)_{1.5} (PEI/PSS)₁ multilayers, the fluorescence intensity shows an exponential increase to ca. 11400, and the lag time before the exponential behaviour is increased by around 4 hours. However, in the case of bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)₄ multilayers, the fluorescence intensity is increased to only ca. 600.

In the bacterial patches coated with (ALG/CHI)_{5.5}, and (ALG/CHI)_{1.5} (PEI/PSS)₁ multilayers CFUs appear at 8 hours, and the number of released bacteria is slightly lower in the latter. The release of bacteria from the bacterial patch corresponds with the exponential increase of fluorescence intensity in both cases. Thus, the exponential rise of fluorescence

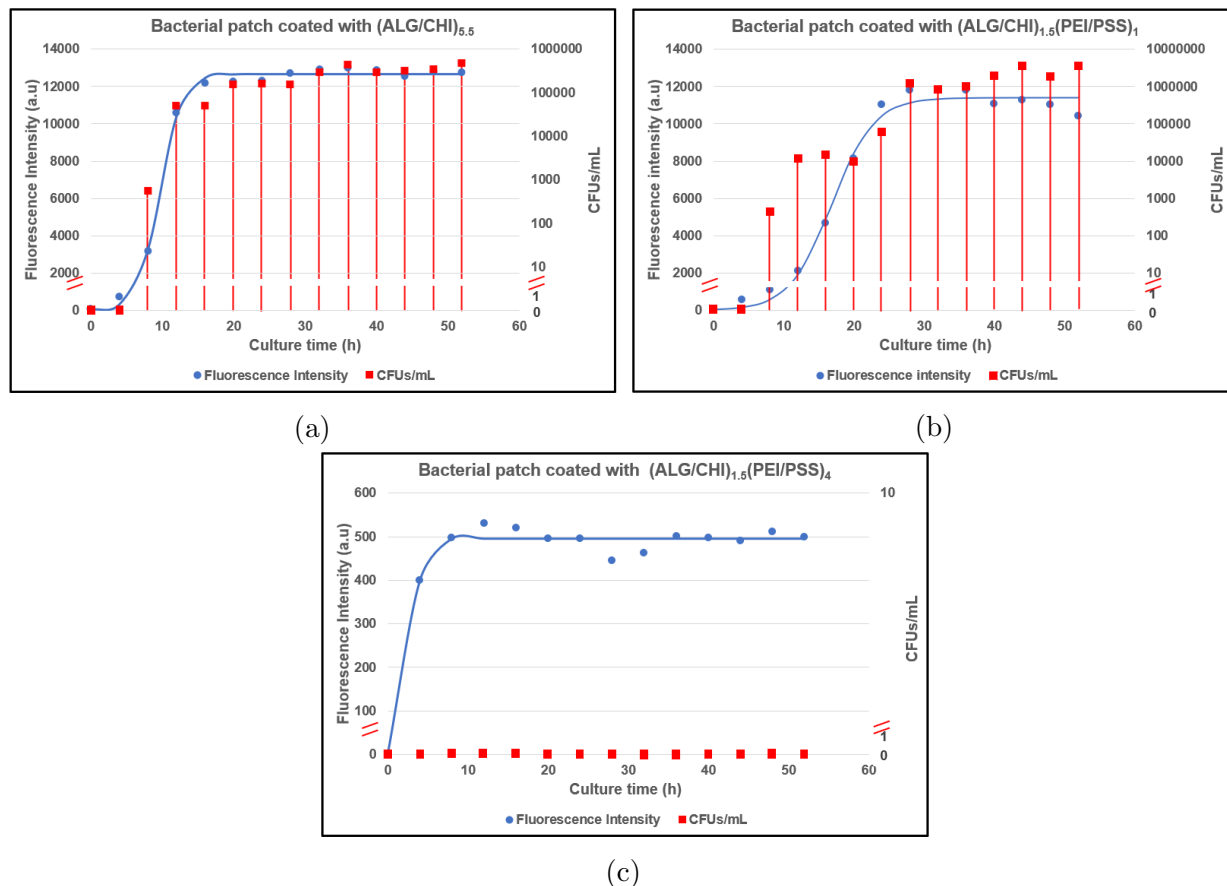


Figure 4.11: Fluorescence intensity and CFUs/ mL measurements for bacterial patches coated with (a) (ALG/CHI)_{5.5}, (b) (ALG/CHI)_{1.5} (PEI/PSS)₁, and (c) (ALG/CHI)_{1.5} (PEI/PSS)₄. The right hand side axis in each plot is divided into linear and logarithmic scales.

intensity to ca. 11400, and 12600, respectively, can be associated with the growth of released bacteria in the supernatant. However, the bacteria is not released at all from the bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)₄ multilayers for at least 52 hours. Therefore, the limited fluorescence intensity of bacteria in Fig. 4.11c corresponds to the growth of entrapped bacteria inside the patch.

The bacterial patches coated with (ALG/CHI)_{5.5}, and (ALG/CHI)_{1.5} (PEI/PSS)₁ multilayers show similar results, with slightly lower bacterial activity, number of released bacteria and increased lag time in the case of using (PEI/PSS) multilayers. Furthermore, these bacterial patches are only able to prevent release of bacteria for 4 hours, whereas bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)₄ multilayers prevent the release of bacteria for at least 52 hours, while maintaining the metabolic activity of bacteria inside the patch. However, the metabolic activity of bacteria is much less than free bacteria, which shows the

limited growth of bacteria inside the patch. This can be associated with the limited available space for bacteria inside the patch, in addition to the antibacterial effect of multilayers.

In conclusion, (PEI/PSS) multilayers are more efficient to limit the release of bacteria in the surrounding medium, compared to (ALG/CHI) multilayers and bacterial patches coated with (ALG/CHI)_{1.5} (PEI/PSS)₄ multilayers can completely suppress the release of bacteria for at least 52 hours, while maintaining a limited bacterial activity inside the patch.

Effect of (PEI/PSS) bilayer number and outer layer polyelectrolyte

To investigate the effect of (PEI/PSS) bilayer number and the outer layer polyelectrolyte, the experiment was repeated on bacterial patches coated with (ALG/CHI)_{1.5} (PEI/PSS)_{*n*}, *n* = 0.5, 1.5, 2.5, 3.5. The results are shown in Fig. 4.12. The fluorescence intensity of bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)_{0.5} is increased exponentially and reaches ca. 12000 after around 36 hours. However, when more (PEI/PSS) bilayers are used, the maximum fluorescence intensity is decreased dramatically in all cases. The maximum fluorescence intensity for bacterial patches coated with (ALG/CHI)_{1.5} (PEI/PSS)_{1.5, 2.5, 3.5} reaches ca. 1050, 860, and 370, respectively. So, the maximum fluorescence intensity decreases by depositing more (PEI/PSS) bilayers. Additionally, by comparing Fig. 4.11c and Fig. 4.12d, it can be seen that the maximum fluorescence intensity is decreased from ca. 600 to ca. 370, when the outer layer is chosen as PEI, instead of PSS.

The bacteria release from the bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)_{0.5} occurs at 24 hours, and the exponential increase in fluorescence intensity can be associated with the released bacteria. However, CFUs are only observed at 24 and 40 hours and not in other time steps. By using more (PEI/PSS) bilayers, the release of bacteria can be prevented for at least 52 hours. Thus, the limited fluorescence activity is associated with the activity of bacteria inside the bacterial patch. Moreover, comparing Fig. 4.11b and Fig. 4.12a shows that when PEI is selected as the outer polyelectrolyte, the bacterial patch can prevent release of bacteria for much longer times, and the number of released bacteria is much lower.

In bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)_{0.5} multilayers, bacteria are released at 24 hours and the metabolic activity of bacteria is maintained. When the number of (PEI/PSS) bilayers are increased to 1.5, 2.5, and 3.5, the release of bacteria is completely hindered for at least 52 hours, while the metabolic activity of bacteria is drastically decreased as well. The decrease in metabolic activity of bacteria can be associated with the confinement of bacteria inside the bacterial patch, which limits the growth of bacteria, and also the antibacterial effect of PEI. As the number of (PEI/PSS) bilayers is increased, the metabolic activity of bacteria inside the bacterial patch is reduced. Thus, increasing PEI content can suppress the metabolic activity of bacteria inside the patch, due to higher antibacterial effects, and adding too many bilayers might suppress the activity of bacteria completely.

The number of released bacteria and bacteria release time from bacterial patch coated

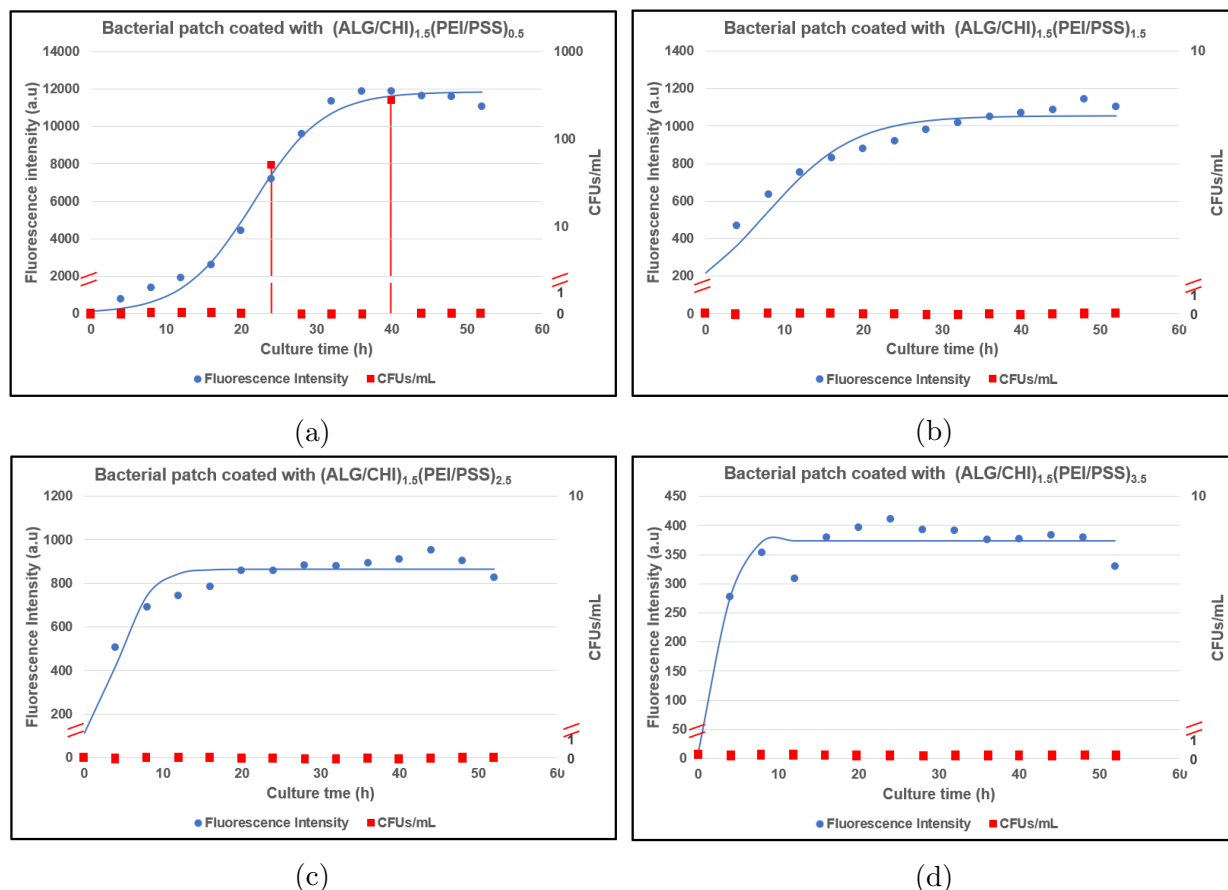


Figure 4.12: Fluorescence intensity of supernatant, and CFUs/mL measurements for bacterial patches coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_n$, where n is equal to 0.5, 1.5, 2.5, 3.5. The right hand side axis in each plot is divided into linear and logarithmic scales.

with $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_{0.5}$ multilayers is much less than the case of bacterial patch coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_1$ multilayers. When PEI is selected as the outer polyelectrolyte, the highly cationic nature of PEI, leads to higher positive charge and consequently, higher antibacterial effect of multilayers against *S. epidermidis*. The antibacterial effect of (PEI/PSS) multilayers can also kill the released bacteria in supernatant. That could be the reason why in bacterial patch coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_{0.5}$ multilayers, metabolic activity of bacteria is maintained, despite much lower CFUs/mL compared to bacterial patch coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_1$ multilayers, and also the fact that CFUs are only observed at two time steps and not in other ones. We hypothesize that the released bacteria contribute to the high fluorescence intensity observed in Fig. 4.12a, but they are killed due to the presence of multilayers incorporating PEI on the outmost layer, and that is why they do not make CFUs on agar plate. The effect of selecting PEI as the outer polyelectrolyte can also be seen by comparing the bacterial patches coated with $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_{3.5}$ and $(\text{ALG}/\text{CHI})_{1.5}(\text{PEI}/\text{PSS})_4$ multilayers. In both cases, bacteria release is completely

suppressed. However, the metabolic activity of bacteria is lower in the former. This shows the higher antibacterial effect of multilayers when PEI is selected as the outer polyelectrolyte.

To conclude, the efficiency of (PEI/PSS) multilayers to limit the bacterial release increases with the number of deposited bilayers. Additionally, depositing PEI as the outer polyelectrolyte can greatly enhance the antibacterial activity of multilayers. So, bacterial patch coated with (ALG/CHI)_{1.5} (PEI/PSS)_{1.5} multilayers is the best choice as it leads to the highest bacterial activity inside the bacterial patch among other bacterial patches, while suppressing the release of bacteria for at least 52 hours.

Chapter 5

Conclusion and Perspectives

In this study, a novel approach for encapsulation of *S. epidermidis* in the form of a bacterial patch was investigated for skin disease treatment. Bacterial patch was prepared by loading the bacteria in a modified porous PC membrane, followed by agarose gel coating and deposition of LbL films incorporating antibacterial polycations on top. The goal was to prevent the release of bacteria from the patch, while maintaining their metabolic activity.

Firstly, it is confirmed that molecules with a size similar to bacterial metabolites can diffuse through agarose gel and that larger molecules diffuse slower in agarose gel. This was done by taking rhodamine B and FITC-dextran as model molecules. Additionally, LbL assembled multilayers on the surface of the patch were characterized. As a result, it was confirmed that the multilayers cannot reach and kill the bacteria encapsulated in the membrane.

LbL films incorporating antibacterial polycations were coated on bacterial patch. (ALG/CHI)_{1.5}(PLL/HA)₄ multilayers showed a similar effect on the bacterial patch compared to (ALG/CHI)_{5.5} multilayers. They both released bacteria after 2 hours culture, while maintaining the bacterial activity inside the patch. Additionally, The number of released bacteria and also the bacterial activity inside the patch increased with culture time. However, (PLL/HA) multilayers go through a linear growth regime in the first several bilayers. On the other hand, too many number of bilayers can lead to unstable multilayers. Considering these two limiting factors, The bacterial patches coated with (ALG/CHI)_{1.5}(PLL/HA)_n, n= 12, 16, 20 were studied. Increasing the (PLL/HA) bilayer number can delay the release of bacteria. At the end, bacterial patch coated with (ALG/CHI)_{1.5}(PLL/HA)₁₆ shows the best efficiency by preventing the release of bacteria for four hours, while preserving their metabolic activity.

PEI with stronger antibacterial activity was incorporated in LbL coatings on bacterial patch. Bacterial patches coated with (ALG/CHI)_{1.5}(PEI/PSS)_{1,4} multilayers were tested. The former bacterial patch shows similar efficiency to the one coated with (ALG/CHI)_{5.5}. On the other hand, the latter limits the metabolic activity of bacteria inside the patch, while preventing the release of bacteria for at least 52 hours. Thus, PEI is more efficient than CHI. Moreover, (ALG/CHI)_{1.5}(PEI/PSS)_n, n= 0.5, 1.5, 2.5, 3.5, were tested to compare the effect

of (PEI/PSS) bilayer number and outer layer polyelectrolyte. Depositing more (PEI/PSS) bilayers suppresses the metabolic activity of bacteria inside the bacterial patch. So, the metabolic activity of bacteria inside the bacterial patch can be tuned with controlling the (PEI/PSS) bilayer number. The antibacterial effect of multilayers are enhanced when they are ended with PEI. So, multilayers ended with PEI show more suppression of bacterial activity inside the patch and delay more the release of bacteria. The bacterial patch coated with $(\text{ALG/CHI})_{1.5}(\text{PEI/PSS})_{1.5}$ showed the best efficiency as it maintained the highest bacterial activity inside the patch, while hindering the release of bacteria for at least 52 hours.

PEI proved to be quite effective as an antibacterial polymer for this application. However, other antibacterial materials can also be studied in future for this purpose. For instance, antimicrobial peptides are widely used for combating bacterial growth in different applications. [91, 136] Moreover, inorganic materials with antibacterial properties, such as rectorite, can also be used in LbL assembled multilayers and they could potentially enhance the efficiency of bacterial patches. [58, 137] Another prospective study would be the efficiency of bacterial patches against different kinds of pathogenic skin bacteria, such as *C. acnes* *in vitro* and *in vivo*.

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