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Institute of Condensed Matter
and Nanosciences

Protein-polyelectrolyte complexes
for the surface immobilization
of bioactive proteins

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en sciences agronomiques et ingénierie biologique par

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Abstract

The immobilization of proteins to impart specific functions to surfaces is topical for chemical engineering, healthcare and diagnosis. Layer-by-Layer (LbL) self-assembly is one of the most used method to immobilize macromolecules on surfaces. It consists in the alternate adsorption of oppositely charged species, resulting in the formation of a multilayer. This method in principle allows any charged object to be immobilized on any surface, from aqueous solutions. However, when it comes to proteins, the promises of versatility, simplicity and universality that the LbL approach holds are unmet due to the heterogeneity of protein properties.

This thesis aims at demonstrating that protein surface properties can be standardized via their complexation with soluble charged polymers, *i.e.* polyelectrolytes (PEs), and that the resulting protein-polyelectrolyte complexes (PPCs) can be used as building blocks for LbL assembly. The results show that using PPCs, LbL assembly becomes independent of the protein electrical properties, as demonstrated for the immobilization of lysozyme. In particular conditions, highly hydrated films that only contain PEs are obtained, even though a protein was used to construct them. Upon dehydration, these films are shown to irreversibly lose their water content. When compared to the LbL assembly of bare proteins, the PPCs-based method is shown to maintain a higher protein bioactivity and to immobilize larger protein amounts, which results in a higher total bioactivity. For applications aiming at delivering proteins, a higher bioactivity is obtained, which is attributed to a self-reorganization of the multilayer upon release. Finally, the method is translated to the creation of a useful functional material, *i.e.* a dressing designed for wound healing, on which an antimicrobial peptide that failed to be immobilized without PPCs formation is immobilized. The LbL assembly of PPCs thus holds much promises in terms of universality. It also allows to create new functionality and structures at interfaces, which will be beneficial for the design of drug delivery systems, biomaterials and sensors.

The author contributed to the following papers:

- Art, J.-F.; vander Straeten, A.; Dupont-Gillain, C. NaCl strongly modifies the physicochemical properties of aluminum hydroxide vaccine adjuvants. **International Journal of Pharmaceutics** 2017, 517 (1-2), 226-233.
- vander Straeten, A.; Bratek-Skicki, A.; Germain, L.; D'Haese, C.; Eloy, P.; Fustin, C.-A.; Dupont-Gillain, C. Protein-Polyelectrolyte Complexes to Improve the Biological Activity of Proteins in Layer-by-Layer Assemblies. **Nanoscale** 2017, 9 (44), 17186–17192.
- Art, J.-F.; vander Straeten, A.; Dupont-Gillain, C. Immobilization of Aluminum Hydroxide Particles on Quartz Crystal Microbalance Sensors to Elucidate Antigen-Adjuvant Interaction Mechanisms in Vaccines. **Analytical Chemistry** 2018, 90 (2), 1168–1176.
- vander Straeten, A.; Bratek-Skicki, A.; Jonas, A. M.; Fustin, C.-A.; Dupont-Gillain, C. Integrating Proteins in Layer-by-Layer Assemblies Independently of Their Electrical Charge. **ACS Nano** 2018, 12 (8), 8372–8381.

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List of abbreviations

l_b	Bjerrum length
l_p^0	Intrinsic persistence length
l_p^e	Electrostatic persistence length
l_p	Persistence length
(GlcNAc) ₃ -MeU	4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside
[Θ]	Molar ellipticity
ΔH_z	Frequency shift
Δm	Mass shift
Δm_w	Mass shift due to water sorption/desorption
a.a.	Amino acid
A ₄₅₀	Optical density at 450 nm wavelength
A ₅₆₂	Optical density at 295 nm wavelength
AFM	Atomic force microscopy
Algi	Alginate
AMP	Antimicrobial peptide
a _w	Water activity
BCA	Bicinchoninic acid
Bdl	Below detection limit
BSA	Bovine serum albumin
CD	Circular dichroism
Chito	Chitosan
CLEAs	Cross-linked enzyme aggregates
C _{shup}	Aromatic shake up
D	Dissipation shift
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol
F	Phenylalanine
GA	Glucoamylase
GOx	Glucose oxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I	Ionic strength
IEP	Isoelectric point
LbL	Layer-by-Layer
LOQ	Quantification limit
MeU	4-methylumbelliferone
o-Ph	o-phthalaldehyde
PAA	Poly(acrylic acid)

PAH	Poly(allylamine hydrochloride)
PBS	Phosphate buffer saline
PDMS	poly(dimethyl siloxane)
PE	Polyelectrolyte
PECs	Polyelectrolyte-polyelectrolyte complexes
PEI	Poly(ethylenimine)
PEM	Polyelectrolyte multilayer
PMAA	Poly(metacrylic acid)
PPCs	Protein-polyelectrolyte complexes
PPCs ^{0.5}	Lyz-PSS _I complexes with charge ratio (-)/(+net)=0.5
PPCs ²	Lyz-PSS _I complexes with charge ratio (-)/(+net)=2
PPCs _{I/a}	LL37-alginate complexes with charge ratio (-)/(+net)=2
PSS	Poly(styrene sulfonate)
PSS _h	Poly(styrene sulfonate) with $M_w=70,000 \text{ g mol}^{-1}$
PSS _I	Poly(styrene sulfonate) with $M_w=7,540 \text{ g mol}^{-1}$
PVS	Poly(vinyl sulfonate)
QCM-D	Quartz crystal microbalance with dissipation monitoring
R _E	Chain end-to-end distance
R _h	Hydrodynamic radius
RH	Relative humidity
R _{RMS}	root-mean-square roughness
SDS	Sodium dodecyl sulfate
ToF-SIMS	Time of flight secondary ion mass spectrometry
TRIS	Tris(hydroxymethyl)aminomethane
W _u	Water uptake
XPS	X-ray photoelectron spectroscopy
α -C	α -chymotrypsin
κ^{-1}	Debye length

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Part I: Context of the research

1. Functional materials

Materials that possess particular properties and functions are at the core of modern technologies. These so-called functional materials are used for an ever-increasing number of applications. For diagnostic purposes, materials that can detect specific molecules are highly promising. Every day, millions of diabetics control their glycemia with portable sensors. In this frame, Shin *et al.* created SnO₂ tubes that allow for the real-time measurement of glycemia, therefore considerably simplifying the control of glucose levels (see *Advanced Functional Materials* cover in Figure I.1a).¹ In the context of diagnosis improvement as well, the differential adhesion of circulating tumor cells compared to normal blood cells has been used to evidence primary and metastatic lesions (see *ACS Nano* cover in Figure I.1b).² Exploiting cell-material interactions is not limited to the above-mentioned field and surgeons regularly fit functional prostheses that promote cell adhesion and differentiation (see *JACD* cover in Figure I.1c).³ Still in healthcare, the field of drug delivery, within which scientists engineer drug carriers to target organs and protect medicine from deactivation, is ever-growing.⁴ An example is depicted in an amusing way on an *Angewandte Chemie* cover (Figure I.1d), where a new way to selectively target tumors was presented.⁵ A last field known as heterogeneous catalysis highly benefits from functional materials since it uses the reactivity of solid materials to transform molecules into useful chemical compounds. Heterogeneous catalysis is widely used in the chemical industry as it offers the opportunity to easily recover both the product and the reactive material, or even to operate in flow. A telling practical example is the reported conversion by a catalyst of biomass-derived fructose into 5-hydroxymethylfurfural (HMF), a molecule that can be used as a biofuel (see *Angewandte Chemie* Cover in Figure I.1e).⁶



Figure I.1 – Covers of scientific journals that illustrate a few major applications of functional materials. (a) *Advanced Functional Materials*: a biosensor based on SnO_2 tubes allows the real-time measurement of glycemia. (b) *ACS Nano*: the cell-material interaction is used to diagnose primary and metastatic lesions. (c) *JIACD*: a biocompatible auricular prosthesis is placed. (d) *Angewandte Chemie*: Tumors are targeted via chemotherapeutic agents delivered from vesicles. (e) *Angewandte Chemie*: solid reactivity is used to transform fructose into fuel.^{1–3,5,6}

The common thread between all the above-mentioned applications is that functionality is driven by the ability of the material to interact with its surrounding environment. Heterogeneous catalysis uses the particular reactivity of the surface to convert molecules while sensors transduce surface events into processable signals for the detection of chemical compounds. Similarly, cells sense and adapt to specific cues on the material surface, which can, in turn, be engineered to control their fate. The region that separates the bulk material from the surrounding environment is thus critical for the design of new functional materials. Strategies that aim at modifying material surfaces to create functionality have thus become prevalent and a myriad of methods were developed over the last century.⁷

Speaking of functionality, we do not need to look far to get inspiration; living organisms are the best: harvesting photons, transforming molecules and transporting information are their everyday routine. The question is thus: how comes that life is so good at creating functionality? One element of answer proposed by Otto *et al.* is that functionality arises from structure – living organisms are highly functional because they are highly structured on many length scales.^{8,9} For example, cells are compartmentalized in many subcellular proteomes and each one of these possesses different functions.¹⁰ Cell functionalities arise both from the structure at the atomic level, *i.e.* from (macro)molecules, and from the association of these molecules at larger length scales (see Figure I.2).

Atoms can be organized in an infinite number of molecules. For biological systems, this notably results in biomacromolecules, *i.e.* nucleic acids, carbohydrates, lipids and polypeptides. Nucleic acids are mostly used by living organisms to store information. Carbohydrates and lipids are used to store energy and as structural molecules, and polypeptides can perform a vast array of function such as chemical transformation, information mediation and structural support. As far as functionality is concerned, polypeptides, especially proteins, are the biomacromolecules that hold the best promises. These are very complex polymers made of twenty-two different amino acids (a.a.) that are polymerized into a well-defined sequence. A comprehensive analogy written by Norde *et al.* is that a.a. are letters associated together to make a word, *i.e.* a polypeptide. Just as an infinite number of words can be written by using the twenty-six letters of the alphabet, an endless number of polypeptides can be created by associating twenty-two a.a. As with words, only a fraction of the a.a. sequences that are formed will be meaningful, *i.e.* will yield a polypeptide that can fold into a functional structure.¹¹ This folding and its subsequent functionality depend on the polymer length and the distribution of polar and apolar, cationic and anionic, rigid and flexible a.a. within the polypeptide. Depending on their length, polypeptides are considered as proteins, peptides or oligopeptides, from the longer to the shorter chains.¹²

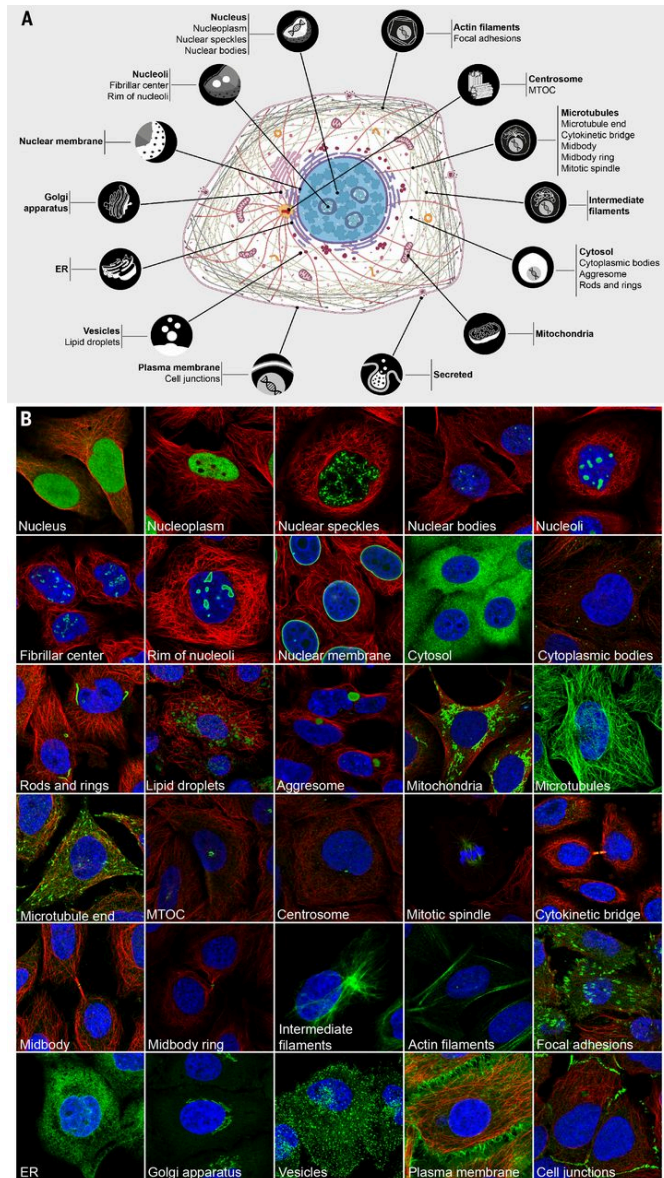


Figure 1.2 – (A) Schematic overview of the cell. Thirteen subcellular proteomes, as well as a proteome of secreted proteins, were defined in the Cell Atlas by determining the localization of proteins to 30 subcellular structures. – (B) Subcellular structures annotated in the Cell Atlas by immune-fluorescence microscopy. Examples of proteins (green) localizing to each annotated structure in the representative set of human cell lines used in the Cell Atlas. Microtubules are marked with an antibody against tubulin (red); the nucleus is counterstained with DAPI (blue). The side of an image is 64 μm . Figure reproduced from Thul *et al.* with permission of the AAAS.¹⁰

Enzymes are proteins that can accelerate chemical reactions by $>10^{17}$ -fold.¹³ They serve as structural scaffolds that selectively recognize and bind their substrate. After binding, the enzyme provides an environment that differs from the bulk, which allows the reaction to be faster. The substrate binding to the enzyme is usually compared to a lock-and-key system, where the substrate structure perfectly matches the cavity created by the a.a. folding. It is substantial to stress that this conventional view has now shifted to a much more complex view of the enzyme. Indeed, it has been demonstrated that enzymes can change their conformation to adapt to the substrate. Long-range electrostatics were shown to have a critical importance in the enzyme ability to accelerate reactions. Therefore, the structure is not only important at the binding site, but also far from it. Finally, networks of a.a. that are thermodynamically coupled are present in the enzyme. The consequence is that any change to the enzyme can cause a significant variation of their enzymatic activity. For example, if the enzyme is attached to a surface *via* a covalent bond that is located far away from its catalytic site, it still may cause a dramatic change in its enzymatic activity. These features are illustrated in Figure I.3 (adapted from Agarwal).¹⁴ Proteins that do not catalyze any reaction also possess functions that are related to their structure. Exactly as for enzymes, these are highly dynamic and interconnected. For example, some signal proteins are responsible for information transmission between distant functional surfaces. The substrate binding on one side is propagated through the protein, which causes a conformational change on another site of the protein. Similarly, allosteric regulation in various proteins emerges from non-local interactions between residues.¹⁵

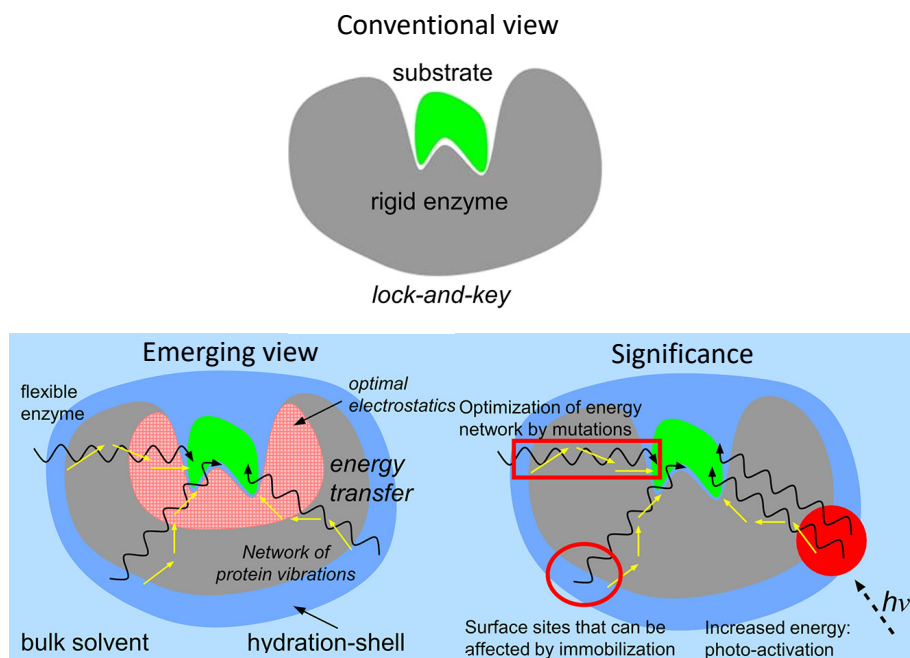


Figure I.3 – The conventional view for the origin of enzyme activity is the rigid structural lock-and-key analogy. With the emerging view, enzymes are now considered as highly dynamic macromolecules. The interconnection among protein structure, dynamics, and function is now considered to be a key feature of enzyme activity. The active site region provides an environment with optimal electrostatics to promote the catalyzed reaction. Several enzymes contain conserved networks of residues that connect surface regions to the active site. These networks provide thermodynamic coupling between the hydration shell and bulk solvent, and the catalyzed reaction. As a consequence, any change in the protein surface can result in significantly different enzymatic activities Reprinted with permission from Agarwal *et al.* Copyright 2019 American Chemical Society.¹⁴

Since proteins are highly functional, their immobilization on a material surface is topical for many reasons, and all fields that were presented hereabove may benefit from protein immobilization (see Figure I.4):^{7,16,17}

- a) For diagnosis, a field known as biosensing has been growing for the last two decades. The aim is to create sensors that combine biological elements of recognition with a physical transducer. The resulting device, called biosensor, is capable of detecting specific molecules. In Figure I.4a, the binding event of a molecule to an immobilized protein is transduced by the supporting material (transducer) into a processable signal. The surface of the material

can be engineered to avoid unspecific interactions, therefore decreasing the risk of false negative/positive.

- b) For the design of biocompatible materials, *i.e.* biomaterials, the immobilization of proteins can have a tremendous impact. As represented in Figure I.4b, proteins, once immobilized on surfaces, can be recognized/sensed by cells. This allows to improve the material biocompatibility through, amongst others, the improvement of cell attachment or differentiation. For example, prostheses coated with proteins from the extracellular matrix were shown to lead to better osseointegration and lower foreign body response.
- c) Proteins are potential prominent medicines and their delivery to various locations in the body has led to critical advances in healthcare. The delivery of proteins that are trapped in particles or immobilized on the particle surface is illustrated in Figure I.4c. Alternatively, their ability to interact specifically with certain molecules also allows to target cells or organs with a very high precision. For example, nanoparticles coated with proteins and targeting tumor cells have been used to deliver chemotherapeutic agents with a high precision.
- d) Enzymes are proteins which can catalyze the transformation of molecules with an unequalled specificity, at low temperature and in aqueous solvent. Their immobilization in a reactor allows to easily separate the enzyme from the product and to operate under flow (Figure I.4d.1). Protein immobilization can also improve their stability against deactivation in elevated temperature or the presence of solvent. Interestingly, the immobilization of two different enzymes that work in tandem allows creating cascade reactions, *i.e.* one enzyme converts a molecule into the substrate of another enzyme (Figure I.4d.2). Cascade reaction between the immobilized enzyme and a reactive supporting material can also be obtained. A last benefit of enzyme immobilization is the improvement of enzyme bioactivity through engineering of the supporting material (Figure I.4d.3). Channeling effects can for example be used to enhance enzyme tandem reactions (improved product surface diffusion).

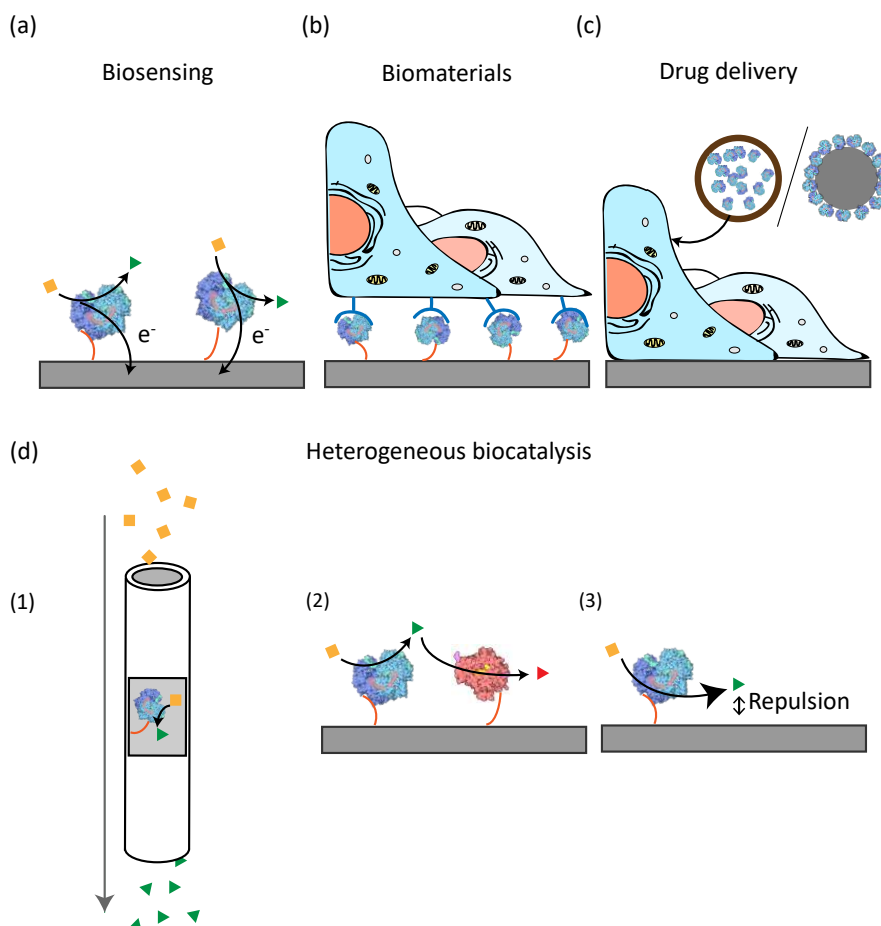


Figure I.4 – Illustration of the advantages that promote protein immobilization in (a) biosensing, (b) biomaterials, (c) drug delivery and (d) heterogeneous biocatalysis. (d.1) shows how the protein immobilization allows to operate in flow, (d.1) illustrates a tandem reaction between two enzymes that are immobilized and (d.3) shows how the engineering of the substrate properties can influence the enzyme reaction rate.

Proteins have thus become one of the most used functional macromolecules and their interfacial immobilization has a huge potential for all applications requiring functionality at interfaces. Moreover, tremendous advances were recently made with respect to their recombinant expression and purification.¹⁸ Proteins have thus become affordable materials and new proteins or protein mimics are steadily available.

2. Protein immobilization methods

In the following, an overview of the existing methods that are used to immobilize proteins is presented. It is worth mentioning that a classification of these methods is not straightforward, and it may vary depending on the application that is aimed. For instance, in the field of drug delivery, the entrapment of proteins in particles is considered an immobilization method as it allows to phase separate, concentrate and handle proteins. Contrarily, protein entrapment is never described as a way to create biosensors. For the sake of comprehensiveness, a method of protein immobilization will be defined as a way to phase separate the proteins in a controlled manner.

2.1. Physical adsorption

Proteins are highly surface active and usually, they adsorb spontaneously on almost any surface. The reason is that proteins are amphiphilic and easily optimize their interactions with surfaces.¹⁷ Because of their complex nature, protein adsorption is an intricate phenomenon involving many different interaction types such as electrostatics, dispersion and hydrophobic forces, and conformational changes. It is thus challenging to predict the outcome of their interaction with a surface. In this view, the convenient terms *hard* and *soft* proteins have been introduced, which indicate the strength of their internal structure.¹⁹ A *soft* protein will easily change its conformation upon adsorption, in contrast to a *hard* protein. Since conformational change is one of the major driving forces, *hard* proteins will be less likely to adsorb on surfaces when compared to *soft* proteins. This tendency of proteins to unfold may cause the protein to lose its bioactivity. Indeed, as already explained, protein activity is closely related to its structure. Moreover, the protein amount that is immobilized is limited to the surface area of the supporting material and molecular crowding usually limits the surface loading.²⁰ Another issue is that protein adsorption is under dynamic equilibrium and thus protein removal from the surface is very likely, especially upon condition change.²⁰ The physical adsorption is thus not the most appropriate method in a view to functionalize surfaces with active proteins. However, due to its simplicity, this method is still used in many applications, mostly for the widely used ELISA.²¹ Another field that uses physical adsorption is tissue engineering

where cellular cues are sent through the adsorption of proteins from the extra-cellular matrix.²²

2.2. Covalent binding

Functional groups present on the protein can be used to covalently link a protein to a solid surface. Procedures using acrylamide, carboxy/diimide, cyanogen bromide and glutaraldehyde were reported in many papers.²³ The advantages are that a higher surface loading and a lower protein leaching can be obtained.²³ Still, many different issues are related to covalent binding. First, protein loading is limited to the surface area. Importantly, covalent immobilization requires reactive chemical functions on the material, which consequently reduces the versatility of the method. Lastly, reaction of the linker with the protein may lead to protein inactivation.²⁴ As explained hereabove, protein structure is critical and even the slightest change can affect that activity. For example, the old fashion approach consisting in positioning the covalent bond far from the active site may cause a loss of the protein function.¹⁴

2.3. Entrapment in particles

Strategies that aim at immobilizing proteins by either trapping them into networks of molecules or into core-shell particles have been widely investigated. There are three different ways to immobilize proteins in particles and these are represented in Figure I.5. The first is the entrapment in polymeric networks (Figure I.5a). For example, sol-gel chemistry has been widely used to immobilize proteins into matrices prepared from metal-alkoxides.²⁵ Another procedure, rather biomimetic, consists in the encapsulation of proteins in lipid-based vesicle (Figure I.5b).²⁶ A method related to the former is the protein self-immobilization which is achieved by the formation of cross-linked enzyme aggregates (CLEAs; Figure I.5c).²⁷ In the latter case, freestanding aggregates of potentially active proteins are obtained. Compared to the physical adsorption and the covalent binding, these strategies present major assets. First, the loading is not restricted to the material surface and the whole volume can be filled with proteins.²⁸ Consequently, highly active materials can be obtained. Except for CLEAs, the

absence of chemical reaction with the protein limits protein inactivation. However, the issue with these methods is that these are usually restricted to particles. It is thus not straightforward to use them to create continuous flow reactor or bioactive prosthesis coatings. Moreover, for sol-gel, pore size has to be carefully controlled so that the protein does not leach out or to allow diffusion of the enzyme substrate into the network/particle.



Figure I.5 - Protein immobilization strategies: (a) entrapment, (b) encapsulation, (c) and self-immobilization. Proteins are represented by circles. Adapted from Braddy *et al.* with permission from Springer Nature.²⁷

2.4. Polymer brushes

Polymer brushes have been widely used to immobilize proteins.²⁹ These are constructed by grafting a polymer to a surface or by directly polymerizing it from the interface (grafting from).^{30–32} When polymer brushes are exposed to proteins, they either repel or adsorb them depending on the condition, the polymer used and the protein. When the interaction between the protein and the brush is favorable, three case scenarios are observed, i.e. three adsorption positions: (a) *primary adsorption*, at the brush substrate interface, (b) *secondary adsorption*, at the brush liquid interface and (c) *tertiary adsorption*, where protein adsorbs throughout the brush due to attractive interaction between the proteins and polymeric chains (see Figure I.6).³³

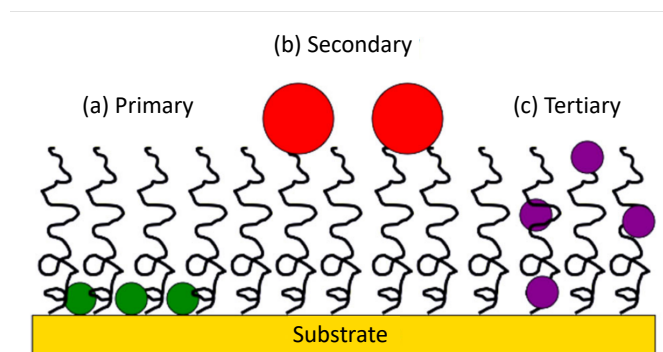


Figure I.6 – Schematic of PE brushes on a surface and protein adsorption position: (a) primary adsorption, at the brush substrate interface, (b) secondary adsorption, at the brush liquid interface and (c) tertiary adsorption, where protein adsorbs throughout the brush due to attractive interaction between the proteins and polymeric chains. Proteins are represented as the green, red or purple circles. Adapted from Taylor *et al.* with permission from Springer Nature.³³

2.5. Self-assemblies

Living organisms are highly functional because they are highly structured. At the molecular level, the folding of polypeptides is at the origin of their functionality. Now, looking at the macromolecular scale, it appears that nucleic acids, saccharides, lipids and proteins are organized at a higher scale to create functional structures. For example, lipids are used to design compartments as they naturally form vesicles in aqueous medium. Nucleic acids and polypeptides can self-assemble to achieve many functions in the living organisms (replication, structure, etc.). By combining structures that are prescribed at the atomic level, many different systems were already engineered. For example, Ke *et al.* have shown that DNA bricks could be designed so that they assemble together to form defined 3D macromolecular structures. This elegant method demonstrates how far the synthesis of defined building blocks and the prediction of the structure resulting from their self-assembly (see Figure I.7).³⁴

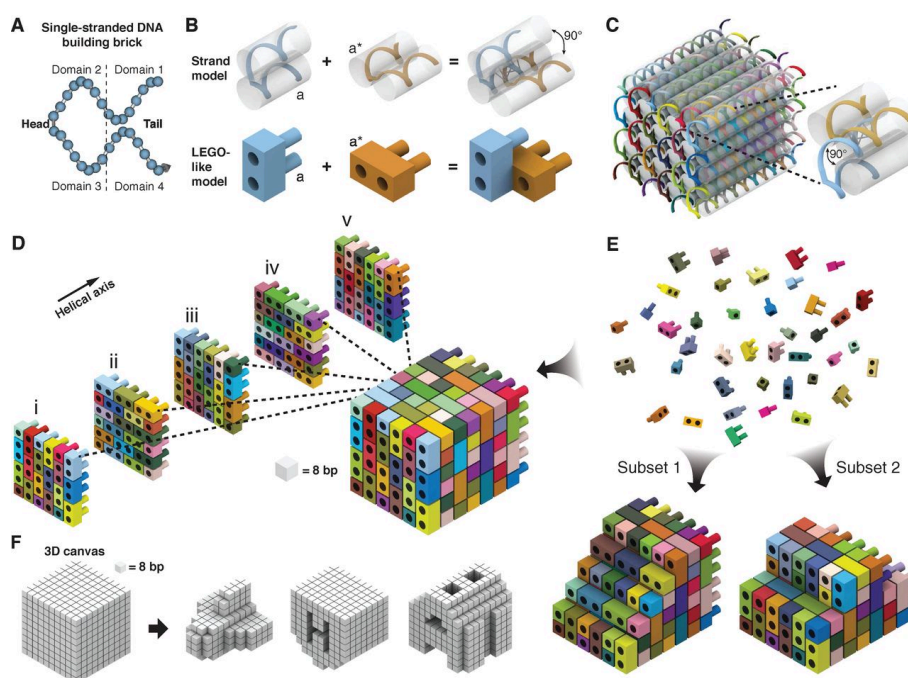


Figure 1.7 – Bricks with hundreds of distinct sequences self-assemble into prescribed 3D shapes in one step. Each 32-nucleotide brick is a modular component; it binds to four local neighbors and can be removed or added independently. Reproduced from Ke *et al.* with permission from AAAS.³⁴

Importantly, when macromolecules interact to form large aggregates, *i.e.* self-assemble, a myriad of different structures can be obtained depending on the conditions of assembly. These structures are usually out of equilibrium. Out of equilibrium means that different metastable (or transient) states can be obtained depending on the path that is followed to create the system. Indeed, all systems do not necessarily evolve spontaneously towards the state of lower energy, *i.e.* they can be trapped in non-equilibrium states.³⁵ In these cases, time or energy is needed to overcome the energy barrier necessary for the system to reach its state of lower energy. In other words, through different self-assembly pathways, different structure can be obtained. Yet, functionality arises from structure and therefore, different functionalities can be obtained depending on the self-assembly pathway. Actually, this idea is very intuitive: if one would decide to mix together all the constituents of a bacteria, it would not spontaneously self-assemble into a functional cell: the path followed for assembly matters. This idea of a

network of molecules interacting to give emergent properties has led to a new field of research named “system chemistry”.³⁵ It consists in exploring the different structures that can be obtained in all states of the energy landscape.³⁶

With respect to protein immobilization, it appears that by carefully selecting the molecules to self-assemble and the way to mix them, structures that immobilize proteins can spontaneously be formed either at interfaces or in solution. In this way, self-assemble multilayered structures can be constructed by alternating the adsorption of layers of material on top of each other, *i.e.* using the Layer-by-Layer (LbL) approach.^{37,38} In solution, the proteins can self-assembled with polyelectrolytes to form a so-called protein-polyelectrolyte complex (PPC). The former is widely used for the surface immobilization of proteins, while the latter found applications in drug delivery and protein separation technologies.^{37–39} These two self-assemblies will be extensively discussed in the following.

3. Layer-by-Layer self-assembly

In the modern scientific community, the LbL nomenclature exists since the late 1960’s and usually stands for the alternate adsorption of oppositely charged materials. The driving force of the multilayer growth is the surface charge overcompensation by the new layer of material that is adsorbed. This results in a surface charge inversion, which allows adsorbing a next layer of oppositely charged material. Charged soluble polymers, *i.e.* polyelectrolytes (PEs), are the most widely used materials. Their LbL assembly was first formalized and demonstrated by Decher *et al.* in 1992 by alternately dipping a surface into a solution of positively and negatively charged PE (see Figure I.8).⁴⁰ The assembly is achieved according to the following sequence of steps: first, a surface is immersed in a solution of a positively charged PE, *i.e.* a polycation, which adsorbs readily. Then, a washing step enables to remove any loosely attached PE. Then a negatively charged PE, *i.e.* a polyanion, is adsorbed on the now positively charged surface and subsequently washed as well (see Figure I.8). By repeating this procedure, a PE multilayer (PEM), whose thickness can be precisely controlled by the number of adsorption cycles, is obtained.⁴¹ This self-assembled layered structure can be obtained

only by alternating the adsorption of PEs. The simultaneous adsorption of the very same components would fail to self-assemble into a multilayer. Out-of-equilibrium self-assemblies acquire here their full meaning. Indeed, the ability to create these multilayered architectures at interfaces has emerged from the pathway that was used. In terms of simplicity and versatility, the LbL assembly of PE hold the best promises for surface functionalization, especially compared to methods such as covalent binding. However, in terms of functionality it is usually restricted to the chemical functions brought by the PEs and the multilayered structure (the latter gives particular light-material interactions for instance) that is obtained. Therefore, over the past thirty years, a myriad of other charged materials such as polymers, nanoparticles, lipids, nanosheets, DNA, proteins, etc. has been assembled using the LbL approach and the method has taken many different forms.⁷

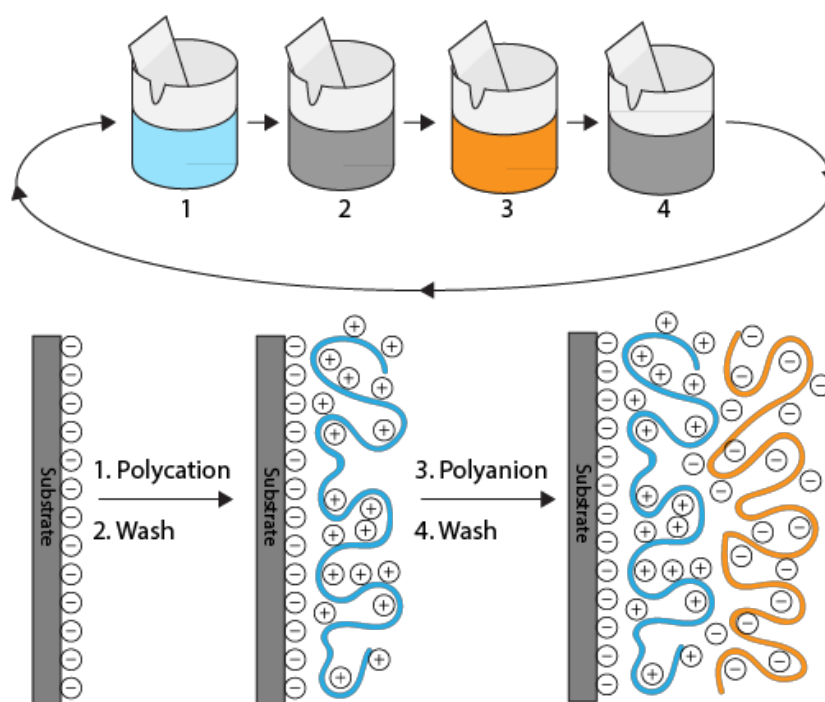


Figure I.8 – Adapted from Decher *et al.*⁴²: Schematic of the film deposition process using slides and beakers. Steps 1 and 3 represent the adsorption of a polycation and polyanion, respectively, and steps 2 and 4 are washing steps. Counterions are omitted for clarity. The polyion conformation and layer interpenetration are an idealization of the surface charge reversal with each adsorption step.

Proteins were amongst the first building blocks to be LbL assembled and, only three years after that the first paper of Decher *et al.* was published on the LbL assembly of PEs, seven different globular proteins were immobilized by alternate adsorption with PEs on surfaces.^{37,38} Indeed, proteins are very similar to PEs since they are charged and usually soluble in water. The basic principles of the LbL assembly of PEs were applied, *i.e.* alternate adsorption of oppositely charged building blocks and charge overcompensation. In order to fully grasp how the LbL assembly of protein with PE has evolved since its first use, and what are the challenges that are associated with this method, an extensive literature review was conducted. Over 365 papers were selected in Scopus using “LbL”, “Layer-by-Layer”, “protein”, “multilayer” or “assembly” keywords. Amongst those, 143 were selected for presenting the LbL assembly of a protein with a PE (entrapment of protein in multilayered vesicles, diffusion of proteins in pre-formed solely PE-based multilayers and functionalization of LbLs with proteins were excluded). Then, all papers presenting sufficient data to conclude that a multilayer is growing were selected, which represents 64 references that reported 91 different systems immobilizing 25 different proteins. All reported successful LbL assemblies were summarized, with the respective pH and salt condition used, in Table I.1 (p40).

From this literature review, it appears that, within the ten years that followed the first protein immobilization by alternate adsorption with a PE, the LbL assembly of 17 supplementary globular proteins assembled with different PEs was reported. The vast majority of these proteins were enzymes. The main goal of these papers was to understand how proteins can be assembled by LbL with a PE and what is the effect of their immobilization on both their bioactivity and conformation. With that effort, systems that are useful for biosensing and heterogeneous biocatalysis were developed. In 2001, type-1 collagen, a fibrillar protein from the extracellular matrix, was immobilized to promote interactions with cells. In the next five years, the LbL assembly of proteins of the extracellular matrix has been developed to create new biomaterials.⁴³ However, up to now, only a few more proteins of the extracellular matrix were assembled with a PE, *i.e.* type-1 and type-4 collagen, laminin, fibronectin and type-A gelatin (see Table I.1, p40).

Importantly, the majority of these reported “successful” LbL assembly lacks a complete investigation of the multilayer growth, and the formation of a LbL is assumed based solely on the alternate adsorption of oppositely charged species (which is purely speculative). Interestingly, when analyzing with Scopus the number of documents published with “Layer-by-Layer AND (protein OR biomaterial)” in their title, abstract or keywords, it appears that a constant number of new documents is published since 2010. On the contrary, the number of new documents published with only “Layer-by-Layer” in their title, abstract or keywords has increased each year since 2010. This suggests that the field of LbL assembly for protein immobilization did not expand as well as the LbL field (see Figure I.9a). This observation is actually consistent with the decreasing number of new proteins that were immobilized using the LbL approach between 1990 and 2017 (see Figure I.9b hereunder and Table I.1 p40, this cumulative number of new protein is based on the 143 papers reporting the LbL assembly of a protein with a PE), revealing the limits of the conventional LbL method in immobilizing proteins.

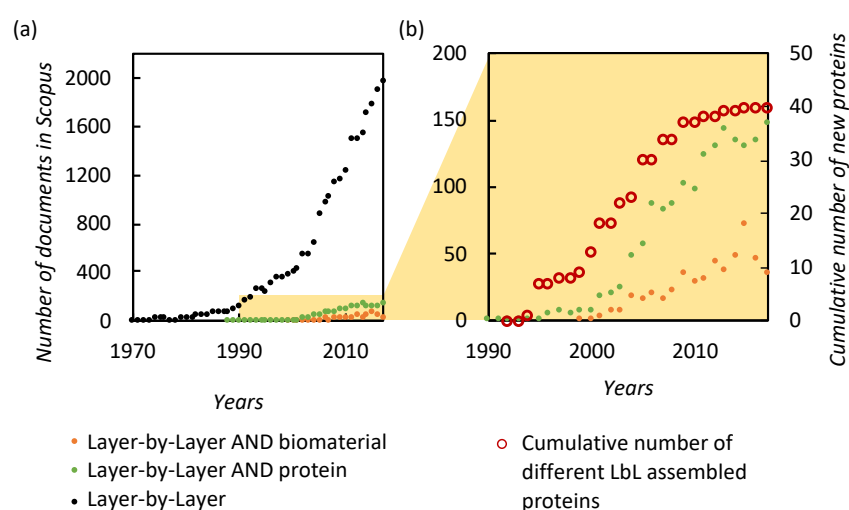


Figure I.9 – (a) Evolution of the number of documents found in Scopus with the following word(s) in their title, abstract or keywords: Layer-by-Layer, Layer-by-Layer AND biomaterial, Layer-by-Layer AND protein. (b) Cumulative number of new proteins that have been reported to be LbL assembled with a PE. Results presented together with a zoom corresponding to the yellow square of the left panel.

The gap between the potential and the current state of the art of LbL assembly of proteins with PEs is clear and until now, there is no clear general way to immobilize any given protein by the LbL method. The difficulty is that the failure to grow a multilayer by alternating the adsorption of a protein with a PE is hardly reported, and usually only successful assemblies are presented. However, few papers clearly show that the protein polyampholyte nature and complex 3D structure results in a lack of charge overcompensation upon multilayer growth. A telling example was presented by Zhang *et al.* for the assembly LbL of hyaluronic acid with type-I collagen. The surface charge was measured at each step of the multilayer construction and results are reproduced in Figure I.10a. They have shown that, as the multilayer grows, the charge overcompensation, *i.e.* the number of unpaired charges, decreases.⁴⁴ In order to further show that the protein heterogeneity causes a failure of the LbL growth, Mauquoy *et al.* assembled LbL two proteins, namely type-I collagen and fibronectin. In this paper three different buffers were tested as well as the influence of collagen denaturation and of a poly(ethylenimine) (PEI) anchoring layer. The total multilayer hydrated mass (expressed as a frequency shift) was measured as a function of the number of steps and results are reproduced in Figure I.10b. It appears that all conditions led to a failure to grow a LbL assembly.⁴⁵

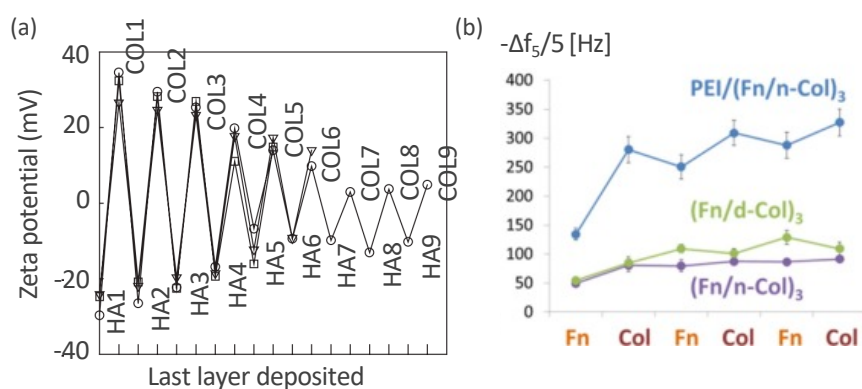


Figure I.10 – (a) Evolution of the ζ potential for three independent PEI/(HA/COL)_n multilayer films during their construction. Reproduced from Zhang *et al.* with permission of Elsevier.⁴⁴ – (b) LbL assembly in phosphate buffer saline (PBS) of native collagen type-1 (n-Col, in the Figure, Col1 throughout the thesis) with fibronectin (Fn) on a PEI anchoring layer (blue), denatured collagen type-1 (d-Col) with Fn (green) or Col with Fn (purple). Reproduced from Mauquoy *et al.* with permission of Elsevier.⁴⁵

In order to illustrate the wide variety of protein structures that exist, some of the proteins that are most used in LbL assemblies are shown at scale in Figure I.11. Since charges are critical for their assembly LbL, protein surfaces were colored red for negative residues and blue for positive residues at physiological pH (details to obtain these graphics are presented in Chapter 2). It appears that, for this rather small sample of proteins, a wide variety of size, shape and charge distribution is obtained. In all cases, the polyampholyte nature of proteins appears, with both positive and negative charges being present. To illustrate the charge distribution of type I collagen while keeping the same scale as for other proteins, a collagen-like peptide is shown. Nevertheless, the low-resolution envelope structure of type I collagen is presented in the bottom panel with all other proteins at the relevant lower resolution scale. The difference of size is striking, once more illustrating how diverse the protein structure can be. For the sake of comparison, a PE made with an aliphatic carbon chain is shown at scale for a polymerization degree of 340. From these molecular representations, it is clear that proteins are immensely more heterogenous and ordered than PEs.

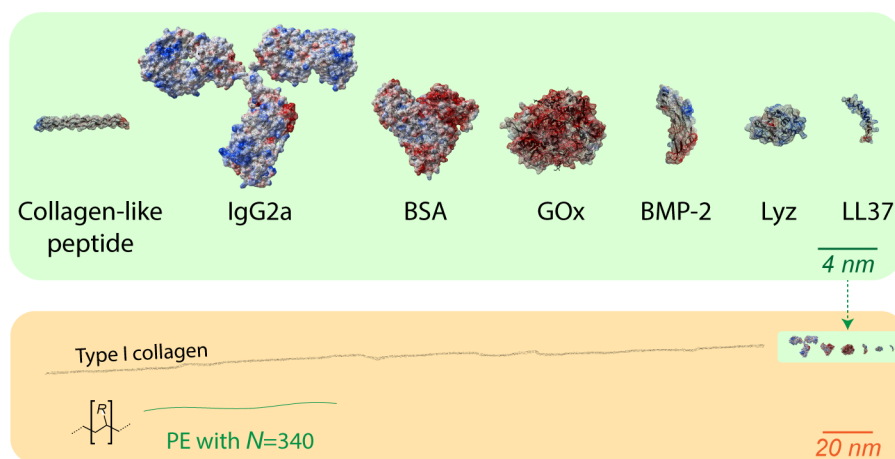


Figure I.11 - Molecular structure of type I collagen (PDB: 3HQV), collagen-like peptide (PDB: 1CGD), IgG2A (PDB: 1IGT), BSA (PDB: 4F5S), GOx (PDB: 1GAL), BMP-2 (PDB: 3BMP), Lyz (PDB: 1HEL) and LL37 (PDB: 2K6O). The electrostatic surface potential is calculated using the Chimera software. The protein surface is colored red for negative potential and blue for positive potential. These molecular structures were produced using the UCSF Chimera package.

Due to this heterogeneity, proteins are extremely difficult to assemble LbL with a PE. Nevertheless, in a view to facilitate the use of the LbL method for protein immobilization, *i.e.* to guide the choice of the assembly conditions and the PE nature, a generic approach is presented in Figure I.12. The first step is to define the specifications of the application that is aimed. These specifications can be divided into two categories: the nature of the materials that are used and the functionalities that are desired. For the former, features of the PE and the protein (biodegradability, biocompatibility etc.) should be identified. For the latter, either multilayers that are stable (biosensing or biocatalysis) or that release their content in a controlled manner (drug delivery) are needed. These characteristics depend on the choice of the PE, but also the building conditions (pH, salt concentration etc.). Once the specifications of the system are identified, the choice of the PE and the building conditions can be motivated *via* three distinct approaches. These are based on the extensive literature review that was carried out (presented in Table I.1, p40). These approaches are discussed hereunder and are represented in Figure I.12.

1. Since protein structure (much higher ordering level when compared to PEs) is thought to be the major limiting factor, the first approach is to find a protein structural analog that was already successfully immobilized, and to use the same adsorption conditions and PE. In this view, the synthetic table of assembly conditions and protein/PE nature presented in Table I.1 (p40) can be used. The reader willing to immobilize a new protein by the LbL method is thus invited to find structural analogs of that protein in this Table.
2. If no structural analog exists or if the reported system does not self-assemble in a multilayer, the prediction of the conditions that are necessary to grow a film based on the mechanisms of protein-PE LbL assembly may prove useful. All reported molecular driving forces of the LbL assembly have been reviewed extensively by Borges *et al.*⁴⁶ However, proteins were evidenced to exhibit a particular behavior when assembled with PE using the LbL approach. This will be discussed hereafter (section 3.1).

3. Unconventional LbL assemblies were reported to allow the successful immobilization of proteins. These permit to circumvent issues relative to the LbL assembly of proteins with PE and should be investigated as an alternative. Importantly, these LbL assemblies usually possess new features compared to classical LbL assemblies, which might be useful depending on the application. These unconventional LbL assemblies will be presented in section 3.3. The method developed in this thesis falls into this last category and will provide a new alternative to the LbL assembly of proteins.

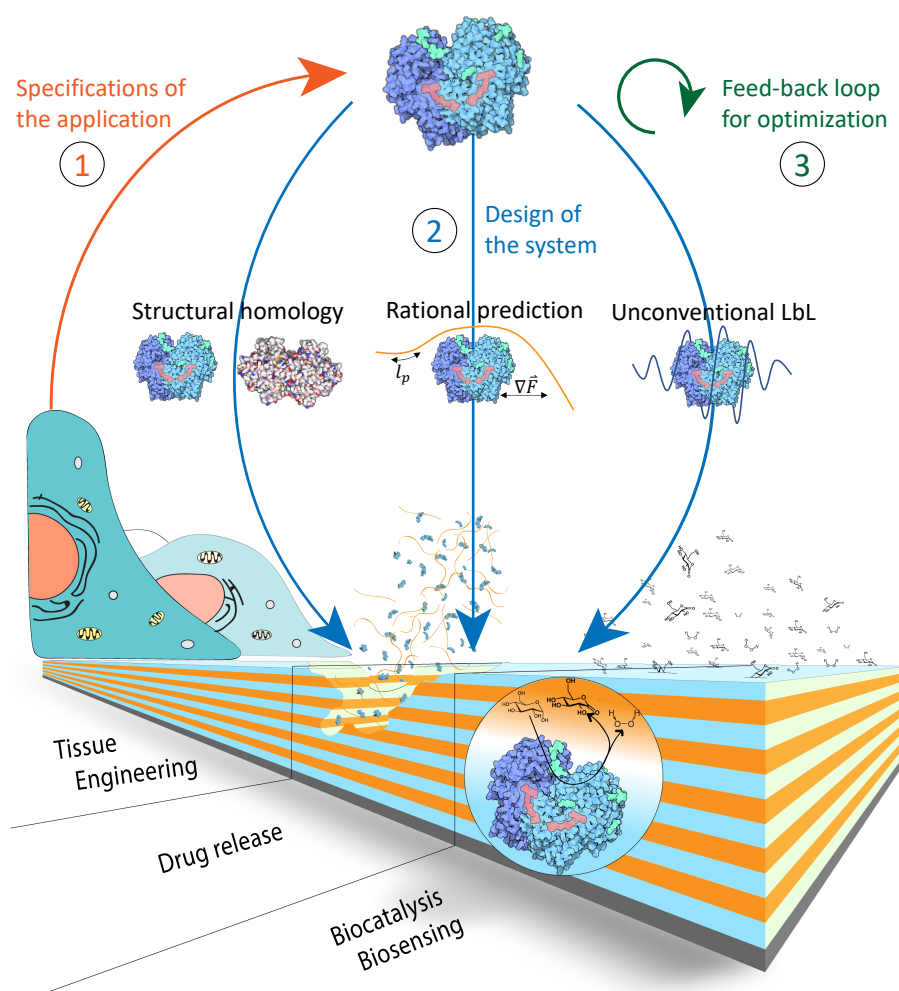


Figure I.12 – Strategies that can be used to choose the right conditions and the right protein-PE couple for the LbL assembly of proteins.

3.1. Mechanisms of the LbL assembly of proteins with PE

In the following, the effect of extrinsic parameters, *i.e.* that are not specific to the desired multilayer (pH, nature and concentration of salts, temperature, concentration of protein and PE), and intrinsic parameters, *i.e.* that defines the multilayer (nature and characteristics of PE, nature and concentration of the protein, effect of solid substrate) on the protein-PE LbL assembly will be reviewed. In order to both avoid the effect of surface geometry and to allow the use of analytical methods such as quartz crystal microbalance with dissipation monitoring (QCM-D), atomic force microscopy (AFM), streaming potential measurement and X-ray photoelectron spectroscopy (XPS), the studies reviewed in this section were mainly conducted on planar surfaces (with a few exceptions regarding the ζ -potential measurement of LbL-coated particles).

3.1.1 *Extrinsic parameters*

a) pH of the solution

pH was found to be a critical parameter in the very first paper reporting the LbL assembly of proteins with PE. It was shown that the pH of the protein solution has to be set apart from the isoelectric point (IEP) so that proteins are sufficiently charged in order to favorably interact with PE.⁴⁷ If the pH of the solution is adjusted lower than protein IEP, the protein will take a net positive charge. In this condition, the protein has to be LbL-assembled with a negative PE (see Figure I.13). If the pH of the solution is set above protein IEP, the opposite stands and the protein has to be LbL assembled with a positive PE. This basic rule was represented in Figure I.13 for bovine serum albumin (BSA) that has a theoretical IEP of 5.78 and an experimental IEP of 4.7.⁴⁸ The BSA charge was computed as a function of pH using its 3D structure (PDB structure of BSA: 4F5S) and the PDB2PQR server. It shows that the protein absolute charge increases when the pH of the protein solution deviates from the IEP (see Figure I.13). This is also represented by molecular graphics of BSA at different pH values in Figure I.13. The electrostatic surface potential of BSA was calculated using the Chimera software and colored red for negative potential, *i.e.* negative charge on the BSA backbone, or blue for positive potential, *i.e.* positive charge on the BSA backbone. BSA appears

mainly positively charged in acidic pH and mainly negatively charged in alkaline pH. Importantly, around BSA IEP, the net charge is small while both positive and negative charges are present. Since the assembly relies on electrostatic interactions with a homogeneously charged PE, it is expected that working at a pH near the IEP of the protein would be detrimental for the LbL growth. The pH that is used for the LbL construction has thus to be chosen a few units above or below IEP. This is supported by the conditions chosen for the construction of 63 different protein-based LbL films as summarized from Table I.1 (p40). $|pH - IEP|$ was computed in each case, and this literature analysis is presented in Figure I.14. It appears that the vast majority of pH conditions that are used for the LbL construction with proteins are at least one pH unit above or below the protein IEP. This confirms that pH has to be fixed away from the protein IEP, and suggests that this condition should be maintained when designing a new system. This condition was represented as the shaded grey area in Figure I.14. Importantly, the protein was systematically alternately adsorbed with an oppositely charged PE. Only one paper, reported the LbL assembly of a protein that has the same charge as the PE.⁴⁹

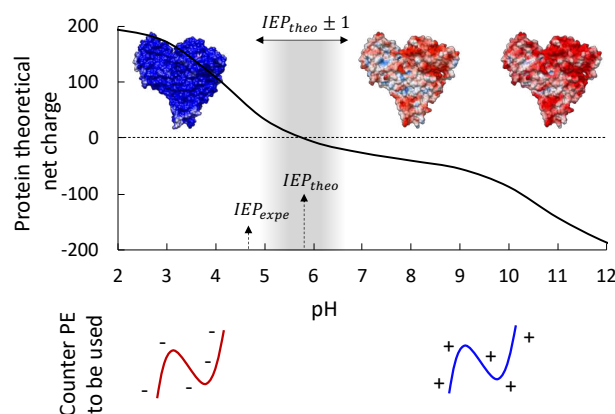


Figure I.13 – BSA charge as a function of pH as computed from the PDB2PQR online tool and using the 4F5S PDB structure. The electrostatic surface potential of BSA is also calculated using the Chimera software. BSA is colored red for negative potential and blue for positive potential. The result is that, to perform LbL assembly, a counter polyanion has to be used below BSA IEP, and a counter polycation has to be used above BSA IEP. Of course, the BSA structure changes with pH and the conformation presented here only illustrate the BSA surface charge variation with pH.⁵⁰

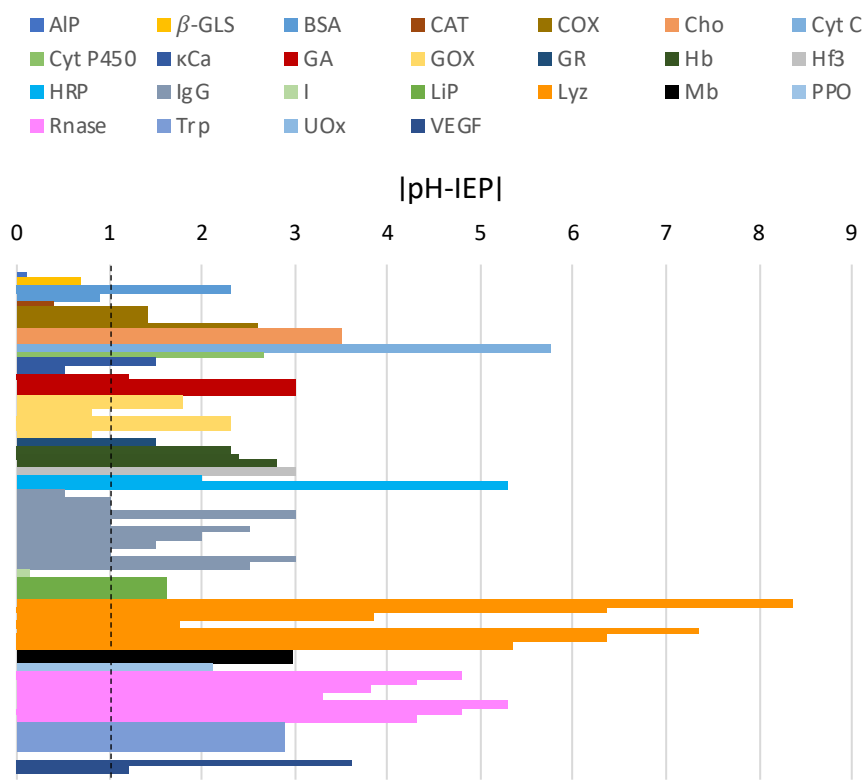


Figure I.14 – $|pH-IEP|$ was calculated for proteins successfully assembled using the LbL approach (data from Table I.1, p40) – the pH value is the one selected for the protein adsorption step). Each color represents a LbL assembled protein (see Table I.1, p40 for the protein abbreviations). The protein IEP reported in the paper was used. If no IEP was reported, the theoretical IEP of the given protein was computed using PDB2PQR. In all cases, the protein was oppositely charged to the PE. (64 references reported the successful immobilization of 25 different proteins in 91 different pH and I conditions).

With respect to the great protein charge variation with pH (as demonstrated in Figure I.13), it has been reported that the higher the absolute net charge, the better the LbL assembly. Indeed, it was shown that more favorable electrostatic interactions improve LbL assembly.^{51,52} In a similar way, it was shown that $|pH - IEP|$ correlates with the protein amount that is adsorbed on an oppositely charged multilayer.⁵³ If a weak polyacid is chosen, the balance between protein and PE ionization governs LbL assembly. For example, it was shown that for Lyz assembled with poly(methacrylic acid) (PMAA), a weak polyacid, the immobilized amount highly depends on PMAA ionization degree.⁵⁴ Among others, it was shown that when PMAA has a high

ionization degree, *i.e.* bears more positive charges, soluble Lyz/PMAA complexes form, thereby removing Lyz from the multilayer. This results in the failure to grow a LbL film.

The possible complexation in solution between proteins and PE that bears the same net charge is well known. This is attributed to the presence of positively charged patches on the protein, even if the protein net charge is negative.³⁹ The question that flows naturally from this is: do proteins and PE of the same charge assemble into a LbL film? Interestingly, the LbL assembly of negatively charged heme proteins with negatively charged dextran sulfate was reported.⁴⁹ However, it might be that the N₂ drying between each deposition step ensured LbL growth. Indeed, it appears in Table I.1 that an exponential LbL growth (*i.e.* the mass increment increases at each step of the multilayer growth) is observed in most of the studies in which N₂ is used to dry the film between deposition steps. In contrast, when no drying step is used, the growth is usually linear (see Table I.1, 40). The complete change in the structure of the multilayer upon N₂ drying is supported by papers that show that when a PE layer is dried, further adsorption by a new immersion in the same solution is made possible (the same PE is thus adsorbed).⁵⁵ This latter case has been attributed to a rearrangement of the last adsorbed layer. It is thus clear that different LbL films are obtained when a drying step is applied between deposition steps. This might represent a valuable alternative if no LbL growth is achieved.

The pH value of LbL assembly has to be carefully selected so that protein denaturation does not occur. For example, hemoglobin assembled with poly(styrene sulfonate) (PSS) at pH 4.5 gets denatured while hemoglobin assembled at pH 9.2 with PEI retains its native structure.⁴⁷ Protein aggregation may also occur at given pH values as shown for the assembly of collagen with hyaluronic acid. In that case, the assembly has to be carried out at pH 4 to avoid fibril formation.⁵⁶ The pH of the multilayer construction has also to be selected with respect to the targeted application. Indeed, any change in condition may disrupt the formed film, especially when the PE or protein charge is decreased. In order to stabilize LbL films upon pH change, many papers reported on crosslinking strategies. Brynda *et al.* found that IgG

could be assembled with dextran sulfate at pH 4 but an ELISA assay carried out at pH 7.4 disrupted the multilayer. The LbL film was thus crosslinked with glutaraldehyde after that the multilayer was constructed.⁵⁷ It is worth mentioning that while cross-linking improves stability, it may be detrimental to the protein functionality since it reacts with the amine functions protein.

A last crucial point, which is also related to protein stability, concerns the choice of the pH of LbL build-up depending on the pH of the application that is aimed. If $|pH - IEP|$ is maximized to immobilize a large protein amount, it could lead to the construction of a multilayer which is too unstable (the only exception being for drug delivery systems where a burst release is sometimes required). Indeed, the vast majority of proteins have an IEP which is close to the physiological pH (around pH 7, see Figure I.15). This suggests that lower or higher pH values should be chosen for LbL construction to maximize $|pH - IEP|$. However, the pH of the majority of applications is also found between pH 5 and pH 8. This was represented for biomaterials in Figure I.15 where the pH of different body parts is shown (data are taken from Lu *et al.*⁵⁸). This means that, after multilayer formation at a pH value far from the IEP, when the multilayer is placed in the conditions required for the application, the net charge of the protein decreases. The consequence is that the multilayer is likely to be disrupted. As an alternative, it could be interesting to construct the multilayer at the pH of the application. A lower protein amount would be immobilized, but a higher stability could be reached since the pH then does not change upon exposure to the medium. This comment is also valid for salt concentration, a parameter that will be discussed hereunder.

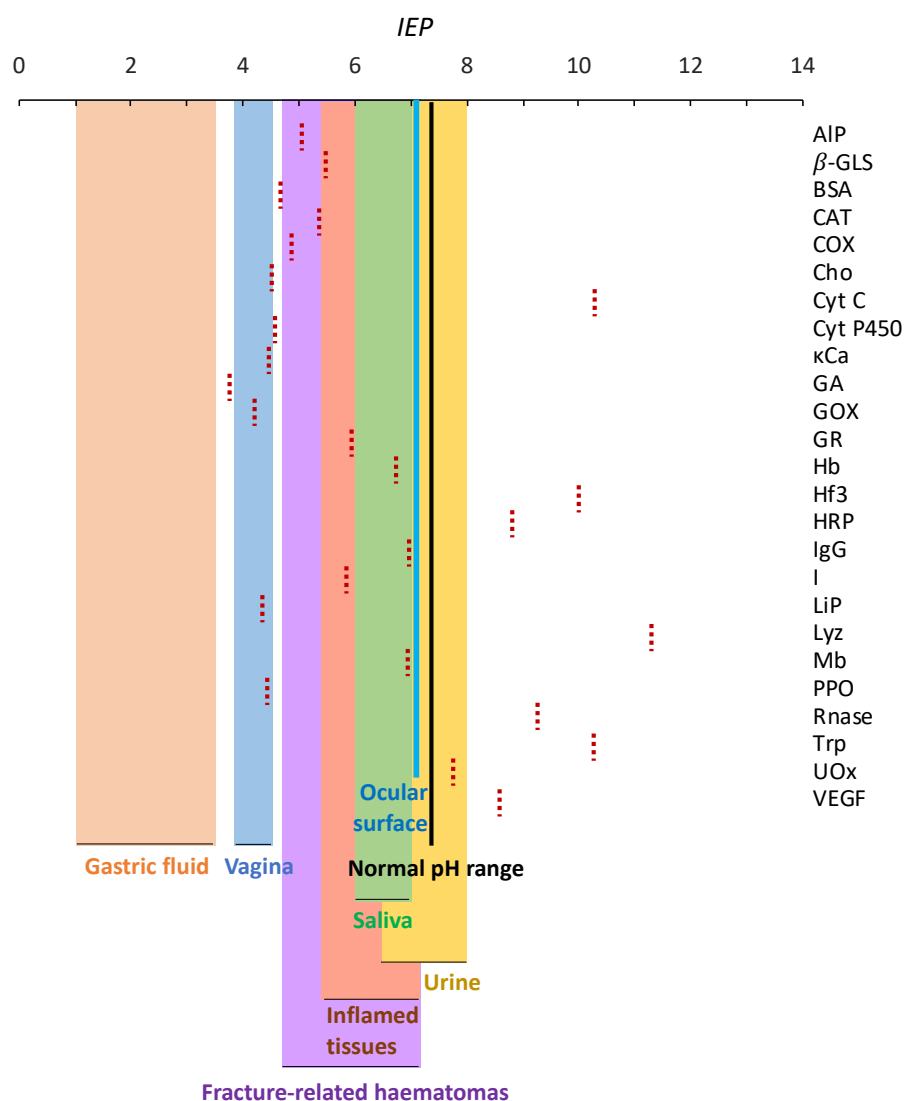


Figure I.15 – IEP of proteins that were successfully LbL assembled represented as the dotted red line (data from Table I.1, p40). pH range of some body parts (pH data are taken from Lu *et al.* ⁵⁸).

b) Presence of salts in solution

The presence of salts in solution has a tremendous influence on the multilayer growth. Similarly to pH, its effect on LbL construction of proteins with PE has been investigated since the first papers published on the subject. Ions dissolved in solution with proteins and PE have an influence on two

length scales: on short distances, *i.e.* ion pairing, and on long distances, *i.e.* Debye screening.

On short distances, it has been evidenced that ions are condensed on the charged functions of proteins and PE. These condensed ions, *i.e.* counter-ions, are released when the protein and the PE complex each other. Their release produces an entropy gain, which favors the cooperative formation of ionic pairs between the protein and the PE. Curiously, this driving force is usually not discussed in LbL assemblies with proteins. It is however considered as one of the major associative force driving the complexation of oppositely charged species.⁵⁹ As it is usually described in more details for soluble or suspended PPCs (*i.e.* not at interfaces), this driving force will be presented in the section related to PPCs.

On long distances (at the molecular scale), the electrostatic screening of charges play an important role in the LbL construction.^{59–61} This screening can be evaluated by computing the Debye length (κ^{-1}), which represents the distance over which electrostatic interactions are significant. κ^{-1} was computed as a function of salt concentration, which was expressed as the square root of the ionic strength (I) (see Figure I.16, details on computation will be presented in Chapter 2 of Part IV:). An exponential decay of κ^{-1} as a function of I is obtained. The Bjerrum length (l_b) which represents the distance at which electrostatic interaction between two charges is equal in magnitude to the thermal energy scale, is also represented as the dashed line on Figure I.16. When κ^{-1} reaches values below l_b , the electrostatic contribution to the LbL growth is thus considered negligible.

Salt concentration also influences PE conformation, which is a key parameter in LbL construction. As represented in Figure I.16, when salt concentration increases, *i.e.* κ^{-1} decreases, the electrostatic repulsion between charged monomers decreases and the PE becomes more flexible and adopts a less extended, more coiled structure in solution. The consequence is that polymer loops are formed upon adsorption on the surface for intermediate salt concentrations. In this way, Caruso *et al.* studied the effect of various MnCl_2 amounts on the LbL deposition of PSS with anti-IgG on a

Poly(allylamine hydrochloride)-PSS ([PAH-PSS]₂) precursor film.⁶² They determined that increasing amounts of both PSS and anti-IgG were deposited with increasing MnCl₂ concentration, *i.e.* 0, 10 and 100 mM MnCl₂. This observation was attributed to the more coiled PSS conformation as salt concentration rises. It was proposed that this coil conformation increases the surface available for anti-IgG adsorption, which was confirmed by the increased surface roughness with increased MnCl₂ concentration.⁶²

The increased protein amount with increased salt concentration has also been attributed to the decreased lateral repulsion between adsorbed proteins. If the salt concentration decreases, κ^{-1} takes increasingly higher values and charged proteins repel each other. This results in a decreased protein density in each layer of a LbL film at low salt concentration.⁴⁹

Contrarily to what is observed when the salt concentration is increased from low to medium, the excessive charge screening at high I results in the impossibility to grow a film by alternate adsorption. For instance type 1 collagen alternate adsorption with hyaluronic acid did not lead to any LbL growth at high salt concentration.⁵⁶ The same result was observed for keratin/poly(diallyldimethylammonium chloride) (PDADMAC) assembly that showed a decreased amount of immobilized keratin as salt concentration rose.⁶³ In a similar way, it has been shown that human serum albumin adsorption on a multilayer ending with oppositely charged poly(vinyl sulfonate) (PVS) was drastically reduced when adsorption was carried-out in 1M NaCl instead of PBS buffer (I around 0.015 M).⁵³ Additionally to charge screening, recent data suggest that the impossibility to grow a LbL in concentrated salt solution is due to a decreased strength of the entropic contribution to ion pairing.⁶⁴

In conclusion, the effect of salt on the LbL growth is non-monotonic (see Figure I.16). When salt concentration increases from low to medium, the PE becomes more flexible and lateral repulsion between proteins is screened. This results in an increased amount of protein incorporated in the LbL film as salt concentration rises. However, when salt concentration increases from medium to high, the electrostatic attraction decreases and the LbL growth is

no longer possible. This was nicely shown by He *et al.* who studied the effect of a wide range of salt concentrations, and have shown that both the activity of the protein and the surface concentration of protein in LbL increased then decreased with I (fixed with NaCl), passing through a maximum at about 300 mM NaCl.⁴⁹

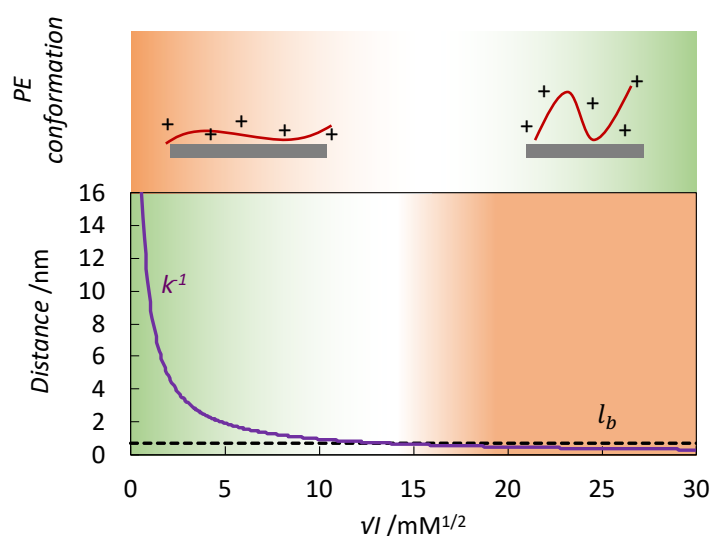


Figure I.16 – κ^{-1} (solid purple line) and the l_b are calculated as a function of salt concentration, which is expressed as the square root of I. Schematics of the adsorbed PE conformation at low and high I are also presented above. The favorable contributions to the LbL growth are presented in green while unfavorable contributions are presented red.

Based on Table I.1, all salt concentrations of the protein solutions that led to the successful LbL assembly of proteins with PE were collected and summarized as presented in Figure I.17a. In many studies, the reported condition was actually either not well defined or not even mentioned. This high proportion of missing information is stressed in Figure I.17a, where all missing data are presented as dashed blue squares. These raw data clearly suggest that the majority of the successful LbL assemblies were conducted at intermediate salt concentration. In order to clearly evidence this, the occurrence of each condition used in the range of less than 1, 1 to 49, 50 to 199 and more than 200 mM of salt was computed. Results are presented as histograms of the salt concentration in the protein solution and in the PE solution in Figure I.17b. It appears that LbL assemblies conducted at medium

salt concentration are found twice more than others for successful LbL assembly. This analysis clearly supports the fact that LbL assembly proceeds better at intermediate salt concentration.

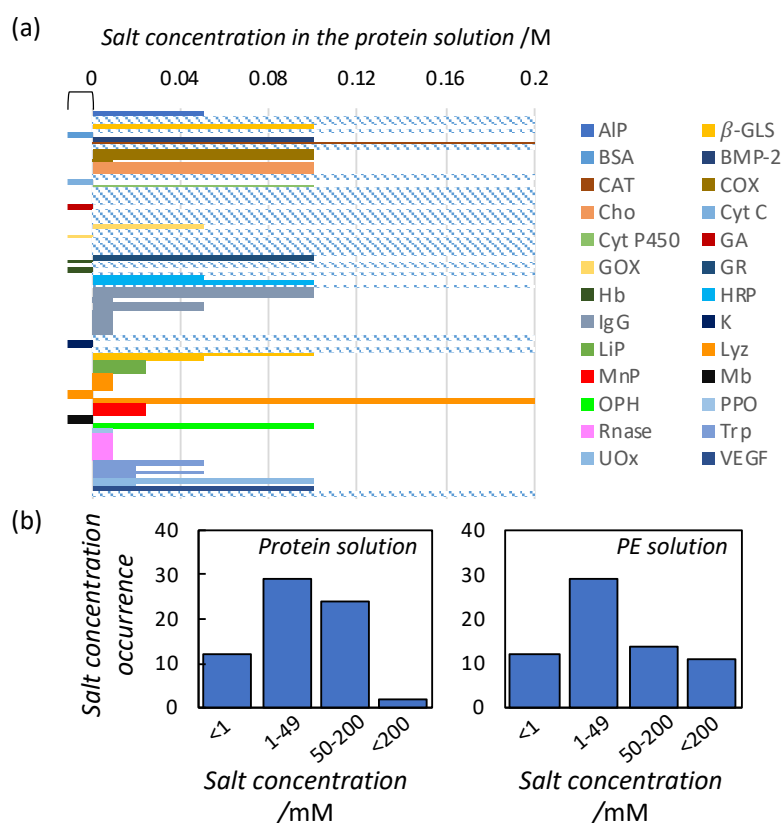


Figure I.17 – (a) Overview of salt concentrations in the protein solution (alphabetical order) that were used to successfully immobilize various proteins. Lines on the left of the graph, under the header “0”, are systems for which no salt was added. Dashed blue lines are data for which the pH was given (see Figure I.14), but the salt concentration was either missing or not sufficiently explicit. (see Table I.1, p40 for the protein abbreviations). (b) Histograms of the occurrence of the salt concentration used in the protein solution or PE solution. These histograms were constructed based on the values of (a), thus only based on studies in which salt concentration is explicitly reported.

Multilayer stability as a function of salt concentration was also investigated. Indeed, it is common to construct multilayers in conditions that are different from the ones of their application. Usually, when I is increased above a certain value, the electrostatic screening of charges disrupts the LbL film. Interestingly, the stability in salt solutions can be improved by forming

hydrogen bonds in the multilayer. This can be done by changing the pH and protonating the PE. For example, a poly(acrylic acid) (PAA)-Lyz multilayer was reported to be unstable when salt concentration was increased to physiological concentration at neutral pH while it was stable when I was increased at pH 5. This observation was attributed to the lower PAA ionization degree at lower pH resulting in the ability to form hydrogen bonds that stabilized the film.⁵⁴

c) Temperature

To our knowledge, the effect of temperature on the LbL assembly of proteins with PEs was not investigated. However, such assembly shares common features with LbL assemblies solely based on PEs (PEMs), and similar mechanisms are at play.

When a surface is immersed in a PE solution, the PE adsorbs on the surface through diffusion-driven kinetics. The rate of diffusion, which increases with temperature, thus dictates the time needed to reach equilibrium (the concentration also plays a role, which will be discussed later). When the temperature increases, the adsorption equilibrium is reached faster. This effect, which is observed in PEMs, is also expected in protein-PE LbL assemblies.

For some PEMs, PE adsorption for a given deposition step is not limited to the surface of the multilayer that is already formed and the PE can diffuse inside the multilayer. This PE diffusion in adjacent layers is also temperature dependent. Actually, the degree of PE interpenetration between layers is dictated by the ability of the PE to diffuse in the PEM, which depends on the PE-PE couple and on the conditions that are used.⁶⁰ If the PE interpenetration is constant at each step of the multilayer growth, the total multilayer mass increases linearly with the number of adsorption steps. Contrarily, if the deposited PE diffuses in the whole multilayer, then the total multilayer mass increases exponentially with the number of adsorption steps. Multilayers are thus highly dynamic and constantly reorganize upon adsorption of new charged materials.⁶⁵ The effect of temperature is that diffusion, *i.e.* interpenetration, in the PEM is improved, which results in a higher mass

increment at each deposition step.⁶⁶ This may even lead to a transition between a linear and an exponential growth of the multilayer as temperature rises. With respect to protein-PE LbL assemblies, it is less likely that the protein may diffuse within the film given protein size. The PE used as the counterpolyion might, however, diffuse in the multilayer, which could improve LbL growth when temperature increases.

It is important to remind that protein fibrillation may occur at high temperature, which limits the temperature operation window. For instance, Mhanna *et al.* carried out collagen adsorption at 4°C to avoid its fibrillation.⁶⁷ Another related issue is that temperature affects protein quaternary structure and subsequently its activity.

d) Concentration of the protein solution

By analogy with PE-PE LbL assembly, one can assume that protein-PE growth is partially governed by substrate diffusion. As for temperature, the increase of protein concentration in the solution that is used for the adsorption allows equilibrium to be reached faster. This parameter was only investigated in a few papers. It was reported that the amount of immobilized protein scales with protein concentration up to concentrations in the range of 1 mg mL⁻¹.⁴⁹ He *et al.* tested the effect of different protein concentrations on their adsorption on a LbL film. The same adsorption time was used for all concentrations that were tested. A higher protein amount was observed on the multilayer surface when the concentration increased. This result was attributed to an incomplete surface saturation at low protein concentration, for the incubation time that was tested.⁴⁹

3.1.2 Intrinsic parameters

The rational prediction of LbL growth between proteins and PEs is very challenging due to the wide variety of parameters at play, and more importantly, to due to the very high heterogeneity of proteins. Very few papers investigated systematically how the LbL construction is affected by changing its composition, *i.e.* the PE nature (chemical nature, persistence length, charge) and the protein nature, as well as by changing the substrate.

a) The nature of the PE

When PEs are assembled LbL with proteins, they should be highly flexible to accommodate for the protein structure and form loops at the interface. The PE flexibility can be evaluated by its persistence length (l_p), which represents the length over which correlation between PE segments is lost.⁶⁸ l_p is usually decomposed in the intrinsic persistence length (l_p^0) and the electrostatic persistence length (l_p^e). l_p^0 represents the stiffening coming from steric hindrance and hybridization state of the atoms. l_p^e represents the stiffening due to electrostatic repulsion between charged monomers. The latter parameter is thus related to κ^{-1} .

PEs with a small l_0 must then be selected to ensure PE flexibility. Then, l_e must also be kept with a small value. This can be achieved by increasing the salt concentration, *i.e.* by decreasing κ^{-1} . This will decrease the electrostatic repulsion between monomers and thereby increase PE flexibility. Polymers with a decreased number of charged monomers can also be chosen. For instance, the degree of charged monomers can be controlled in chitosan by its deacetylation degree. Finally, weak PEs can be chosen as pH can then be used to tune their ionization degree. When the PE is fully ionized, the repulsion between its monomers will stiffen the PE.

The second feature of PEs that has to be considered is the nature of the charged group. At the molecular level, the association between oppositely charged PEs occurs through the cooperative formation of ionic pairs.⁶⁰ The free energy of this association, *i.e.* of complexation, is generally accepted to originate from an entropic contribution due to the loss of counterions and, to a lesser extent, from the enthalpic contribution coming from water perturbation.⁵⁹ Depending on the nature of the charged group and the counter-ion the interaction between the polyanion and the polycation may thus vary. In this way, it has been shown in solution that PE-PE free energy of complexation scales with the Hoffmeister series. A PE side chain with a carboxylic acid function will thus interact stronger than a PE with a sulfonate function with a polycation.⁵⁹

The ability of PEs to form hydrogen bonds with proteins is also critical for LbL formation. Lee *et al.* observed a linear growth for the LbL assembly of κ -casein with PSS. In contrast, when κ -casein is LbL-assembled with PAA, an exponential growth followed by a linear growth is observed.⁶⁹ Much thicker multilayers are thus obtained when PAA is used as shown in Figure I.18. This effect was attributed to the ability of PAA to form hydrogen bonds with κ -casein. The same effect was observed for Lyz LbL-assembled with PAA at various pH values.⁵⁴

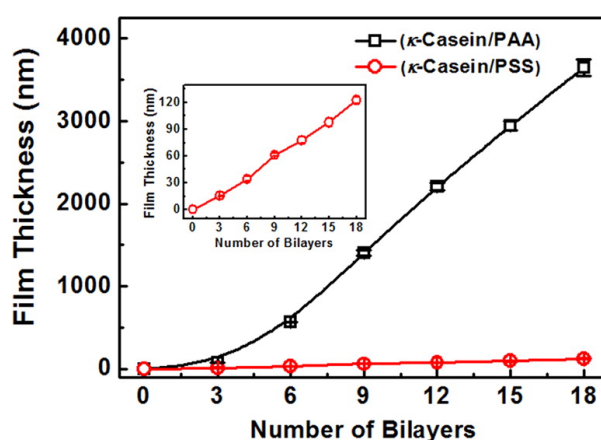


Figure I.18 – Film growth behavior of (κ -casein/PAA) and (κ -casein/PSS) multilayered films assembled at pH 3 on silicon substrates by the dip-based LbL deposition. Inset: focus on (κ -casein/PSS) multilayers, showing the linear growth. Reprinted with permission from Lee *et al.* Copyright 2019 American Chemical Society.⁶⁹

Finally, the hydrophobicity of the PE plays an important role in the LbL construction. For instance, Cytochrome C was successfully LbL assembled with PSS and poly(ester sulfonic acid) at a pH close to its IEP. The authors suggested that the hydrophobic interactions were involved in the build-up process since the protein net charge was close to zero.⁷⁰ Importantly, the authors stressed that the LbL construction is, however, not only based on one type of interaction but rather on an interplay of all of them.⁷⁰ The possible protein-PE interactions arise from van der Waals forces, dipolar or hydrogen bonds, electrostatic forces and hydrophobic effects.⁷¹ It is also worth to stress that protein interactions with hydrophobic PE could denature them. Indeed, proteins are usually made with a hydrophilic shell and a

hydrophobic core. PE interaction with apolar side chains of amino acid residues that are buried in the protein could alter protein structure and therefore its bioactivity.

b) The nature of the protein

The effect of the protein nature on LbL assembly is almost a case-by-case due to the extremely large number of different structures that are obtained. Even though some parameters such as geometrical features, tendency to unfold upon adsorption (soft *versus* hard protein) or charge distribution may help the prediction of their LbL assembly, the absence of a systematic reporting of the failure to grow a LbL makes it hard to find general rules. From the synthesis of all proteins successfully immobilized (see Table I.1, p40), it appears that the vast majority of these are globular. It is not straightforward to conclude whether this observation is due to a difficulty to immobilize or to a lower interest in fibrillar proteins. As long as negative results are not systematically reported, the analysis of a large set of data from literature will be biased by the impossibility to differentiate correlation (a set of protein is more interesting than another) from causality (a set of protein is easier to immobilize than another).

c) The substrate

The substrate chemistry is an important feature that highly dictates the LbL growth. However, this parameter is usually underestimated and the growth obtained for instance on a gold QCM-D sensor is extrapolated to other substrate chemical surface properties without double checking that the same growth is still observed. The issue is that it is sometimes difficult, if not impossible, to obtain the same information with different methods since specific substrates are required. The advantage that QCM-D offers is that the sensor can be coated with different materials. In this way, it was shown that collagen type-I/chondroitin sulfate growth on a QCM-D gold sensor is completely different from its growth on a poly(dimethyl siloxane) (PDMS)-coated sensor.⁶⁷ These results are shown in Figure I.19 and it appears that, on a gold sensor, collagen type-I adsorption is important for the first step and decreases in subsequent layers while, on a PDMS-coated sensor, the collagen type-I -chondroitin sulfate growth follows an exponential path.⁶⁷ A less time-

consuming approach that is used in most cases to circumvent a possible effect of the substrate nature is the standardization of the surface through the construction of at least two PE-PE bilayers on the substrate before further LbL construction.^{49,52,72}

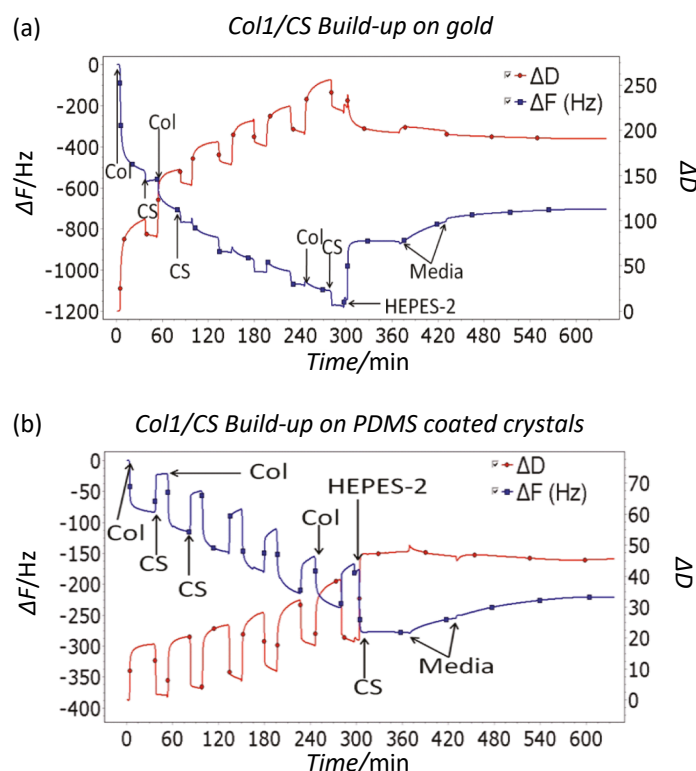


Figure I.19 – collagen type-I (Col1)- chondroitin sulfate (CS) build-up on (a) gold sensor or (b) PDMS coated sensor. Reprinted with permission from Mhanna *et al.* Copyright 2019 American Chemical Society.⁶⁷

3.2. Protein bioactivity in the LbL assembly

For most applications based on proteins, keeping their bioactivity is a key factor. The effect of their immobilization *via* LbL assembly will be reviewed hereafter.

The first proteins that were LbL-assembled with PEs are enzymes. Papers reporting their assembly thus investigated their activity. It was rapidly evidenced that one of the main parameter limiting enzyme activity in LbLs is the substrate diffusion through the film.⁷³ For example, glucose oxidase

(GOx) assembled with a PE on top of glucoamylase (GA), also assembled with a PE, showed different activities as a function of the nature of the last layer and the presence of a spacer between layers. In this case, a higher activity was observed when the enzymes are in the order of the cascade reaction, *i.e.* GA on the top that hydrolyses starch to glucose, which then reacts with GOx further in the multilayer. With respect to the influence of the spacer, it was shown that thicker spacers led to a higher activity, which may sound counterintuitive. It was proposed that, in this particular case, a better enzyme-PE assembly is obtained on the spacer, or that H₂O₂ produced by GOx, which inhibits both GA and GOx, diffuses more easily in a thin spacer.⁷³ It is however important to point out that this paper lacks experimental evidence on the stability of the enzyme in the layer. The measured enzymatic activity can be due to enzymes that are released from the film. The same group later reported the LbL assembly of GOx with PEI and PDADMAC. In this case, the activity was limited by substrate diffusion after the deposition of 2 layers of GOx-PEI. It was also shown that by changing the buffer, 40% of GOx can be released and that the enzyme that is released keeps about 80% of its native activity. Moreover, it was shown that GOx thermostability was improved through immobilization in LbL films.⁷⁴ Another example of how diffusion can influence activity in multilayers was reported with the diffusion of IgG in a LbL film formed with an anti-IgG and PSS.⁶² It was shown that IgG is able to diffuse in the PSS-anti-IgG multilayer but fails to diffuse when each anti-IgG layer is separated by [PSS[PAH-PSS₂]].

Another class of proteins that has been integrated in multilayers are proteins from the extracellular matrix. Most of these studies reported the LbL assembly of collagen with a counter polyanion. It was consistently reported that collagen improves the biocompatibility by promoting cell adhesion and/or differentiation, and is able to limit the cytotoxicity of the substrate. For example, it was reported that collagen type-I/PAA LbL assemblies promote cell attachment and survival on, otherwise, cytotoxic nanoparticles.⁷⁵ The order of the layers is important. Indeed, it was reported for collagen type-I/hyaluronic acid multilayers that chondrosarcoma cells produce their own extracellular matrix on collagen type-I -terminated films whereas such production is not found on hyaluronic acid -terminated ones.

While the vast majority of the obtained multilayers has found applications in biosensing, few studies reported the release of active proteins.^{76–79} Especially, coated polymer microneedles are used for the transdermal delivery of protein, and implantable materials coated with growth factors improve both the osteointegration and the cellular response.^{76,80–86}

3.3. Unconventional LbL-based method for protein surface immobilization

A way that was proposed to improve the LbL assembly of proteins with PEs is rather old. It was found in 2001 by Jin *et al.* that the LbL assembly of catalase with PSS results in poor film growth and poor enzymatic activity (see Figure I.20).⁸⁷ As an alternative, microcrystals of catalase were created and coated with PSS. The resulting PSS-coated catalase crystal was then LbL-assembled with PAH. This methodology resulted in a much better film growth compared to uncoated catalase (soluble, *i.e.* not the crystalline form, see Figure I.20).⁸⁷ It was also demonstrated that uncoated catalase crystals could not be assembled LbL with PAH without the prior formation of a PSS coating. It was proposed that the formation of a PSS coating allowed the LbL construction to rely on the PSS-PAH interaction rather than on the PSS-catalase interaction. This was proposed to be at the origin of the multilayer growth improvement. Especially, Jin *et al.* suggested that the higher negative charge density of PSS deposited on the enzyme crystals compared to the bare catalase molecules enabled a better LbL assembly. In other words, when the LbL assembly relies on PE-PE interaction rather than on protein-PE interaction, the multilayer growth is improved. A severe pitfall of this method is that protein crystals have to be obtained.

Actually, LbL build-up with components mixed with polyelectrolytes prior to assembly has been proposed in the early ages of the LbL method development.^{88–91} This was proven to be effective for polyelectrolyte–polyelectrolyte complexes (PECs).⁹² Polyallylamine–poly(acrylic acid) (PAH–PAA) complexes, assembled in solution at different polymer ratios, were immobilized with poly(styrene sulfonate) (PSS) by the LbL method, giving new film morphologies.

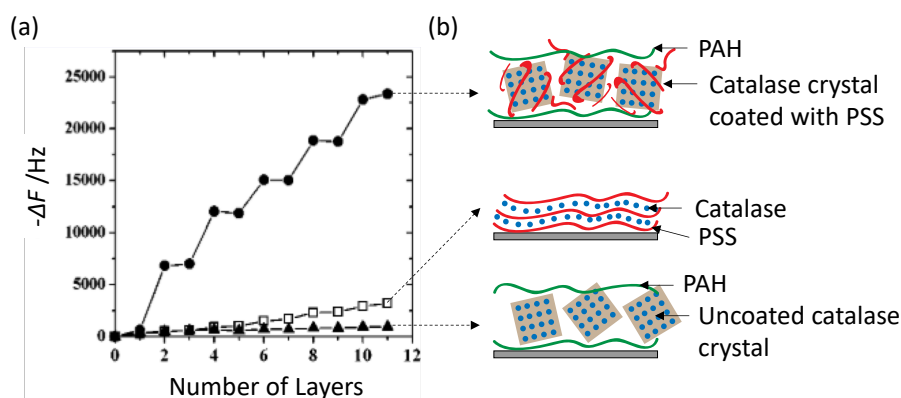


Figure I.20 – (a) The frequency shift measured by QCM-D as a function of the number of layers for PSS-coated catalase crystals (circles), uncoated catalase crystal (square), solubilized catalase (triangles). These are alternately adsorbed with PAH or PSS. Reprinted with permission from Jin *et al.* Copyright 2019 American Chemical Society.⁸⁷ – (b) schematic of each multilayer.

3.4. Literature review of all LbL assembled proteins

Table I.1 – Summary of all proteins successfully assembled using the LbL approach. Systems are reported as **protein/PE** and conditions are reported as *condition for protein assembly/condition for PE assembly* (if these conditions are different from each other). pH and salt used are separated by a coma. Only papers that demonstrate a LbL growth are reported.

	Protein	System and conditions	Ref	Surface	PEM growth
Globular	Acyase (Acy)	Acy/PEI <i>pH 8, tricine buffer</i>	93	Planar	n.d.
	Alcaline phosphatase (ALP)	ALP/tannic acid <i>pH 5, 0.05M acetate</i>	94	Planar	n.d.
	Alpha-amylase	α-A/PEI <i>pH 6.9, 20 mM NaPB buffer with 6.7 mM NaCl</i>	93	Planar	n.d.
	Anti-platelet GP IIb/IIIa	Anti-platelet GP IIb/IIIa/chitosan <i>pH 8 buffer, n.d./ n.d., 0.2 wt % acetic acid</i>	95	Planar	Linear
	Bacteriorhodopsin (BRh)	BRh/poly-L-lysine <i>pH 7.2, DI water</i>	96	Planar	n.d.
	Beta-glucosidase (B-GLS)	B-GLS/PSS <i>pH 4.8, 0.1M Acetate buffer/ pH 4.8, 0.5M NaCl</i>	97	Spherical latex particles	n.d.
	BSA	BSA/PDADMAC <i>pH 7, n.d. PBS/ pH n.d., 0.5M NaCl or pH 5.6, DI water/</i>	98	Planar	n.d.

	Protein	System and conditions	Ref	Surface	PEM growth
Globular		<i>pH n.d., 0.5M NaCl</i>			
	Bone Morphogenetic Protein-2 (BMP-2)	BMP-2/PAA <i>pH5, 100 mM sodium acetate</i>	99	Scaffold	Linear
	Catalase (CAT)	CAT/PSS <i>pH 5, 1M Potassium acetate</i>	100	Planar	Linear
		CAT/star-PDMAEMA <i>pH 7, n.d./ pH 6, 0.1 M NaCl</i>	101,102	Planar	Linear, hybrid with GOx
	Cholesterol oxidase (COX)	COX/PEI <i>pH 6.3, 0.1M phosphate buffer/ pH 6.3, 2M KCl</i>	103	Planar	Sigmoidal, saturation after 10 bilayers
		COX/PDADMAC <i>pH 6.3, 0.1M phosphate buffer/ n.d.</i>	104	Planar	n.d.
		COX/PAH <i>pH 7.5, 0.01M Tris HCl buffer</i>	105	Planar	Linear
	Choline oxidase (ChO)	Cho/ PDADMAC <i>pH 8, 0.1M PBS/ n.d.</i>	106	On carbon nanotubes	n.d.
		Cho/PDADMAC Cho/PEI <i>pH 8, 0.1M phosphate buffer/ pH 7.4, Dulbecco's buffer</i>	107	On electrode	Indirect evidence
	Cytochrome C (Cyt C)	Cyt C/PSS <i>pH 4.5, 4.8, pH adjusted with HCl</i>	47	Planar	n.d.
	Cytochrome P450 (Cyt P450)	Cyt P450/PEI <i>n.d., 0.1M PBS (4°C)/n.d.</i>	108	Planar	Linear
	κ- casein (Ca)	κCa/PAA <i>pH 3, n.d.</i> κCa/PSS <i>pH 3, n.d.</i>	69	Planar Planar	Exponential then linear Linear
	Basic Fibroblast growth factor (bFGF)	bFGF/alginate <i>pH 5, sodium acetate buffer</i>	109	Planar	Exponential
		bFGF/chondroitin sulfate <i>pH 7.4, PBS/ pH 5.6, 0.1M NaCl</i>	110	Planar	Linear
	Glucoamylase (GA)	GA/PEI <i>pH 5, DI water</i>	111	Planar	n.d.
		GA/PEI <i>pH 6.5, n.d.</i> GA/PDADMAC <i>pH 6.8, n.d.</i>	47	Planar Planar	n.d.
	Glucose dehydrogenase (GDH)	GDH/PSS GDH/PASA <i>n.d.</i>	112	Planar	Linear
	Glucose oxidase (GOX)	GOX/PAH <i>pH 6.8, 0.05M HEPES buffer/ pH 4.8, 0.5M NaCl</i>	113	Spherical particles	n.d.
		GOX/Star PDMAEMA <i>pH 6, n.d./ pH 6, 0.1M NaCl</i>	114	Planar	n.d.

	Protein	System and conditions	Ref	Surface	PEM growth
Globular		GOX/PEI <i>pH 5, DI water</i>	111	Planar	n.d.
		GOX/PEI <i>pH 6.5, n.d.</i>	47,74	Planar	Linear
		GOX/PDADMAC <i>pH 6.8, n.d.</i>		Planar	n.d.
		GOX/star-PDMAEMA <i>pH 6, n.d./ pH 6, 0.1 M NaCl</i>	101,102	Planar	Linear, hybrid with CAT
	Glucose 6-phosphate dehydrogenase (G6PDH2)	G6PDH2/PEI-Fc <i>pH 7.5, 0.1M Tris-HCl buffer containing 3.3mM MgCl2/DI water</i>	115	Planar	Indirect evidence
	glutathione reductase (GR)	GR/PEI-Fc <i>pH 7.5, 0.1M Tris-HCl buffer containing 3.3mM MgCl2/DI water</i>	115	Planar	Indirect evidence
	Hemoglobin (Hb)	Hb/PSS <i>pH 4.5, pH adjusted with HCl</i>	47	Planar	Linear
		Hb/PEI <i>pH 9.2, n.d.</i>		Planar	Linear
		Hb/ dextran sulfate <i>DI water</i>	49	Planar	Slightly exponential - drying between steps with N2
	Histone f3 (Hf3)	Hf3/PSS <i>pH 7, n.d.</i>	47	Planar	n.d.
	Horseradish peroxidase (HRP)	HRP/PSS <i>pH 6.8, 0.05M HEPES buffer/ pH 4.8, 0.5M NaCl</i>	116	Spherical particles	n.d.
		HRP/QPVP <i>pH 7.5,0.1M PBS/ n.d.</i>	117	Planar	n.d.
		HRP/PSS <i>pH 5.5, acetate buffer/ n.d., 0.5 M NaCl</i>	118	Planar	Linear QCM-D
		HRP/ redox active PE <i>n.d.</i>	119	On electrode	Indirect evidence
	IgG	IgG/PSS <i>pH 7.5, I=0.1M (0.05M HEPES + NaCl) or pH 6, I=0.1M (0.05M MES + NaCl)/ pH 6, 0.01 M MnCl2</i>	62	Planar	Linear
		IgG/dextran sulfate <i>pH 4, 0.05 M citrate</i>	57	Planar	n.d.
		IgG/PSS <i>pH 6, 0.05M MES/ pH n.d., 0.5M NaCl</i>	98	Planar	n.d.
IgG/PMAA <i>pH 4.5, pH 5, pH5.5 pH 6,0.01 M phosphate buffer</i>		54	Planar	n.d.	

	Protein	System and conditions	Ref	Surface	PEM growth
Globular		IgG/PAA <i>pH 4, pH 4.5,</i> <i>0.01 M phosphate buffer</i>		Planar	n.d.
	IgG n.d.	Anti-HRP/dextran sulfate <i>pH 4, 0.03M citrate</i>	120	Planar	Linear
	Keratin (K)	K/PDADMAC K/PAA <i>DI</i>	63,121	Planar	n.d.
	Lactate oxidase (LAX)	LAX/PVP-Os-AA <i>PBS/ n.d.</i>	122	Planar	Linear
	Laccase (Lac)	Lac/PAH-Os <i>pH 4.7, 0.1M acetate buffer/ pH 8, n.d.</i>	123	Planar	Only two layers
		Lac/PDADMAC <i>pH 4.5, 0.05 M sodium acetate</i>	124	Planar	Linear
	Lignin peroxidase (LiP)	LiP/PDADMAC LiP/PEI LiP/PAH <i>pH 6, 24 or 40 mM acetate buffer</i>	125	Planar	Slightly linear, huge desorption observed
	Lysozyme (Lyz)	Lyz/PSS <i>pH 3 (citrate buffer), pH 5 (acetate buffer), pH 7.5 (HEPES buffer), pH 9.6 (carbonate buffer), I=0 mM, I=10 mM, I=200 mM, I=800 mM</i>	This thesis	Planar	Linear
		Lyz/PSS <i>pH 4, pH adjusted with HCl</i>	47	Planar	Linear
		Lyz/PSS <i>pH 6, n.d.</i>	126	Planar	Linear
		Lyz/PMAA <i>pH 5, pH 5.5, pH 6, pH 6.5, from 0 to 4 M NaCl</i>	54,127	Planar	Linear up to 10 layers
		Lyz/PAA <i>pH 4, pH 4.5, pH 5, pH 5.5 pH 6, from 0 to 2.4 M NaCl</i>			
	Manganese peroxidase (MnP)	MnP/PDADMAC MnP/PEI MnP/PAH <i>pH 6, 24 or 40 mM acetate buffer</i>	125	Planar	Slightly linear, huge desorption observed
	Myoglobin (Mb)	Mb/PSS <i>pH 4, 0.001 M NaCl</i>	127	Planar	Linear growth
		Mb/ dextran sulfate <i>DI water</i>	49	Planar	Slightly exponential - drying between steps with N2
	Organophosphorous hydrolase (OPH)	OPH/PTAA	128,129	Planar	n.d.

Part I

	Protein	System and conditions	Ref	Surface	PEM growth
Globular		<i>pH 7.3 (0.1 M KH₂PO₄ and 0.1 M NaOH) /pH 8.8 (0.1 M C₄H₁₁NO₃ and 0.1 M HCl)</i>			
	Polyphenol oxidase (PPO)	PPO/PAH <i>pH 6.6, 0.01M Tris buffer</i>	130	Planar	Linear
		PPO/Chitosan <i>pH n.d., PBS</i>	131	Planar	Exponential
		PPO/PDADMAC <i>pH 7, PBS buffer</i>	132	n.d.	n.d.
	Ribonuclease A (bovine pancreas) (RNase)	RNase/PMAA <i>pH 4.5, pH 5, pH5.5 pH 6, 0.01 M phosphate buffer</i>	54	Planar	n.d.
		RNase/PAA <i>pH 4, pH 4.5, pH 5, 0.01 M phosphate buffer</i>		Planar	n.d.
	Trypsin (Trp)	Trp/PSS <i>pH 7.4, 0.05M Tris or 0.02M CaCl₂</i>	133	Planar	n.d.
		Trp/alginate <i>pH 7.4, 0.05M Tris or 0.02M CaCl₂</i>		Planar	n.d.
	Uricase (UOx)	UOx/PAH <i>n.d., 100 mM borate buffer/ n.d., Dulbecco's phosphate-buffered</i>	134	Planar	Linear
	Urease (U)	U/PDADMAC <i>pH 7.2, 0.02 M Tris-HCl buffer</i>	124	Planar	Linear
Peptide	Insulin	VEGF/PAA <i>pH5, 100 mM sodium</i>	99	Scaffold	Linear
		VEGF/heparin <i>pH 7.4, PBS /pH 4.2, DI</i>	135	Planar	Indirect evidence
Fibrous	Insulin	Insulin/Star PDMAEMA <i>pH 6, n.d./ pH 6, 0.1M NaCl</i>	114	n.d.	n.d.
		Insulin/Star PDMAEMA <i>pH 7, n.d./ pH 6, 0.1M NaCl</i>	101,102	Planar	Linear, hybrid with GOX/star-PDMAEMA, Cat/star-PDMAEMA
	Collagen Type-1 (Col1)	Col1/PSS <i>pH 4.2, pH adjusted with HCl/ pH 4, pH adjusted with HCl</i>	136	Planar	Sigmoidal, saturation at 10 bilayers
		Col1/HA <i>pH 4, acetic acid/ pH 4, DI adjusted with HCl</i>	56	Planar	Linear up to 5 bilayers
		Col1/HA <i>pH 4, 0.012M HCl + 0.1M acetate buffer/ pH 4, 0.082M acetic acid</i>	137	Planar	Linear up to 9 bilayers
		Col1/HA <i>pH 4.7, n.d. acetate buffer</i>	138	Planar	Exponential

Protein	System and conditions	Ref	Surface	PEM growth	
Fibrous	Col1/He (heparin) <i>pH 4, 0.2M acetic acid buffer/ pH 7, DI</i>	139	Planar	n.d.	
	Col1/chondroitin sulfate <i>pH 3, 0.2M NaCl/n.d., 0.5M NaCl</i>	140	n.d.	n.d.	
	Col1/chondroitin sulfate <i>pH 3, 150 mM NaCl, 1 mM HCl/ pH 6, 150 mM NaCl</i>	67	Planar	Saturate after 6 bilayers	
	Col1/heparin <i>pH 3, 150 mM NaCl, 1 mM HCl/ pH 6, 150 mM NaCl</i>		Planar	Linear	
	Col1/heparin <i>pH 4., acetic buffer solution, 0.14 M NaCl</i>	141	Planar	Exponential - drying between steps with N2	
	Col1/heparin <i>n.d. 0.2 M acetic acid/ pH 7, DI</i>	142	Planar	Linear	
	Col1/PDADMAC <i>n.d., 0.1 M acetic acid/ pH10, DI</i>	143	Planar	Linear	
	Collagen Type-4 (Col4)	Col4/PAA <i>pH 4, adjusted with HCl</i>	75	Nano-particles	Linear up to 10 bilayers
	Fibronectin (Fn)	Fn/Poly-D-lysine <i>pH 7.4, 0.1M PBS</i>	72	Planar	Linear up to 8 bilayers
	Gelatin type-A (Ge)	Ge/PEI <i>n.d./pH 7.4, n.d.</i>	144	Planar	Linear up to 8 bilayers
Ge/chitosan <i>n.d./pH 4, 0.1 M acetic acid and 0.14M NaCl</i>			Planar	Linear up to 8 bilayers	
Laminin-1 (Lm1)	Lm1/chitosan <i>pH 7.4, PBS/ pH 4, 0.1 M acetic acid and 0.14M NaCl</i>	144	Planar	Linear up to 8 bilayers	
	Lm1(?) /Poly-D-lysine <i>pH 7.4, 0.1M PBS</i>	72	Planar	Linear up to 8 bilayers	

List of abbreviations used in the table: PAA: Poly(acrylic acid). PAH: Poly(allylamine hydrochloride). PASA: Poly(anilinesulfonic acid). PDADMAC: Poly(diallyldimethylammonium chloride). PDMAEMA: Poly(dimethylaminoethyl methacrylate). PEI: Poly(ethylenimine). PEI-Fc: PEI-ferrocene carboxylic acid. PMAA: Poly(methacrylic acid). PMAA: Poly(methacrylic acid). PSS: Poly(styrene sulfonate); PTAA: Poly(thiophene-3-acetic acid). PVP: Polyvinylpyrrolidone. PVP-Os-AA: Polyvinylpyrrolidone-Os-acrylic acid. QPVP: Quaternized poly(4-vinylpyridine).

4. Protein-polyelectrolyte complexes

When oppositely charged species are mixed together, they spontaneously self-assemble. A well-known example is the complexation of two oppositely charged PE, which forms a PEC (as schematized in Figure I.21). Since proteins are charged, they can also form a complex upon mixing with an oppositely charged PE, they form a so-called protein-polyelectrolyte complex (PPC – schematized in Figure I.21). PPCs are very interesting for two reasons. First, protein complexation with a PE might represent a valuable way to immobilize proteins in particles. Secondly, the driving forces for PPCs formation are akin to the ones driving LbL formation. The study of PPCs formation is thus highly valuable as it can shed light onto the LbL assembly of proteins with PEs.¹⁴⁵

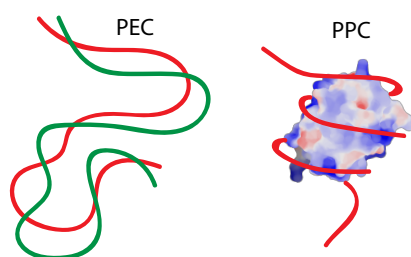


Figure I.21 – Schematic of a PEC and a PPC. The red and green lines represent oppositely charged PE. A globular protein complexed with a PE is schematized on the right. The different colors on the protein suggest the heterogeneous charge distribution.

The first papers published on PPCs can be found back from 1952 to 1955. They reported that BSA complexation with PE depends on PE nature, pH, I, polymer-to-protein ratio and polymer molar mass, and that trypsin complexation has a strong effect on its bioactivity.^{146,147} It is impressive to realize that, at the time, the most important parameters governing PPCs formation were already identified. From 1955 to 1963, these papers were frequently cited and inspired other similar studies.¹⁴⁸ Surprisingly, afterwards, the subject went dormant for more than two decades until a few new papers emerged in the 1990's.¹⁴⁹ Then the subject boomed, especially with the two notable reviews from Dubin *et al.* in 2005 and 2013.^{39,150} The reasons for this resurgence of interest are multiple: the first is that PPCs are of interest for various applications such as separation and purification, biocatalysis, biosensing, drug delivery or wound healing.³⁹ Especially, the

increased protein commercial availability has made these common materials. The second reason for the regain of interest is that computer simulation has become steadily more powerful and accessible. Papers that enlighten PPCs formation using the theory of interaction have been flourishing.³⁹ Together with the new broad range of experimental techniques available, the understanding of PPCs formation has thus evolved rapidly.³⁹

The forces that drive the complexation of two oppositely charged macromolecules in solution are similar to the ones driving LbL assembly.¹⁴⁵ In that respect, it was shown that the salt dependence of PEC formation can be used to predict PEMs growth kinetic.¹⁴⁵ Yet, three major differences result from the fact that PEMs are considered far from equilibrium assemblies while PECs and PPCs are in, or close, to an equilibrium state (if PPC precipitates, it is identified as an arrested “glass”, if it forms a coacervate, two liquid phases coexist).^{145,151} The first is that the association of oppositely charged species in solution has a small entropic penalty to the decrease of the Gibbs free energy comparatively to the one related to PEMs formation. Indeed, when PEs are adsorbed on surfaces, an important entropic energy cost is associated with the decrease of the PE freedom degrees. Secondly, the LbL assembly requires charge inversion at each step of the PEM growth while PEC formation is only based on the favorable interaction between two charged species. Finally, the concentration of each charged species highly dictates the resulting PEC charge and morphology. In contrast, the influence of concentration on PEMs growth is mainly kinetic. The consequence of these differences is that, when oppositely charged species are mixed in solution, they almost systematically form a complex.³⁹ The association is so favorable that binding of proteins to PE of the same net charge has been reported numerous times.³⁹ This was attributed to the presence of charged patches on the protein that are oppositely charged to PE, even if the protein net charge is of the same sign as the one of the PE.³⁹

PPCs formation was proven to originate from electrostatic interactions, even if hydrophobic effects sometimes contribute.³⁹ According to Dubin *et al.* “there is a surprising range of interpretations of electrostatically driven complexation”.¹⁵⁰ If these forces are loosely defined as the effects involving

oppositely charged groups, then salt bridges and ion pairs should be included. These are directionally specific short-range forces and, according to Dubin *et al.*, the existence of such atomically defined complexes is questionable because (1) usually, PPCs are not biological cognate systems (it means that protein-PE interaction cannot be compared to a ligand-receptor interaction); (2) the PE precise fitting to the protein charge distribution would require too much energy; (3) the exact local arrangement of PE would cause a too drastic entropy decrease.¹⁵⁰ They suggest that a large array of local chain configurations with a similar energy, which is the sum of Coulombic interactions (both repulsive and attractive), defines the PPCs. The result is that the driving force of PPCs formation has been labeled electrostatic by almost all researchers of the field and protein interaction with PE is usually discussed in the frame of continuum electrostatic theory.⁵⁹ The weakening of protein-PE interaction as salt concentration rises is thus usually discussed using the Debye-Huckel screening theory.¹⁵⁰

The driving force of the complexation between oppositely charged polyions is however still under debate. Indeed, assuming an electrostatic-based interaction (in the sense of the Debye-Huckel theory), heat should be released upon complexation. For PECs formation, this is observed in a very limited fashion (a few kJ mol^{-1}), which is attributed to water perturbation, *i.e.* change in the H-bonding network upon PEC formation, rather than electrostatic interactions.^{59,152} For example, PSS complexation with PDADMAC releases only 1.7 kJ mol^{-1} . Moreover, continuum electrostatic theory fails to explain differences when complexing a PE with different counter-ions.¹⁵³ In this way, it has been recently demonstrated that PECs formation is mainly entropic, with a small enthalpic contribution originating from water perturbation.⁵⁹

While it is steadily approved that coulombic interactions are a minor driving force of PECs formation and that PE association is more likely due to ion-pairing, things are not as clear for protein complexation with PEs. Indeed, as already mentioned, short-range ion-pairing would require severe PE contortion to adapt to the rather rigid and heterogeneous protein surface. Moreover, the exothermicity of PPCs formation was reported to be much

higher than the one of PECs formation, which was attributed to both electrostatic and hydrophobic interactions.^{154,155} For example, Lyz binding to either PVS or PAA releases respectively 63.6 and 41.8 kJ mol⁻¹.¹⁵⁵ The literature available on the subject suggests that PPC formation is driven by many different factors. It appears that PPC formation can be discussed in the frame of the Debye-Huckel theory, but if not incorrect, this theory may reveal incomplete depending on the protein and PE that are used. Recently, an extensive molecular dynamic simulation of the complexation between poly(ethylene glycol) methyl ether acrylate-*b*-poly(carboxyethyl acrylate) and Lyz was reported. They found that at physiological pH, binding is mediated by coulombic interactions between oppositely charged groups of the PE and Lyz. Importantly, they showed that when the pH is lowered, the coulombic interactions alone fails to explain the experimental data.¹⁵⁶ They added the contribution of hydrogen bonds, van der Waals interactions and water-water interactions to the simulation to explain PPCs stability at low pH. This clearly suggests that coulombic interactions need to be complemented with more entropic-based forces to explain PPCs formation.¹⁵⁶ This is nicely demonstrated in a recent paper from Xu *et al.* that shows that release of counter-ions upon Lyz complexation with dendritic polyglycerol sulfate is the main driving force, excepted below a salt concentration of 25 mM where long range electrostatic interactions are at play.¹⁵⁷

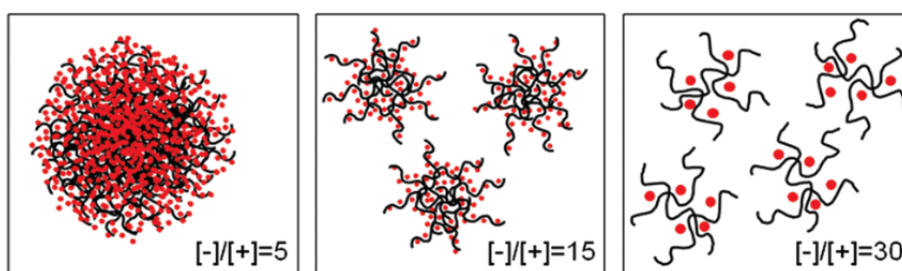


Figure I.22 – Schematic representation of the structure of PPCs formed by addition of SCPI-54k (black lines) to Lyz (red circles). SCPI-54k addition is expressed as a charge ratio. Reprinted with permission from Karayianni *et al.* Copyright 2019 American Chemical Society.¹⁵⁸

Now considering the effect of the protein and PE concentrations, it appears that the main difference with protein-based LbL assemblies is that protein-

PE stoichiometry can be tuned in the case of PPCs. Depending on the protein-to-PE ratio (or the opposite), which is usually expressed as a charge ratio, different PPCs architectures can be obtained.¹⁵⁴ For example, Karayianni *et al.* found that depending on the amount of SCPI-54K added to Lyz (expressed as the ratio of charge coming from SCPI-54K to the net charge of Lyz), different PPCs could be obtained. As shown in Figure I.22, solid-like (precipitated) PPCs are obtained at $[-]/[+]=5$.¹⁵⁸ When $[-]/[+]$ increases to 30, *i.e.* more SCPI-54K is added to Lyz, soluble PPCs are obtained.

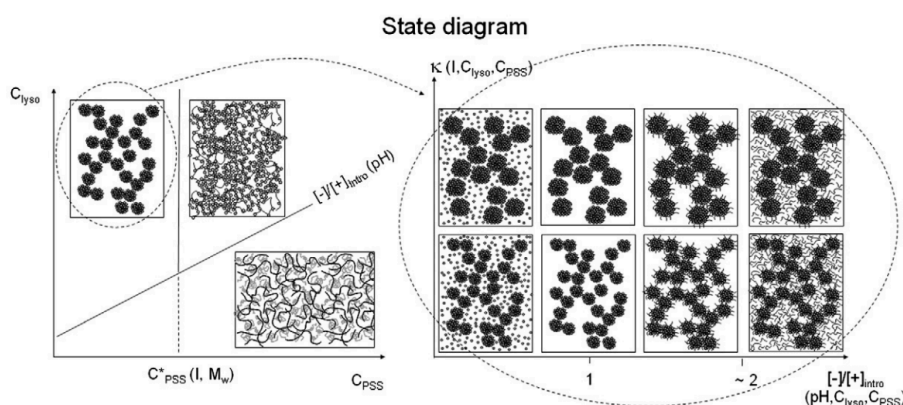


Figure I.23 – Structures of the PPCs obtained by mixing Lyz with PSS as a function of concentration and parameters that can be used to tune the complexation. Reprint from Cousin *et al.* with permission from Elsevier.¹⁵⁹

PPCs formation thus depends on many different parameters. These were extensively reviewed by Cousin *et al.* for Lyz complexation with PSS, *i.e.* the most studied system for PPCs formation.¹⁵⁹ Figure I.23 summarizes qualitatively how different PPCs architectures can be obtained depending on the amount of PSS added to Lyz. This is expressed as $[-]/[+]$, where $[-]$ are charges coming from PSS, while $[+]$ are charges coming from Lyz.

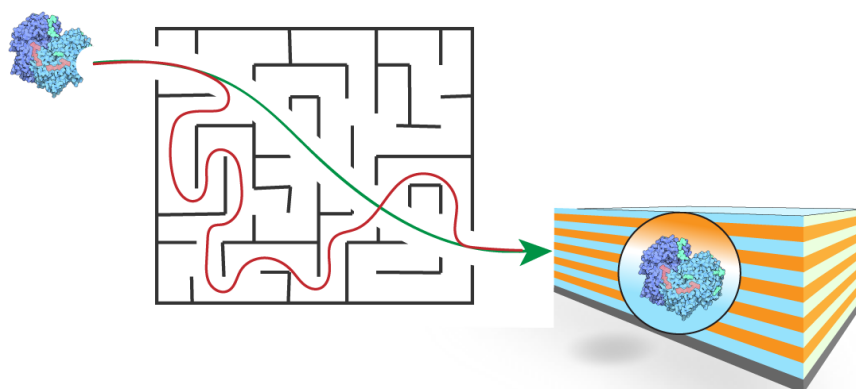
The top left graphic in Figure I.23 shows that depending on the amount of Lyz and PSS that is added, *i.e.* the $[-]/[+]$ ratio, PPCs that are obtained can be either particles/precipitate or a mixture without any structure. The most interesting object for drug delivery and purification technologies are particles, and the top right panel of Figure I.23 shows the conditions that lead to their formation. To create particles, one must add a similar amount of

positive and negative charges, *i.e.* $[-]/[+]$ is around 1. When $[-]/[+] < 1$, the PSS charges compensate those of Lyz and PPCs are neutral. When PSS is added and $[-]/[+] > 1$, the PPCs take a core-corona structure with the PSS chains dangling outside the complex particule.^{159,160} The latter is thus negatively charged, with an internal structure that is neutral. This graph also shows that when Lyz and PSS concentration increases at a given $[-]/[+]$, bigger PPCs are obtained. In general, precipitates are obtained at $[-]/[+]=1$ in low I solution. Of course, polymer molar weight, protein charge, polymer charge density and I also influence the architecture. For example, when the salt concentration rises, more PE has to be added to precipitate the protein.¹⁵⁴

Part II: Objective and strategy of the thesis

1. Objective of the thesis

After three decades of innovation, LbL assembly is now amongst the most used method to create nanostructured coatings and materials. As shown in the previous section, there is a clear gap between the potential and the current state of the art regarding the incorporation of proteins in LbL assemblies. We truly believe that this underutilization of the LbL approach for surface biofunctionalization originates from technological issues rather than from an underestimation of its potential by the scientific community. **This thesis aims at providing a way to facilitate the use of the LbL method for protein surface immobilization.** The developed method should allow designing self-assembled films with a high bioactivity and with a controlled architecture. This would open perspectives for applications in a variety of fields, including biocatalysis and biomedicine.



2. Strategy of the thesis

The LbL assembly of a protein with a PE, if possible, highly depends on both the pH, I and PE nature, and only a few assembly conditions allow to obtain a sustained LbL growth. This issue is attributed to the large variety of existing proteins and their heterogenous distribution of chemical functions as they are made out of different sequences of a.a. Finding a way to standardize protein surface properties could thus provide a route to assemble any protein with a PE, based on the LbL approach.

This protein surface standardization should be simple to implement and be applicable to any protein. Moreover, the bioactivity of the protein should be maintained. In this view, protein-polyelectrolyte complexes (PPCs) are promising candidates. Indeed, in theory, any PE can form a complex with a protein, provided that they are oppositely charged. The resulting PPCs has a surface charge that depends on the protein-to-polymer ratio. By creating PPCs in solution and controlling their charge, we anticipate that it is possible to assemble them, using the LbL method, with a second PE that has a charge opposite to the one of the PPCs. Importantly, the reported neutral charge of the PPCs core suggests that the resulting LbL assembly would be independent of the protein charge. This could have tremendous implications as it would considerably simplify the identification of conditions for the LbL assembly of proteins.

2.1. PPCs formation

The first step of the thesis consists in determining the nature of the protein and the PE that will be used to demonstrate the feasibility of the approach. Since the aim is to develop a new method, the system should use components that are well characterized. With respect to the choice of the protein, it appears that Lyz has been widely studied both in PPCs and in LbL coatings. This small globular protein (around 3 nm in diameter) is positively charged on a wide range of pH since its IEP is at pH 11.35.¹⁶¹ According to the soft-hard classification of proteins, Lyz unambiguously falls into the hard protein category.¹⁷ Lyz thus hardly changes its conformation upon adsorption on a surface. The coulombic surface potential of Lyz was computed by Seyrek

et al. and their results are reproduced in Figure II.1.¹⁶² The surface potential resulting from a positive and a negative charge is respectively colored in blue or red. It appears that Lyz is globally positively charged. Indeed, Lyz possesses almost only positively charged a.a. (in blue in Figure II.1), excepted for few a.a. residues (in red in Figure II.1). Lyz binds to the gram-positive bacteria cell walls and hydrolyses 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan.¹⁶³ Different methods exist to test Lyz hydrolytic activity.

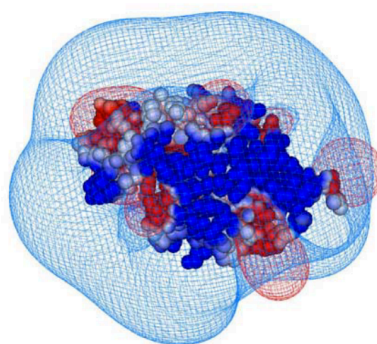


Figure II.1 – The surface potential resulting from a positive and a negative charge is respectively colored in blue or red for Lyz at pH 7 and $I = 0.15$ M.¹⁶²

Lyz complexation with PEs has been widely studied by the group of Boué *et al.* Especially, the complexation of Lyz with PSS has been extensively reviewed in their paper: *The model Lysozyme-PSSNa system for electrostatic complexation: Similarities and differences with complex coacervation*.¹⁶⁴ In this paper, which was presented in the section on *protein-polyelectrolyte complexes*, the macroscopic observations were related to structures at local scales (from 5 Å to 100 nm). When a complex is formed, it takes a structure in which the surface is charged while the core is neutral. Depending on the pH, I , concentration and surface charge, the PPCs either form a new liquid phase, *i.e.* coacervate, or precipitate. In the former case, the neutral cores of the PPCs merge together and form a second fluid phase. In the latter case, the long-range electrostatic repulsion prevents the liquid-liquid phase transition to occur. This leads to complexes that precipitate, *i.e.* particle-like, in solution. In some cases, the particle-like complexes are very small (only

few protein and PE per PPCs) and considered soluble PPCs.¹⁶⁴ In a view to alternate the adsorption of PPCs with an oppositely charged PE, charged objects/particles is needed and coacervation should be avoided. When Lyz is complexed with PSS, coacervation is hardly observed and a stable turbid suspension is always obtained.¹⁶⁴ These results suggest that these PPCs could be assembled using the LbL approach with a counter-PE over a wide range of conditions. PSS will thus be used to complex Lyz.

The first step was to create PPCs by mixing Lyz with enough PSS in such a way that the resulting complexes are negatively charged (see Figure II.2). Since turbid solutions are obtained, turbidimetry was used to monitor PPCs formation. Using this simple method, PPCs made of PSS with different molar mass were tested (see Appendix 2, p225). As a matter of comparison, the Lyz-PAA complex formation was also investigated (see Appendix 2, p225). Based on these preliminary results, it was decided that PSS with a low molar mass should be used (PSS_l). Another important parameter is their size, which was measured using dynamic light scattering (DLS). In order to assemble Lyz-PSS PPCs with a positively charged PE using the LbL approach, it should be verified that these PPCs are negatively charged. Therefore, their ζ -potential was measured using streaming potential measurements. The working principle of DLS and streaming potential measurements are described in the next section (materials and methods, p61). The results related to PPCs size and surface electrical properties are presented in Chapter 1 and deepened in Chapter 2.

2.2. PPCs integration in multilayers

The choice of the counter-PE that is alternately adsorbed with PPCs was motivated by the literature existing on the construction of simple PEMs. Since PSS will in principle be found in the corona of the Lyz-PSS complexes, PAH was chosen. PAH-PSS multilayers are indeed one of the most documented LbL assemblies.⁴² PAH is a weak polybase and it has a pKa that ranges from pH 8 to 9 (The pKa shifts to higher values compared to isolated amine functions because of charge proximity).¹⁶⁵ The LbL assembly of PPCs with PAH is schematized in Figure II.2. The methods for the measurement of the total hydrated mass and of the amount of protein in the assemblies are

reported in the next section (Materials and methods, p61). The potential of PPCs as a building block for LbL assembly is presented in Chapter 1, while the mechanism of their LbL assembly with an oppositely charged PE is revealed in Chapter 2.

2.3. Properties of the multilayer

Since three different species are used here to construct the multilayer (Lyz-PSS complexes and PAH, or more generally PPCs and a counter PE) instead of two in the more classical LbL approach (protein and PE), completely different architectures are expected to be observed. This may profoundly affect the water content of the layers, which is a key factor for layer functionality. This was investigated by measuring the swelling/deswelling behavior of the multilayers when the relative humidity of the surrounding air changes. These results are presented in Chapter 3 (see Figure II.2 of general strategy of the thesis).

2.4. Bioactivity of the protein

The ability to maintain the bioactivity of the protein is critical for all applications requiring proteins at interfaces. Lyz possesses a strong hydrolytic activity against 1,4-beta-linkages in peptidoglycans. These are found in bacteria walls. The hydrolytic activity of Lyz can be easily measured via the decrease of the turbidity of a lyophilized bacteria suspension. These tests are presented in Chapter 1 to demonstrate that Lyz maintains its enzymatic activity after immobilization. An alternative that allows a better sensitivity to be obtained is the use of a small synthetic chitotriose, which releases a fluorescent molecule upon its cleavage by Lyz. Lyz hydrolytic activity against this synthetic molecule was also tested and results are shown in Chapter 1.

For biosensing and heterogenous biocatalysis, the multilayer should be stable at the interface while maintaining a high bioactivity. For drug delivery systems and, to a lesser extent, for biomaterials, the protein should be released in a controlled manner while keeping its bioactivity. LbL films are known to be stable or unstable depending on the conditions of construction or on the medium to which they are exposed. PPCs-based multilayers were

constructed in different conditions and exposed to different media. The conditions that led to stable or unstable multilayers were evidenced, and the activity of the stable or released Lyz was investigated using the small synthetic chitotriose as the enzyme substrate. These results are presented in Chapter 4 (Figure II.2).

2.5. Application to the design of a drug delivery system

The last two decades have seen a strong increase in the number of bacteria that are resistant to common antibiotics.¹⁶⁶ In a view to create antimicrobial surfaces, the once-successful surface immobilization of antibiotics is being abandoned.¹⁶⁷ In the meantime, it has been found that ancient components of the innate system, *i.e.* antimicrobial peptides (AMPs), can be used efficiently against bacterial and fungal pathogens.^{168–170} Besides their antibacterial activity, AMPs are able to modulate the immune response, making them good candidates as therapeutic agents.^{171,172} Many groups of AMPs are known, of which defensins and cathelicidins have been the most studied.¹⁷³ LL37 is a small AMP produced by the human immune system.¹⁷⁴ Interestingly, LL37 also exerts different immunomodulatory functions such as modulation of pro-inflammatory response, chemotaxis, influence on cell proliferation and differentiation, promotion of wound healing and angiogenesis, etc.¹⁷⁵ LL37 thus has much potential for improved wound healing. In this way, the last Chapter investigates the use of the PPCs-based LbL assembly developed in the thesis to immobilize active LL37 on dressings designed for wound healing (see Figure II.2). Two naturally-occurring PEs were used. The positively charged LL37 was complexed with the negatively charged alginate, and the charge of the resulting PPCs was adjusted so that it can be assembled with positively-charged chitosan using the LbL method. The amount of immobilized LL37 and the release profile from the dressing were investigated, as reported in Chapter 5.

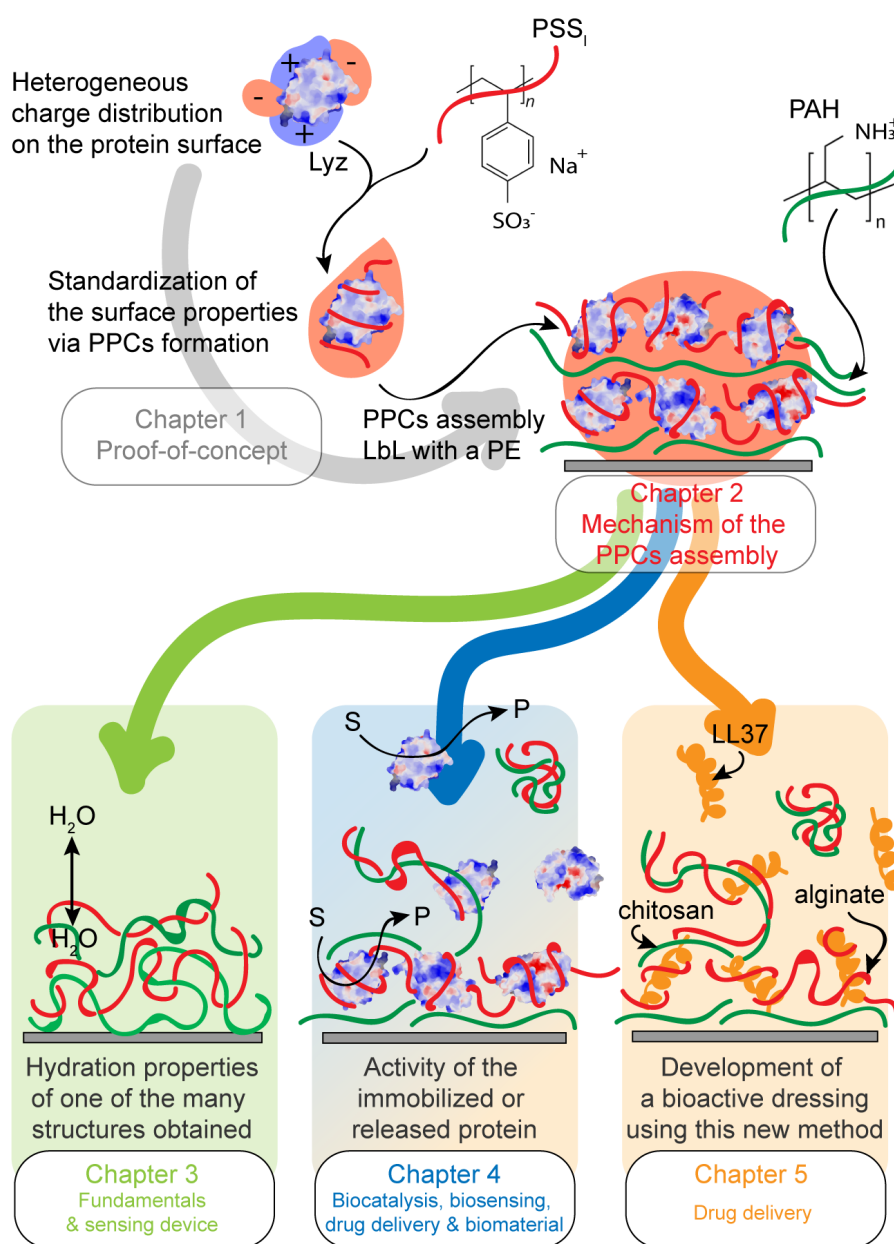


Figure II.2 – Strategy of the thesis. Lyz is first complexed with PSS. The polymer-to-protein ratio is adjusted in such a way that the resulting PPCs are negatively charged. Then, PPCs are alternately adsorbed with PAH, a positively charged PE. Then, properties of the obtained multilayers are investigated, especially, their swelling/deswelling behavior. The bioactivity of immobilized Lyz is explored as well as its bioactivity after its release. Finally, the PPCs-based method is used to immobilize LL37, a bioactive peptide, on dressings designed for wound healing.

Part III: General methods

In this part, the main methods that were used throughout this thesis are presented. Emphasis is put on the working principle and the information it provides. The operating details are presented in the materials and methods section of each Chapter.

1. PPCs formation

The assessment of PPC formation was monitored using turbidimetry, UV-dosage, DLS and streaming potential (zeta-potential) measurements. In the following, DLS and electrophoretic mobility will be briefly presented.

1.1. Dynamic light scattering

DLS (also known as Quasi Elastic Light Scattering or Photon Correlation Spectroscopy) is particularly utilized to determine the hydrodynamic radius from diffusivities. A suspension of particle is illuminated with a laser and the scattered light intensity as a function of time (scattered light density) is measured (at 90° in our case – see Figure III.1). Then, the correlation function is computed. It evaluates how the different positions of the scattered light density plot are related. Small particles move rapidly while big particles move slowly. The consequence is that the correlation is rapidly lost for small particles and slowly lost for big particles. This feature is shown in Figure III.1. Once the correlation function has been found, different mathematical analyses allow to obtain the diffusion coefficient, which is then derived into a size using the Stokes-Einstein equation. The choice between the mathematical methods is motivated by the polydispersity of the suspension (monodisperse v.s. polydisperse). In this thesis, polydisperse solution were always assumed and a CONTIN algorithm was used (uses an inverse Laplace transform).

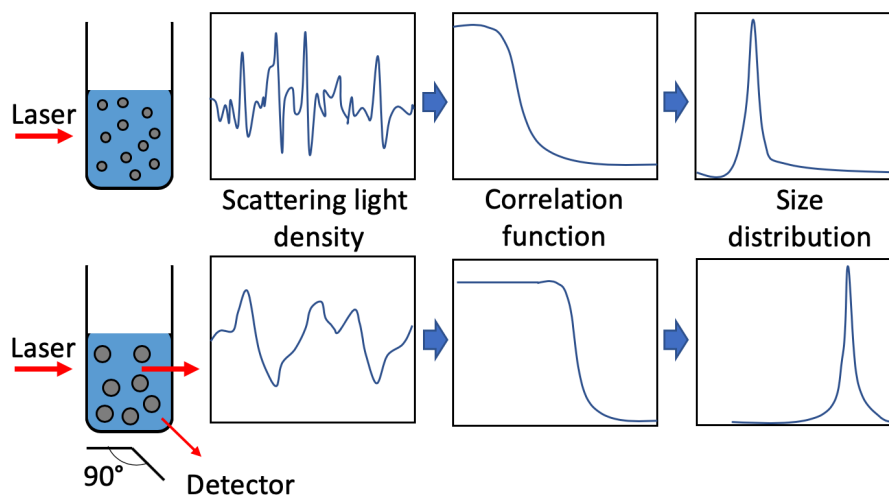


Figure III.1 – Schematic of the DLS principle applied to a suspension of small or large particles.

Since the size is derived from the Stokes-Einstein equation, it means that it represents the hydrodynamic radius of the particle. Moreover, this equation implies a Brownian movement, which is not especially the case for a suspension of charged colloid. This is particularly the case at low I when particles of the same charge strongly repulse each other. This effect was always verified in the thesis. To do so, suspensions were first filtered, then the size distribution was measured again on the instrument. It was verified that the population with a radius higher than the filter size disappeared after filtration.

Usually, 10 measurements were recorded for one sample. For instance, Figure III.2 shows all size distributions that were measured for the PPCs used in Chapter 1 (this will be presented in detail in Chapter 1). It appears that PPCs with a size that is around $0.25\ \mu\text{m}$ are detected. In order to verify that it is not an artifact of measurement due to the charge borne by PPCs, the suspension was filtered on a $0.25\ \mu\text{m}$ filter. Results are presented in the bottom panel of Figure III.2. It appears that, after filtration, all PPCs with a diameter higher than the filter mesh size are removed. This confirms that the measurement initially obtained was not biased by the charge of PPCs. This simple control procedure was repeated for all DLS measurements.

With respect to the precision of the measurements, the distribution that is obtained is based on a mathematical model which fits the correlation functions. If one or two radius distribution are obtained, it means that the mathematical model has fitted the correlation with respectively one or two spheres that have the given radius. Interestingly, for rod-like particles, the fitting could give two populations that correspond to the radius and the length of the particle. Excepted in Chapter 1, only the largest population detected was presented.

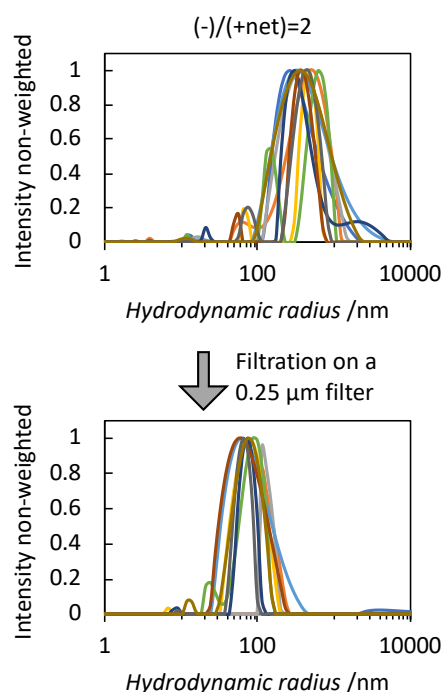


Figure III.2 -Typical size distribution obtained by DLS for PPCs with a $(-)/(+net)=2$ charge stoichiometry = 2, constructed at pH 7.5 and $I=10$ mM . Measurements were performed before (top) and after (bottom) filtration on a $0.25\ \mu\text{m}$ filter. These data are presented in Chapter 1.

1.2. Electrophoretic mobility

The ζ -potential, *i.e.* the electrokinetic potential, describes the charging behavior at interfaces. As presented in the strategy, we aim at creating and analyzing the surface of PPCs that are solid-like, *i.e.* similar to precipitates in a solution. The measurement of ζ -potential *via* electrophoretic mobility will thus be described in the context of solid-liquid interface. This description also

stands for very small charged objects such as proteins. When a charged surface is put in contact with a liquid, it causes a distribution of species that is different at the interface compared to the bulk of the solution. As represented in Figure III.3 for a negatively charged particle, positively charged species accumulate at the interface. In the frame of the electrical double layer model, the solution is divided into a stationary phase and a diffuse layer. The stationary layer comprises ions that are strongly adsorbed on the surface through short-range interactions. The diffuse layer is made of ions that compensate the surface charge *via* coulombic forces. This second layer is diffuse as ions move due to thermal motion and coulombic attraction. By applying a voltage in the solution, charged particles are put in motion and their velocity is measured using a Doppler anemometer. According to the Smoluchowski theory, the ζ -potential of the particle can be found using its velocity in the applied electrical field, the density and the dielectric permittivity of the fluid. The surface charge density that is obtained originates from the boundary between the moving particle and the solution. This boundary is known as the shear plane (see Figure III.3). Electrophoretic mobility measurement thus allows to assess the number of charges per unit area that are present at the shear plane, *i.e.* the ζ -potential. The surface potential (Ψ_s) of the solid is thus not experimentally accessible.¹⁷⁶

This method has been widely used to study the charge of PPCs. Especially, Kovačević *et al.* extensively investigated Lyz complexation with PSS.¹⁷⁷ They used this method to assess the charge of PPCs as a function of PSS-to-Lyz ratio. It appears that when PSS is added to Lyz, PPCs are first positively charged due to the excess of Lyz charge. Then, when PSS is further added, the PPCs charge is inversed and takes negative values due to the PSS excess. These data are perfectly consistent with others and suggest that this method is appropriate to measure the charge of PPCs.^{177,178}

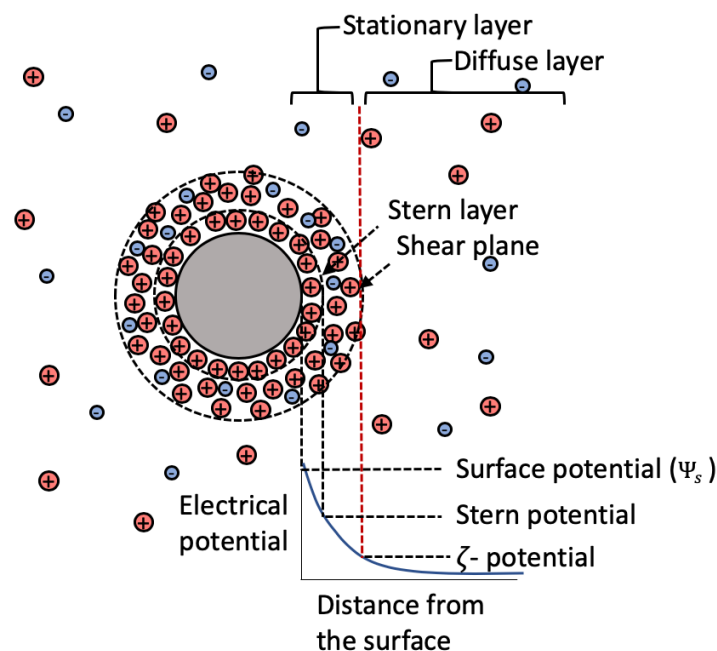


Figure III.3 – Model of the electrical double layer at the solid-liquid interface. Adapted from Luxbacher.¹⁷⁶

2. Multilayer formation

A number of different characterization methods were used in this thesis. Many of these, such as scanning electron microscopy (SEM), AFM and XPS, have become very common and will not be described here. In order to respectively assess the multilayer construction and to quantify the mass of protein, two methods were thoroughly used throughout this thesis. These are the QCM-D monitoring and the bichinchoninic acid assay.

2.1. Quartz crystal microbalance with dissipation monitoring

QCM-D is an acoustic method that allows the real-time measurement of surface adsorption phenomenon. Quartz crystal possesses piezoelectric properties, which means that the application of a voltage results in mechanical deformation. Therefore, by alternating voltage, an oscillatory motion can be obtained. The QCM-D measures the resonant frequency (f_0 , in Hz) of a quartz crystal which is in-between two gold electrodes (Figure

III.4a). A schematical cross section of the crystal which shows the gold electrode (green) and the acoustic wave is presented in Figure III.4b.¹⁷⁹

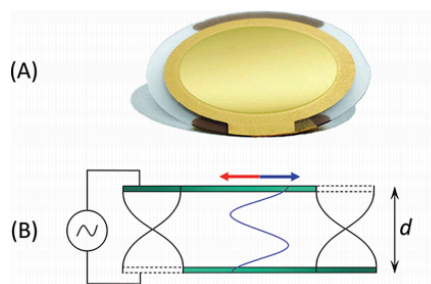


Figure III.4 - (a) Picture of a QCM-D sensor coated with gold. (b) representation of the propagating acoustic wave. Reprinted with permission from Reviakine *et al.* Copyright 2019 American Chemical Society.¹⁷⁹

Upon adsorption of any material on the gold surface, the resonant frequency changes. If the deposited material is thin, homogeneous and rigid, the shift of frequency (Δf) can be directly related to the adsorbed mass (Δm) according to the Sauerbrey equation (Equation (III.1)):¹⁷⁹

$$\Delta m = \frac{-c}{n} \Delta f \quad \text{with } c = \frac{t_q \rho_q}{f_0} \quad (\text{III.1})$$

where n is the harmonic number, c is the mass sensitivity constant (property intrinsic to the crystal), t_q the thickness of the crystal and ρ_q the crystal volumetric mass density. A way that is used to assess the film viscoelastic properties, and *in fine* the Sauerbrey applicability, is the measure of the dissipation (D). To do so, the device stops to apply an alternate voltage and the oscillation are left to decay. Given that quartz is piezoelectric, a voltage is generated, and the decay can be measured. D is extracted from these data as shown by the red curve in Figure III.5. If D takes low values, it means that the sensor has rapidly absorbed the energy. Just as an acoustic wave is rapidly absorbed by a soft material while it resonates for a longer time in a hard material, the latter decay rate can be related to the viscoelastic properties of the sensor. By measuring the shift of D , the viscoelastic changes that are due to the adsorbed material can be probed.

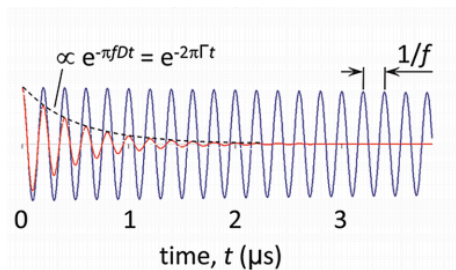


Figure III.5 - Representation of the main parameters derived from the sensor vibration. Reprinted with permission from Reviakine *et al.* Copyright 2019 American Chemical Society.¹⁷⁹

According to Reviakine *et al.*, the Sauerbrey equation can be used if $\left| \Delta D_n / \left(\frac{\Delta f}{n} \right) \right| \ll 4 \times 10^{-4}$ Hz for a 5 MHz crystal or if the different overtones are superimpose after normalization by the overtone number (each of the two criteria can be applied, though the criteria of low dispersion in $\frac{\Delta f}{n}$ is more stringent).¹⁷⁹ If this condition is not met, other models that take into account viscoelasticity of the adsorbed material have to be used. These will not be presented here since they are not trivial and will not be used in this thesis.

In the following, two typical results obtained using QCM-D are presented. $\Delta f/n$ and D were measured after rinsing with buffer at each step of the multilayer growth. Results are presented for PPCs assembled with PAH at pH 7.5 and $I=10$ mM or $I=0$ mM in Figure III.6a and b respectively. The interpretation of these data will be presented for the 7th overtone in respectively Chapter 1 and 2. The purpose here is to illustrate two kind of cases: one where the Sauerbrey model cannot be used and another where it can be used. When the multilayer is constructed at pH 7.5 and $I=10$ mM (Figure III.6a), the different normalized overtones split. This suggests that the viscoelastic properties change as the multilayer grows and that the Sauerbrey model cannot be used to accurately measure the mass. These results, which are presented in Chapter 1, were presented as $\Delta f_7/7$ (*i.e.* under the form of raw data) to avoid any unrealistic mass approximation. It is worth mentioning that the Sauerbrey equation can still be used in this condition by rigorously specifying that it gives the lower limit value. When the multilayer is constructed at pH 7.5 and $I=0$ mM (Figure III.6b), the different normalized overtones are superimposed. The Sauerbrey equation

can be used to assess the mass of the multilayer. It can be pointed that a small overtone splitting is observed upon adsorption of the PPCs, *i.e.* when the $\Delta f/n$ decreases. The multilayer mass might thus be underestimated upon PPCs adsorption. For the sake of clarity, all overtones were not systematically presented in the thesis. Nevertheless, they were systematically measured to ensure the appropriate use of the Sauerbrey equation when required.

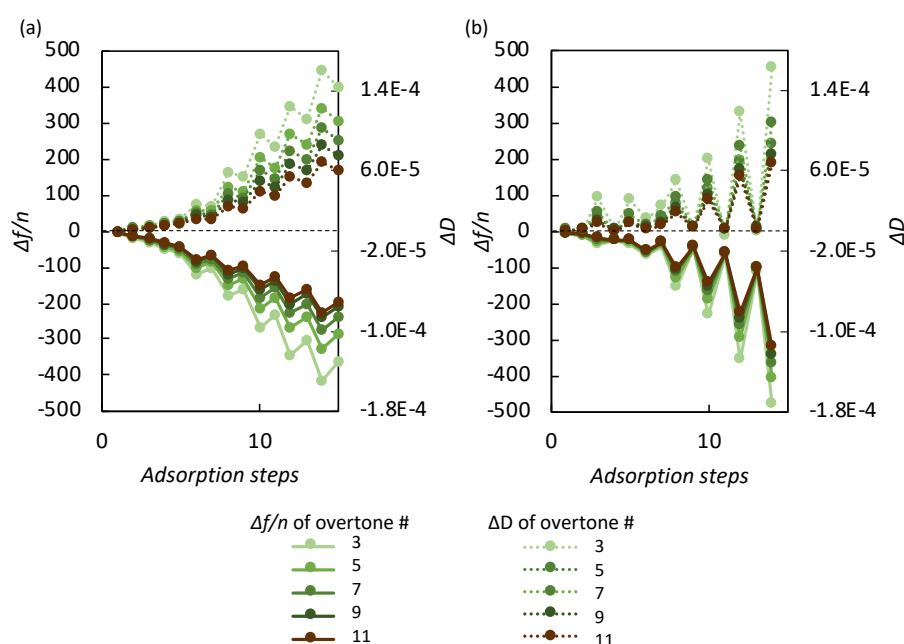


Figure III.6 -Typical results obtained using QCM-D to monitor the construction of a multilayer - Alternate adsorption of PPCs with PAH at pH 7.5 and (a) $I=0$ mM or (b) $I=10$ mM.

2.2. Bicinchoninic acid assay

The bicinchoninic acid assay (BCA) assay is a sensitive method that allows the quantification of proteins in the presence of detergent. The principle is that the amide bond of proteins reduces Cu^{2+} to Cu^{+} (Biuret reaction). The resulting Cu^{+} is then detected *via* the formation of an intense purple complex (absorption at 562nm) with the bicinchoninic acid in alkaline condition (see Figure III.7). The color produced by this reaction is stable and increases proportionally to the amount of protein over a wide range of protein concentrations. The advantage of this assay is that a detergent can be added

to dissolve the multilayer and subsequently quantify the amount of protein released. To do so, 1% Sodium dodecyl sulfate (SDS) was used in all samples.¹⁸⁰ The removal of the multilayers by the BCA assay will be demonstrated in Chapter 2.

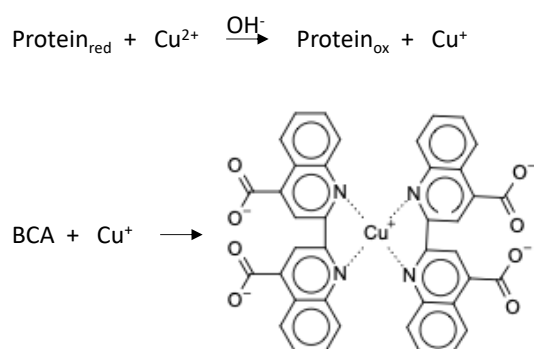
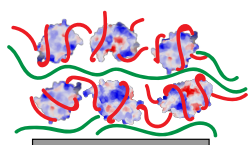


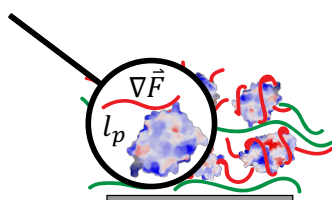
Figure III.7 - Reaction of the bicinchoninic acid assay, the amide bond of the protein reduces Cu^{2+} , which then reacts with the bicinchoninic acid to form a colored complex.¹⁸⁰

3. Outline



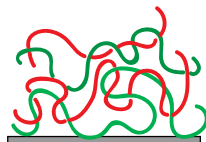
Chapter 1 – Protein–polyelectrolyte complexes to improve the biological activity of proteins in layer-by-layer assemblies

We first investigate the potential of PPCs as building block for the immobilization of proteins using the LbL method. The multilayer growth is measured as well as its mass and bioactivity.



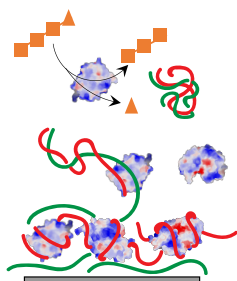
Chapter 2 – Integrating proteins in Layer-by-Layer assemblies independently of their electrical charge

Multilayers that are constructed by simple alternate adsorption of Lyz with PE are compared to the PPCs-based multilayers over a wide range of pH and I conditions. Together with an extensive investigation of PPCs formation in solution, this allows to enlighten the multilayer growth mechanism.



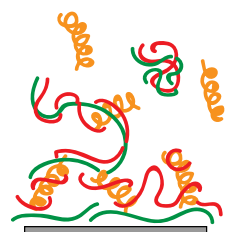
Chapter 3 – Out of equilibrium interfacial self-assembly that irreversibly loses water

Particular features of the multilayers obtained using PPCs are investigated by varying the atmosphere humidity surrounding the thin film.



Chapter 4 – Protein-polyelectrolyte complexes for the delivery of active proteins from self-assemblies

The enzymatic activity of Lyz is investigated in multilayers. To do so, emphasis is put on elucidating the fraction of active Lyz. Is it released or is it stable in the multilayer? If released, is Lyz still complexed?

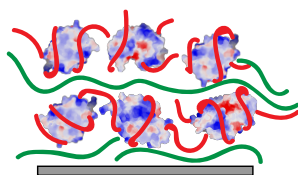


Chapter 5 – Towards LL37 coated bandages to promote wound healing

In this Chapter, we investigate the potential of PPCs-based multilayers to create immunomodulatory and anti-inflammatory coatings. To do so, LL37, a human peptide, is immobilized on bandages using the developed method.

Part IV: Results

1. Protein-polyelectrolyte complexes to improve the biological activity of proteins in Layer-by-Layer assemblies



In this first Chapter, the potential of PPCs as building blocks for the LbL assembly is investigated. To do so, we aim at proving that Lyz can be immobilized and that it is still active using PPCs. Interestingly, it appears that thicker multilayers, with a higher PE fraction, are obtained with PPCs compared to single Lyz molecules. Biological activity is not only maintained, but specific activity is also higher. This is attributed to the more hydrated state of the PPCs-based assemblies.

vander Straeten, A.; Bratek-Skicki, A.; Germain, L.; D'Haese, C.; Eloy, P.; Fustin, C.-A.; Dupont-Gillain, C. Protein-Polyelectrolyte Complexes to Improve the Biological Activity of Proteins in Layer-by-Layer Assemblies. **Nanoscale** 2017, 9 (44), 17186–17192.

1.1. PPCs formation

Lyz was first complexed at pH 7.5 with low molar mass PSS (PSS_i) chains. Complexation was monitored by a turbidimetric assay as a function of $(-)/(+net)$ charge ratio, which is defined as the structural charge ratio, where Lyz charge is +8 and PSS_i charge is -36 (Figure 1a).¹⁸¹ Absorbance at 450 nm (A_{450}) increases with increasing $(-)/(+net)$, *i.e.* with PSS_i addition, until it reaches a plateau at $(-)/(+net)=1.25$. The complexation was further investigated by centrifugation, followed by Lyz UV-dosage (Figure IV.1a and b). The amount of Lyz in the pellet after centrifugation at 9500 g increases following the trend observed by turbidimetry until it reaches a maximum between $(-)/(+net)=1.25$ and 1.5, followed by a slight decrease from $(-)/(+net)=1.5$ to 2. The evolution of the ζ -potential of PPCs depending on $(-)/(+net)$ is presented in Figure IV.1c. Lyz charge compensation by PSS_i occurs close to $(-)/(+net)=1.5$. The PPCs then take an increasingly negative charge as PSS_i is further added. The turbidity plateau and the Lyz maximal amount found in the pellet match Lyz charge compensation by PSS_i, which is consistent with the model and with simulations proposed in literature.^{160,159,182–184} This suggests that, beyond compensation, the complexes take a core-corona structure with a neutral internal domain and negatively charged PSS_i chains dangling outside.¹⁵⁹ Regarding PPCs size, the slight decrease of Lyz amount in the pellet from $(-)/(+net)=1.5$ to 2 (Figure IV.1a) suggests that PPCs density or size decreased. DLS measurements were thus performed (Figure IV.1d) and two populations with different sizes were observed after PSS_i addition to Lyz: one with an apparent hydrodynamic radius (R_h) around 3 nm corresponding to single Lyz molecules, and the other with a larger R_h corresponding to PPCs. Furthermore, R_h decreased from between 1 and 2 μm down to 0.250 μm when $(-)/(+net)$ was increased from 1.5 to 2. A centrifugation carried out at a lower acceleration, *i.e.* 380 g, confirmed that PPCs size decreased above $(-)/(+net)=1.5$ (Figure IV.1b). These observations are in good agreement with complex dissolution, and with the probability to find free protein molecules in solution, as predicted by simulations.¹⁸⁴

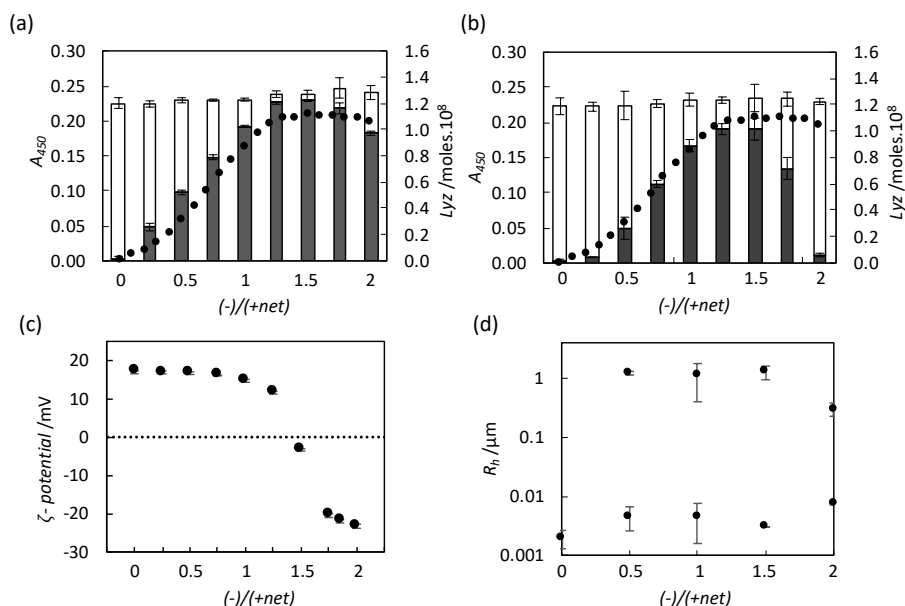


Figure IV.1 – (a) and (b) Normalized A_{450} (●) of a Lyz solution undergoing PSS_i addition in such a way that negative-to-positive charge ratio varies between 0 and 2 at pH 7.5 in HEPES buffer. Lyz amount assessed by UV spectrophotometry in the pellet (solid bars) and in the supernatant (empty bars) after PPCs centrifugation at 9500 g (a) or 380 g (b) for 15 min. (c) ζ -potential dependence of PPCs on their theoretical charge ratio. (d) R_h of PPCs at different charge ratios measured by DLS. Error bars show the standard deviation ($n=3$).

1.2. PPCs² LbL assembly

PPCs with a charge ratio above 1.5 are thus negatively charged. In order to test PPCs immobilization and to verify the role of electrostatic interactions, Lyz- PSS_i complexes with either a $(-)/(+net)$ of 0.5 (PPCs^{0.5}) or of 2 (PPCs²) were assembled with PAH, a polycation, by the LbL method (Figure IV.2a). Layer construction was monitored by QCM-D (Figure IV.2b). For all experiments, two bilayers of PAH assembled with high molar mass PSS (PSS_h) were first deposited in order to minimize the influence of the substrate and to favor further assembly (shaded area in Figure IV.2b).¹⁶ A decrease of the normalized frequency shift of about 40 Hz was recorded. Then, different multilayers were constructed on this $[PAH-PSS_h]_2$ cushion. From the bottom to the top in Figure IV.2b, the following LbL assemblies were constructed; (i) PAH with PPCs^{0.5} $[PAH-PPCs^{0.5}]$, (ii) PAH with Lyz $[PAH-Lyz]$, (iii) Lyz with PSS_h $[Lyz-PSS_h]$, (iv) PAH with PSS_i $[PAH-PSS_h]$, and (v) PAH with PPCs² $[PAH-PPCs^2]$.

Regarding layers integrating PPCs^{0.5} (i), the normalized frequency shift does not change. The positively charged PPCs^{0.5} cannot assemble through electrostatic interactions with PAH, which is also positively charged at pH 7.5. When PAH was adsorbed alternately with positively charged Lyz (ii), no building of the layer was observed either as expected.

The classical LbL assembly that integrates proteins, *i.e.* [Lyz-PSS_h] (iii), and the Lyz-free assembly of PAH with PSS_i (iv) led to a decrease of frequency of respectively 85 Hz and 110 Hz after deposition of five bilayers on the cushion. Finally, for PAH assembled with PPCs² (v), the normalized frequency shift decreased by 240 Hz after five bilayers. It confirms that PPCs² are negatively charged, interact favorably with the positively-charged PAH and are successfully integrated within a multilayer system. In these multilayers, the normalized frequency shift increases after each PAH adsorption step, suggesting that some PPCs² are removed from the multilayer. Further steps of PPCs² adsorption however overcompensate the loss of matter at interface, and the multilayer grows. The frequency shift drop is three-fold higher for multilayers integrating PPCs² (v) than for the classical [Lyz-PSS_h] LbL (iii), which suggests that a thicker film is obtained. The difference between the Lyz-free LbL (iv) and the layers integrating PPCs² (v) can be attributed to a different multilayer architecture and to the presence of both Lyz and PSS_i in the case of PPCs². The *D* measured by QCM-D are respectively of 2×10^{-6} , 2.5×10^{-5} and 9×10^{-5} for [Lyz-PSS_h], [PAH-PSS_i]₅, and [PAH-PPCs²]₅. This suggests that, in particular, the hydration level of multilayers built with PPCs is higher than the one of assemblies built with single Lyz molecule.¹⁸⁵

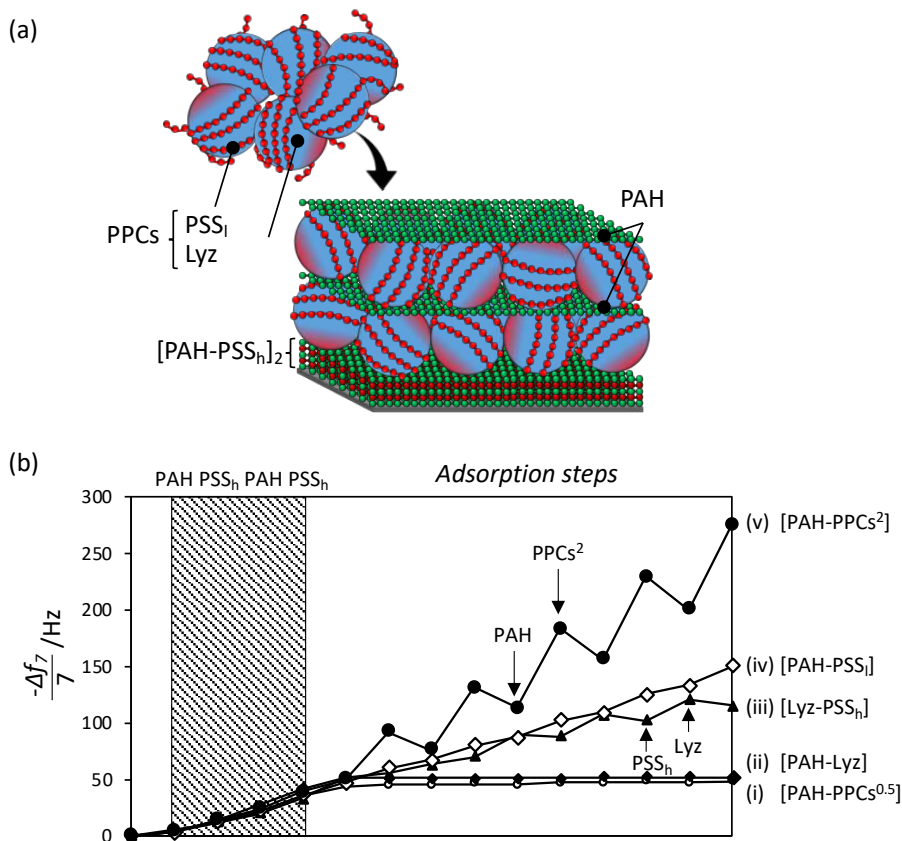


Figure IV.2 – (a) Scheme of the layer-by-layer assembly of PPCs with PAH. (b) Normalized frequency shift $-\frac{\Delta f_2}{7}$ measured by QCM-D after adsorption of a cushion of two PAH-PSS bilayers ([PAH-PSS_h]₂, shaded area) followed by either PAH-PPCs with (-)/(+net)=0.5 ([PAH-PPCs^{0.5}]₅), [PAH-Lyz]₅, [Lyz-PSS_h]₅, or PAH-PSS_l or PAH-PPCs with (-)/(+net)=2 ([PAH-PPCs²]₅) at pH 7.5 in HEPES buffer.

The sequential adsorption of PPCs with (-)/(+net)=2 with PAH enables multilayer construction. However, PSS that is not complexed could participate in the LbL assembly, which could result in the failure to immobilize Lyz. In order to assess Lyz presence in these multilayered systems, XPS experiments were performed. Glass surfaces coated with a thick layer of either PAH or PSS_h prepared by evaporation of a concentrated solution were first analyzed by XPS as control samples. The following assemblies were then built on a PAH-PSS_h bilayer cushion: [PAH-PSS_l]₅ and [PAH-PPCs²]₅. C1s, N1s and S2p spectra are presented in Figure IV.3 for (a) PAH, (b) PSS_h, (c) [PAH-PSS_h]₂-[PAH-PSS_l]₅ and (d) [PAH-PSS_h]₂-[PAH-PPCs²]₅. For PAH, Figure IV.3a

shows a C1s peak that is attributed mainly to two components at 284.8 eV and 286.2 eV, respectively corresponding to aliphatic carbon chains ($\underline{\text{C}}\text{-C,H}$) and amine groups ($\underline{\text{C}}\text{-N}$).¹⁸⁶ The N1s peak can also be decomposed in two components at 399.5 eV and 401.3 eV that are attributed respectively to nitrogen in amine or amide groups, and to protonated nitrogen.¹⁸⁷ A very small S2p contamination is also detected. For the PSS_h sample (Figure IV.3b), the C1s peak can be decomposed with a main component corresponding to aliphatic and aromatic carbon ($\underline{\text{C}}\text{-C,H}$). Another peak corresponding to the aromatic shake-up is observed at 292.1 eV. It also appears that no N1s peak is detected, and that a peak corresponding to the S2p doublet from PSS sulfonate groups is observed, which can be decomposed in two components at 168.4 eV and 169.6 eV.¹⁸⁸ Table IV.1 highlights that, as expected, Cl⁻ is detected as PAH counter ion and Na⁺ as PSS counter ion with a good consistency since 14.1% Cl is detected together with 13.7% total N in PAH samples, and 8.1% Na is detected for 8.2% S in PSS_h samples. Then, multilayers were analyzed. Figure IV.3c shows spectra for [PAH-PSS_h]₂-[PAH-PSS]₅. Two components at 284.8 eV and 286.2 eV, respectively corresponding to aliphatic carbon ($\underline{\text{C}}\text{-C,H}$) and amine groups ($\underline{\text{C}}\text{-N}$) were detected in the C1s peak for [PAH-PSS]₅ multilayers. For multilayers integrating PPCs, i.e. [PAH-PSS_h]₂-[PAH-PPCs²]₅ in Figure IV.3d, a component corresponding to the peptide link is detected at 288.1 eV in the C1s peak. In addition, a higher total nitrogen content together with less protonated nitrogen compared to [PAH-PSS_h]₂-[PAH-PSS]₅, and a new component at 163.9 eV in the S2p peak, corresponding to thiols from cysteines, strongly indicate protein presence. Regarding PSS marker, the S2p peak at 167.9 eV confirms PSS detection. Finally, the remaining protonated component in the N1s peak supports PAH presence. In order to assess each species contribution, one can compute a $[\underline{\text{C}}\text{-(C,H)}]_{\text{Lyz}}/[\text{N-}\underline{\text{C}}=\text{O}]_{\text{Lyz}}$ of 1.7 for Lyz based on its sequence (P00698) and thus assume that $7\% \times 1.7 = 11.7\%$ of the $\underline{\text{C}}\text{-(C,H)}$ component is due to proteins. Then, based on the sulfonate surface atomic concentration detected and the PSS formula, one can assess that PSS contributes up to $3.1\% \times 8 = 24.8\%$ of the $\underline{\text{C}}\text{-(C,H)}$ component. PAH is thus expected to contribute to $[\underline{\text{C}}\text{-(C,H)}]_{\text{PAH}} = [\underline{\text{C}}\text{-(C,H)}]_{\text{tot}} - [\underline{\text{C}}\text{-(C,H)}]_{\text{Lyz}} - [\underline{\text{C}}\text{-(C,H)}]_{\text{PSS}} = 41.6\% - 11.7\% - 24.8\% = 5.1\%$ of the $\underline{\text{C}}\text{-(C,H)}$ component. In order to

confirm the contributions of PAH and Lyz to the detected $\underline{\text{C}}\text{-(C,H)}$ component, one can correlate it to the nitrogen peak decomposition. Indeed, 5.3% of $\underline{\text{C}}\text{-(C,H)}$ measured for PAH theoretically gives 1.8% ($5.3\% \times 1/3$) protonated nitrogen ($\underline{\text{C}}\text{-(C,H)}/\text{NH}^+=3$) and a close 2.1% is detected. Regarding Lyz, 11.7% of $\underline{\text{C}}\text{-(C,H)}$ theoretically gives 8.1% N (based on Lyz sequence, $\underline{\text{C}}\text{-(C,H)}/\text{N}=1.4$) and in agreement with the value of 8.1% found experimentally. It can thus be concluded, based on C1s peak decomposition and on the correlation with other peaks, that proteins, PSS and PAH are all three present in the probed volume of $[\text{PAH-PSS}_h]_2\text{-}[\text{PAH-PPCs}^2]_5$ samples (analyzed depth ~ 10 nm).

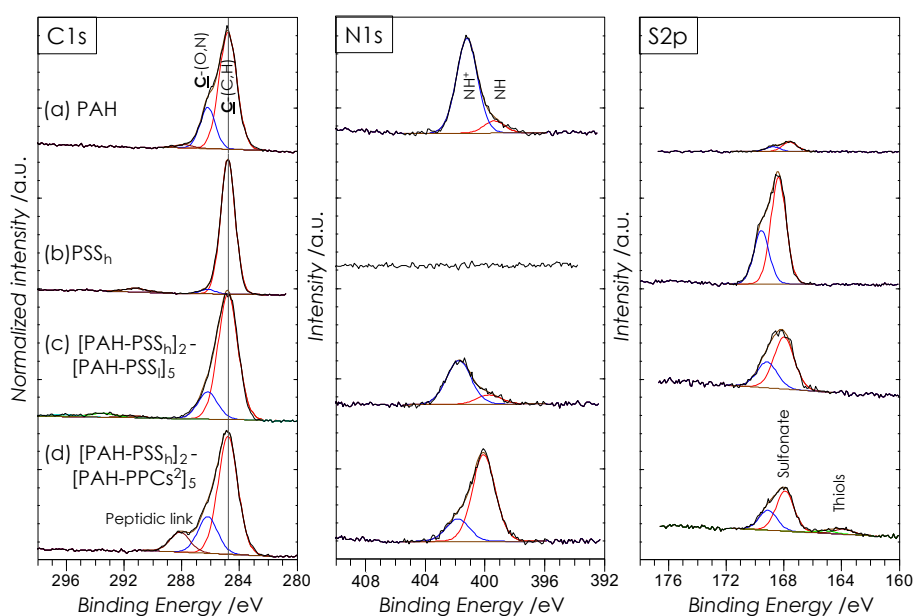


Figure IV.3 – C1s, N1s and S2p peaks recorded by XPS for glass surfaces coated with (a) PAH, (b) PSS_h or with a $[\text{PAH-PSS}_h]_2$ cushion followed by (c) $[\text{PAH-PSS}]_5$ and (d) $[\text{PAH-PPCs}^2]_5$. Spectra are presented with their respective decomposition in components or doublets. The C1s peaks were normalized in such a way that their maxima, i.e. $\underline{\text{C}}\text{-(C,H)}$ component, have the same height. Peak intensities can thus not be directly compared.

Table IV.1 – Surface atomic concentration (in %, excluding H) obtained by XPS on glass surfaces coated with (i) PAH, (ii) PSS_h, or a [PAH-PSS_h]₂ cushion followed by (iii) [PAH-PSS_i]₅ and (iv) [PAH-PPCs²]₅. bdl: below detection limit. C_{shup}: aromatic shake-up.

	O 1s	NH+	NH	C _{shup}	N-C=O	C-(O,N)	C-(C,H)	C _{tot}
PAH	3.6	12.2	1.5	bdl	1.4	15.4	50.9	67.7
PSS _h	18.8	bdl	bdl	2.7	bdl	2.0	58.9	63.6
[PAH-PSS _i] ₅ *	22.7	3.8	0.9	1.8	bd	9.6	44.6	56.0
[PAH-PPCs] ₅	18.1	2.1	8.1	bdl	7.0	12.9	41.6	61.5
	S _{thiol}	S _{sulfonate}	Si	Na	Cl			
PAH	bdl	0.7	0.3	bdl	14.1			
PSS _h	bdl	8.2	0.9	8.1	0.3			
[PAH-PSS _i] ₅ *	bdl	4.4	12	0.2	bdl			
[PAH-PPCs] ₅	0.4	3.1	6.7	bdl	bdl			

*0.4% K is also detected for [PAH-PSS]₅

In order to combine structural and chemical information regarding multilayers, ToF-SIMS was used in the depth profiling mode. The following assemblies, deposited on a PAH-PSS_h bilayer cushion, were analyzed; (a) [PAH-PSS_i]₅, (b) [Lys-PSS_h]_{4.5} and (c) [PAH-PPCs²]₅. Organic depth profiles were recorded and results are presented for SO₃⁻, CN⁻, SiO₂⁻ and CNO⁻ ions in Figure IV.4.

SO₃⁻ ions that are detected are emitted from PSS, CN⁻ ions are emitted from both the protein and the PAH, CNO⁻ ions are emitted from the protein, and SiO₂⁻ ions are emitted from the glass surface underneath the multilayer. A low CNO⁻ is measured in [PAH-PSS_i]₅ compared to [Lys-PSS_h]_{4.5} and [PAH-PPCs²]₅. This confirms that the two latter multilayers contain protein.

In all cases, the ions emitted from components of the multilayer (SO₃⁻, CN⁻ and CNO⁻) decrease, while those coming from the substrate increase (SiO₂⁻) as the exposure to etching ions increases. This confirms that the surface is etched by the ion beam, and that the underneath glass substrate can be

reached. The SiO_2^- plateau, which indicates that the whole multilayer thickness has been etched, is reached after that $5\text{E}+14$ ions cm^{-2} , $3\text{E}+14$ ions cm^{-2} , and $6\text{E}+14$ ions cm^{-2} have been used for respectively $[\text{PAH-PSSi}]_5$, $[\text{Lys-PSSh}]_{4.5}$ and $[\text{PAH-PPCs}^2]_5$.

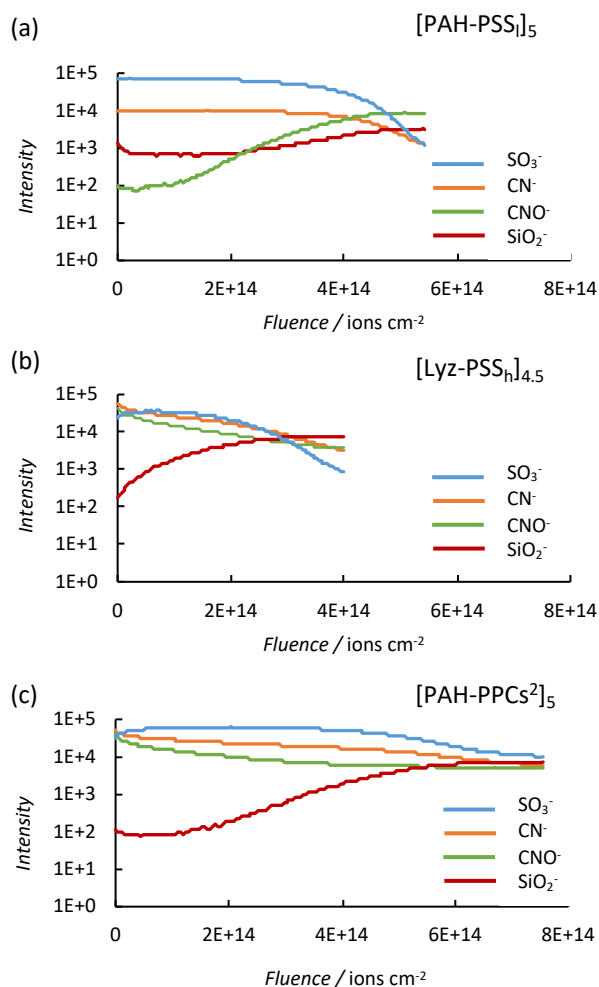


Figure IV.4 – ToF-SIMS analysis, in the depth profiling mode, of glass surfaces coated with a $[\text{PAH-PSSh}]_2$ cushion, followed by (a) $[\text{PAH-PSSi}]_5$, (b) $[\text{Lys-PSSh}]_{4.5}$, (c) $[\text{PAH-PPCs}^2]_5$. The results are presented as background-corrected intensities measured for SO_3^- , CN^- , CNO^- and SiO_2^- ions as a function of the argon cluster fluence.

The layer thickness was determined in the dry state from cross-sections of AFM images after scratching the central area (Figure IV.5a). Layer thicknesses obtained for (i) [PAH-PSSi]₅, (ii) [Lyz-PSS_h]_{4.5} and (iii) [PAH-PPCs²]₅, constructed on a [PAH-PSS_h]₂ cushion, are respectively 14.0 ± 0.3 nm, 7.6 ± 0.5 nm and 14.9 ± 0.5 nm. Hence, [PAH-PPCs²]₅ is almost twice thicker than [Lyz-PSS_h]_{4.5} and slightly but significantly thicker than [PAH-PSSi]₅ (Student test, $\alpha=0.05$). It confirms the lower decrease of frequency shift obtained by QCM-D for [Lyz-PSS_h] multilayers compared to [PAH-PPCs²] multilayers. The smaller difference in thickness measured for [PAH-PSSi]₅ and [PAH-PPCs²]₅ compared to their large difference in frequency shift is explained by a higher hydration level for [PAH-PPCs²]₅ (see *D* here above).

Interestingly, when the ToF-SIMS analysis is performed, the number of ions needed to reach the underneath surface scales with the multilayer thickness measured by AFM. Moreover, from the data obtained by DLS and assuming a packing density of 50% (74% being the highest possible for spheres, the former value was chosen somehow arbitrarily), it appears that around 290 Lyz molecules are present in each PPC² (assuming spherical PPCs²). From the thickness obtained by AFM, it appears that, in average, only one layer of Lyz is added at each PPCs adsorption step. These results clearly demonstrate that the PPCs reorganize upon their LbL assembly, or that a minor undetected fraction of the PPCs², which has a smaller *R_h*, is assembling.

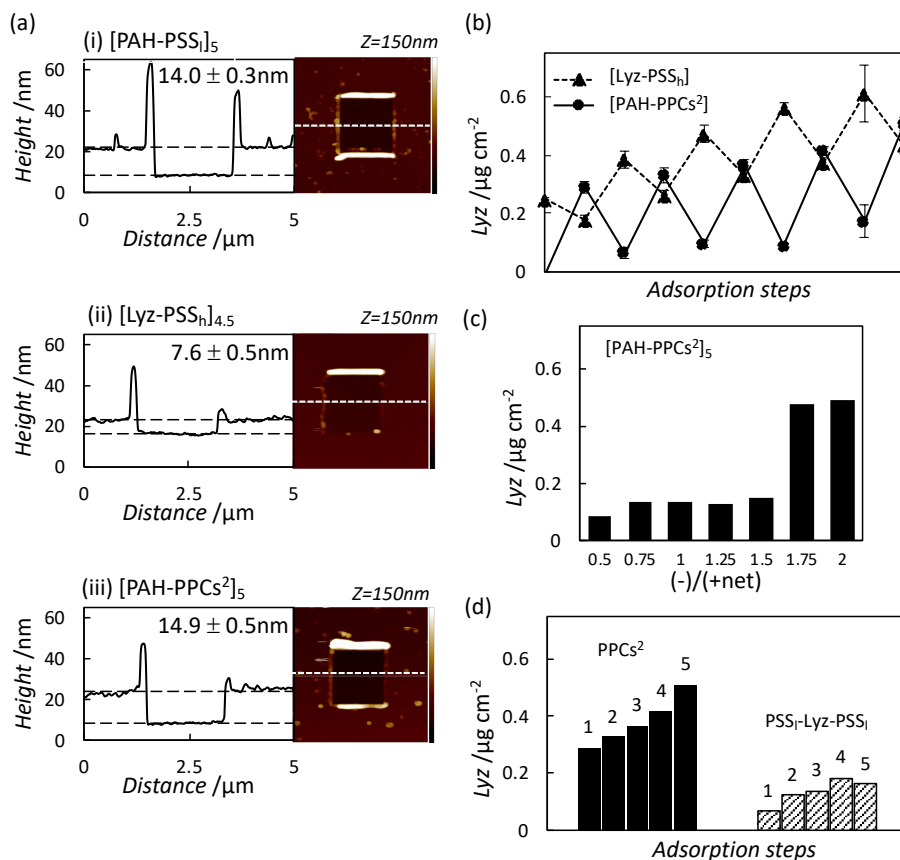


Figure IV.5 – (a) Topographic images (5 μm width and $Z=150\text{nm}$) of glass surfaces coated with a [PAH-PSSh]₂ cushion followed by (i) [PAH-PSSh]₅, (ii) [Lyz-PSSh]_{4.5} or (iii) [PAH-PPCs²]₅ obtained by AFM in contact mode after scratching a $2 \times 2\text{ }\mu\text{m}^2$ central area at high applied load, and cross-sections corresponding to the broken line on topographic images (error bars show the standard deviation $n \geq 2$). Amount of Lyz per cm^2 measured from a BCA assay (error bars show the standard deviation $n=3$). (b) at each step of the building of [Lyz-PSSh]₅ or [PAH-PPCs²]₅, (c) after 5 bilayers of [PAH-PPCs] integrating PPCs with different (-)/(+net), and (d) after each PPCs addition in a [PAH-PPCs²]₅ assembly, or after each sequence of PSSh-Lyz-PSSh addition for a [PAH-PSSh-Lyz-PSSh]₅ assembly.

The amount of Lyz was assessed at different steps of the growth of LbL assemblies using a BCA assay (quantification limit = 70 ng cm^{-2} – Figure IV.5b, c and d). For [Lyz-PSSh] and [PAH-PPCs²] (Figure IV.5b), a saw-tooth profile is observed, which is consistent with QCM-Ds results. However, protein amount is lower in [PAH-PPCs²], which suggests that the larger frequency shift that was observed with QCM-D compared to [Lyz-PSSh] originates from

the PE (PAH, PSS) rather than from Lyz. PPCs with different charge ratios were then used as building blocks for the construction of 5 bilayers (Figure IV.5c). It appears that only PPCs with a charge ratio higher than 1.5 enable good protein integration, which is consistent with ζ -potential measurements that showed PPCs charge inversion at a charge ratio close to 1.5. To highlight the potential of PPCs further, the amount of Lyz incorporated after the construction of [PAH-PPCs²]_n multilayers was compared to the one of [PAH-PSS_i-Lyz-PSS_i]_n multilayers, with the sequence PSS_i-Lyz-PSS_i corresponding to the components of PPCs², but this time assembled at the interface in a LbL fashion (Figure IV.5d). The Lyz amount was measured after each PPCs addition in the case of the [PAH-PPCs²] system, while it was measured after each sequence of PSS_i-Lyz-PSS_i addition for the other system. Lyz incorporation is significantly higher when PPCs are used instead of PSS_i and Lyz from single solutions for LbL build-up. This confirms that PPCs bring diversity to the array of building blocks suitable for LbL assembly, rather than to constitute a shortcut for the deposition of layers of same components.

1.3. Lysozyme enzymatic activity in multilayers

Enzymatic activity was assessed using 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside ((GlcNAc)₃-MeU) as the substrate of Lyz. Glycosidic bond hydrolysis and subsequent release of methylumbelliferone was detected by means of fluorescence spectrometry.¹⁸⁹ Enzymatic activity was assessed after each deposition step for [Lyz-PSS_h]₅ and [PAH-PPCs²]₅, as well as for Lyz adsorbed alone (Figure IV.6a). A saw-tooth profile is observed in agreement with the immobilized Lyz amount (see Figure IV.6b). After the deposition of one protein layer, the activities of both LbL assemblies are equivalent to the one of a monolayer of adsorbed Lyz molecules. Interestingly, while the Lyz amount is lower for [PAH-PPCs²]₅ compared to [Lyz-PSS_h]_{4.5} when more layers are constructed, its enzymatic activity is higher. Indeed, the specific enzymatic activity is equal to $9.0 \pm 1.2 \cdot 10^{-6} \mu\text{mol mg}^{-1} \text{min}^{-1}$ for [PAH-PPCs²]₅ compared to $6.8 \pm 0.8 \cdot 10^{-6} \mu\text{mol mg}^{-1} \text{min}^{-1}$ for [Lyz-PSS_h]_{4.5} (Figure IV.6b). This difference might originate from the presence of more PE in the multilayer, that promotes substrate and/or product diffusion, and from the higher hydration level detected in [PAH-PPCs²]₅.

Finally, enzymatic activity drops close to zero when the last layer is a PE while it increases steadily at each step of Lyz deposition. The last PE layer may change the film architecture and hinder (GlcNAc)₃-MeU diffusion. This is supported by the decrease in D measured by QCM-D at each PE adsorption step (data not shown), which is associated with water release and higher film rigidity.¹⁸⁵ Alternatively, the higher rigidity of the film may lead to a stabilized multilayer under the conditions of enzymatic reaction, while a multilayer that ends with either PPCs or Lyz may release its protein content. In the frame of the latter hypothesis, free Lyz in solution would contribute to the measured enzymatic activity. These hypotheses will be investigated in more details in Chapter 4. In both cases, it opens up interesting perspectives for the control of the enzymatic activity of multilayer films.

In order to measure Lyz enzymatic activity with its natural substrate and to show the method versatility, a reactor was 3D-printed and used to assess multilayers enzymatic activity (Figure IV.6d, see materials and methods for details on printing conditions). It was coated with a [PAH-PSS_h]₂ cushion followed by (i) [PAH-PSS_l]₅ (control) or (ii) [PAH-PPCs²]₅. Results of the enzymatic activity measurements are presented in Figure IV.6c. At 4 °C, Lyz is not expected to show much activity.¹⁹⁰ No decrease in A_{450} , *i.e.* no Lyz activity, was observed for [PAH-PSS_l]₅ multilayers while only a slight decrease was observed for multilayers integrating PPCs². Once the temperature was increased to 40 °C, close to the optimum temperature for Lyz activity, multilayers containing no Lyz (i) showed no activity while multilayers integrating PPCs² (ii) showed a high enzymatic activity, with A_{450} decreasing by $0.030 \pm 0.007 \text{ min}^{-1}$. The microbial cell suspension circulating in the reactor lost most of its turbidity under the action of immobilized Lyz in about 100 min.

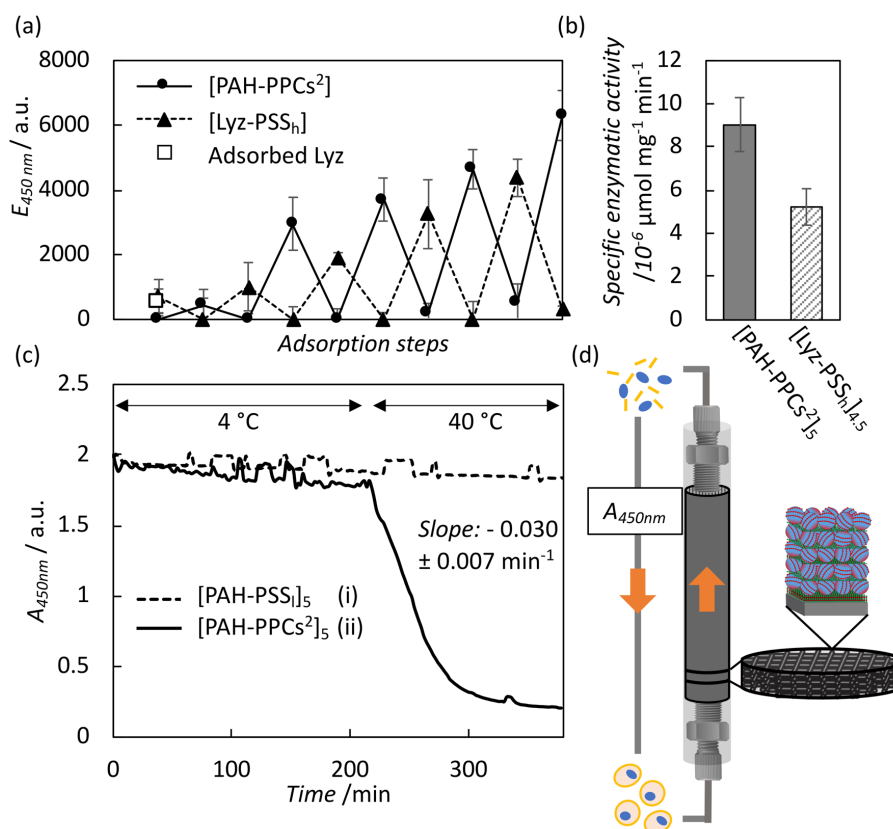


Figure IV.6 – (a) Lyz enzymatic activity measured at each step of the building of [PAH-PPCs₂]₅ and [Lyz-PSS_h]₅, and for Lyz adsorbed alone. Enzymatic activity was measured through the release of the fluorescent MeU from (GlcNAc)₃-MeU glycosidic bond hydrolysis. (b) Specific enzymatic activity of [PAH-PPCs₂]₅ and [Lyz-PSS_h]₅. (c) Enzymatic activity of multilayers coated in a 3D-printed reactor was measured via the decrease of a *Micrococcus lysodeikticus* suspension turbidity. A_{450} decrease was recorded as a function of time at 4 °C then at 40 °C. The reactor was coated with a [PAH-PSS_h]₂ cushion followed by (i) [PAH-PSS_i]₅ or (ii) [PAH-PPCs₂]₅. (d) Scheme of the 3D-printed reactor, the infill pattern and the experimental setup. Error bars show the standard deviation ($n=3$).

1.4. Conclusion

Lyz was complexed with PSS and successfully integrated into multilayers by alternating adsorption of such complexes with PAH. These multilayers were shown to contain Lyz, and to grow thicker compared to a classical Lyz-PSS LbL assembly. The thickness increment is however attributed to the presence of more PE rather than more Lyz in the multilayers. Lyz specific activity was shown to be increased for multilayers integrating PPCs, which could be due

to the more hydrated environment in that case. These new findings offer a potential range of applications with respect to proteins that cannot yet be immobilized efficiently, while keeping their biological activity intact. Furthermore, PPCs add versatility to the LbL systems since new architectures and functions can be tailored, which can be exploited to tune substrate diffusion, layer stability, etc. Applications in biosensing, drug delivery, biomedical sciences or biotechnologies will benefit from this protein immobilization method.

1.5. Materials and methods

1.5.1 *PPCs preparation*

All solutions were buffered with 10 mM HEPES (Sigma-Aldrich, Steinheim, Germany) and pH was adjusted to 7.5 with NaOH. I was adjusted by adding 5.7 mM NaCl (Merck, Darmstadt, Germany) to the buffer. PPCs were prepared by mixing a low molar mass PSS (PSS_I, Mw=7,540 g mol⁻¹, Mw/Mn=1.08, Scientific Polymer, Ontario, USA) solution (0.5 mM negative charge) with a Lyz (Sigma-Aldrich, Steinheim, Germany) solution (0.1 mM net positive charge at pH 7.5). The Lyz solution volume was kept constant while PSS_I and buffer were added in such a way that (-)/(+net) varies between 0 and 2 and that Lyz concentration stays constant, e.g. 1 mL Lyz was mixed with 0.1 mL PSS_I and 0.3 mL buffer to reach a charge ratio of 0.5, except for turbidimetry experiments for which only PSS_I was added to the Lyz solution.

1.5.2 *Turbidimetry*

The optical density at $\lambda=450$ nm (A_{450}) of the suspension was measured using a UV-vis spectrophotometer (DU800, Beckman Coulter, USA). A_{450} was normalized by the dilution factor. Measurements were performed in triplicate.

1.5.3 *Protein UV-dosage*

The PPCs suspension was centrifuged at 9,500 g for 15 min. The supernatant was collected and NaOH 1 N was added both to the pellet and the supernatant to break PPCs down. Protein concentration was then assessed

by measuring absorbance at 295 nm. Measurements were performed in triplicate and the results are presented together with their standard deviation.

1.5.4 Dynamic light scattering

Measurements were performed with a Malvern CGS-3 (Langen, Germany) apparatus equipped with a He-Ne laser with a wavelength of 632.8 nm. The measurements were performed at 25°C at an angle of 90°. The experimental intensity autocorrelation function was measured and analyzed by the CONTIN routine as described elsewhere.¹⁹¹ DLS measurements were repeated at least ten times for 3 replicates and the results are presented together with their standard deviation. R_h refers to the mean apparent R_h calculated for each population in the CONTIN histogram.

1.5.5 ζ -potential

A Malvern Zetasizer Nano ZS instrument (Worcestershire, UK) was used to determine the electrophoretic mobility of Lyz and PPCs using Laser Doppler Velocimetry technique. The ζ -potential was calculated using Henry's equation.¹⁹² Measurements were performed in triplicate and results are presented together with their standard deviation which was always lower than 0.5 mV.

1.5.6 Quartz crystal microbalance with dissipation monitoring

Commercially available 4.95 MHz AT-cut gold-coated quartz sensors (purchased from Q-Sense, Stockholm, Sweden) were used. These sensors were washed with H₂O₂/H₂SO₄ 1:3 (v:v) solution, successively rinsed with water and ethanol, then exposed to UV-ozone prior to measurement. Layer growth on gold-coated sensors was monitored using a QCM-D system (Q-Sense E4 system, Q-Sense, Stockholm, Sweden). The solution flow rate and temperature were respectively set at 20 $\mu\text{L min}^{-1}$ and 20 °C. Layers were constructed by successive adsorption steps from solutions. For all experiments, two bilayers of PAH (Mw=17,500 g mol⁻¹, Sigma-Aldrich, Steinheim, Germany) alternately adsorbed with high molar mass PSS (PSS_h, Mw=70,000 g mol⁻¹, Sigma-Aldrich, Steinheim, Germany) were constructed

on the gold-coated sensor from PAH and PSS_h solutions (0.5 g L⁻¹). Then, five different multilayers were built on this bilayer, with the following solutions: PAH (0.5 g L⁻¹), PSS_h (0.5 g L⁻¹), PSS_i (0.14 mM negative charge), Lyz (0.07 mM positive net charge), PPCs^{0.5} (Lyz 0.07 mM positive net charge and PSS_i 0.035 mM negative net charge), PPCs² (Lyz 0.07 mM positive net charge and PSS_i 0.14 mM negative net charge). Each adsorption step of 40 min was followed by rinsing for 20 min with pure buffer. The changes in frequency and D were recorded and the results for the 7th overtone are presented here.

1.5.7 X-ray photoelectron spectroscopy

XPS measurement were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) using the procedure described elsewhere.²⁹

1.5.8 Atomic force microscopy

The same protocol as for QCM-D was applied for multilayers preparation except that LbL deposition was performed on glass coverslips by complete immersion in the corresponding solution. Adsorption steps for the [PAH-PSS_h]₂ cushion lasted 10 min while other adsorption steps lasted 40 min. Each adsorption was followed by rinsing three times with buffer. AFM images were acquired with a Dimensions Icon AFM from Bruker (Santa Barbara, CA, USA) operated in contact mode in air. Non-conductive silicon nitride tips from Veeco, with a spring constant of 0.32 N m⁻¹, were used. Layer scratching was realized by scanning a 2×2 μm² area at a scan rate of 2 Hz with a loading force set at 230 nN. Then, the scratched area was imaged (5×5 μm²) by scanning at a rate of 0.75 Hz and with the imaging force kept around 11 nN. Thicknesses were obtained by averaging three scratching tests on at least two different samples, and data were pooled to compute standard deviation.

1.5.9 Time-of-Flight Secondary Ion Mass Spectrometry

SIMS in the organic depth profile mode was performed using a TOFSIMS 5 (IONTOF GmbH, Münster, Germany) time-of-flight secondary ion mass spectrometer. The instrument is equipped with an Ar gas cluster ion beam (Ar-GCIB) as sputtering source and a liquid metal ion gun (Bi) as analytical

source both mounted at 45° with respect to the sample surface. The time-of-flight mass analyzer is perpendicular to the sample surface. Depth profiles were carried out in the 'non-interlaced' mode, consisting by period of 3.28 s of 30 keV Bi₅⁺ primary ions pulses for SIMS analysis followed by periods of 3.28 s of continuous Ar-GCIB ion sputtering. Then, a 8.44 s pause is performed during which low energy electrons are sent to the sample in order to recover the initial surface potential. The Ar-GCIB ion source was operated at 5 keV with a d.c. current of 0.35 nA. The Ar-GCIB cluster distribution was centred on Ar₃₀₀₀⁺. For depth profiling, the focussed Ar-GCIB beam of primary ions was rastered over an area of typically 450 x 450 μm². A pulsed beam of 30 keV Bi₅⁺ ions (a.c. current of 0.05 pA) was employed to provide mass spectra from a 200 x 200 μm² area in the centre of the sputter crater. Charge compensation was done by electron flood gun (E_k = 20 eV). All data analyses were carried out using the software supplied by the instrument manufacturer, SurfaceLab (version 6.5).

1.5.10 Bicinchoninic acid assay

Protein amount was assessed by BCA assay (Thermo scientific, Rockford, USA). Multilayers were directly grown in a 96-well plate following the same procedure as for surfaces used for AFM. Multilayers constructed by the sequential adsorption of PPCs² components were also built, *i.e.* [PAH-PSS-Lyz-PSS]_n with solutions having the same concentrations as for PPCs² formation. 20 μL of SDS (1%) was added to solubilize proteins from multilayers. The working reagent (200 μL) was then added and incubated 60 min at 60 °C before absorbance was measured at 562 nm (A₅₆₂). The quantification limit obtain with this procedure is 70 ng cm⁻².

1.5.11 Enzymatic activity tests

Multilayers were directly grown in a 96-well plate following the same procedure as for AFM. Lyz was also adsorbed alone by adding 200 μl of Lyz solution (0.07 mM positive net charge) and incubating for 40 min before washing 3 times with buffer. 20 μL of (GlcNAc)₃-MeU (0.1 g L⁻¹, Steinheim, Germany) in dimethyl sulfoxide (DMSO)/water 2:1 (v:v) stock solution were mixed with 180 μL of acetate buffer (10 mM) at pH 5 and I adjusted to 10

mM with NaCl, and added to each well containing multilayers or adsorbed Lyz. The plate was kept under agitation (110 rpm) for 24h at 40 °C. 180 μ L of reactional media were then transferred to a black 96-well plate and mixed with 50 μ L of 50 mM glycine buffer at pH 10.6 (adjusted with NaOH). Fluorescence (Tecan Infinite 200 Pro, Männedorf, Switzerland) was then measured with excitation and emission wavelengths respectively set at 360 nm and 450 nm.¹⁸⁹ The specific enzymatic activity was computed based on the Lyz mass measured using the BCA assay and the fluorescence of MeU (Sigma-Aldrich, Steinheim, Germany) as a function of its concentration. Results are presented as μ mol of MeU produced per mg of Lyz per minute.

The inner walls of a 3D-printed reactor were coated with multilayers. The surface developed by the reactor was estimated to be around 320 cm² (see below for details on 3D printing parameters). The same protocol as for QCM-D and AFM was applied for multilayer preparation except that LbL deposition was performed by recirculating (40 min) the coating solution (14 mL) in the reactor and by passing through buffer (35 mL) for washing steps. Enzymatic activity was recorded as the decrease in A_{450} of a *Micrococcus lysodeikticus* (1 g L⁻¹, pH 5, acetate 10 mM, 100 mM I) suspension circulating at 5 mL min⁻¹ through the reactor.¹⁹⁰ The enzymatic reaction was first recorded at 4 °C and then at 40 °C. Enzymatic activity was assessed based on the slope between an A_{450} of 1.2 and 0.6. Measurements were performed in triplicate and the result is presented together with its standard deviation.

1.5.12 3D-printed reactor for the measurement of enzymatic activity

The 3D-printed reactors were built by fused deposition modelling in Carbon Black poly(lactic acid) (Machines-3D, France) with a Replicator 2X 3D printer (Makerbot, Germany). The nozzle and the plate were respectively heated to 220 °C and 60 °C. Cylinders of 15 mm diameter and 70 mm length were printed with one shell and a linear infill of 30%. Hence, the developed surface was estimated to be around 320 cm². The reactors were inserted and sealed into a thermoretractable sheath and connections with tubing were insured by A-Lok reducing unions placed at both ends (see Figure IV.7). After coating with multilayers, they were placed vertically into a thermostatic bath and a *Micrococcus lysodeikticus* (1 g L⁻¹) suspension was circulated at 5 mL min⁻¹

from a buffering vessel to the reactor (from the bottom to the top, i.e. orange arrow in Figure S3). A_{450} of the suspensions was then recorded at the reactor outlet in a continuous flow spectrophotometric cell. The microorganism suspension was recirculated through the buffering vessel, the reactor and the spectrophotometric cell for 400 min.

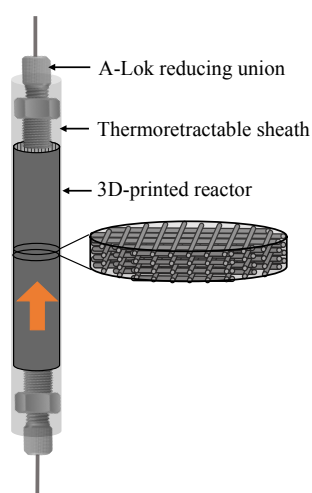
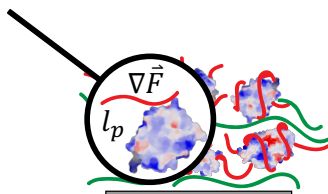


Figure IV.7 – Scheme of the 3D-printed reactor and representation of the infill pattern. The orange arrow shows the flow direction.

2. Integrating proteins in Layer-by-Layer assemblies independently of their electrical charge



In order to extend the PPCs-based LbL assembly to other proteins and to evidence the best conditions of building, it is substantial to investigate the construction mechanism. In this Chapter, Lyz was thus complexed with PSS_i at different I and pH values. The PPCs size and electrical properties were investigated and the forces driving complexation were elucidated, in the light of computations of PE conformation, with a view to further unravel LbL construction mechanisms. QCM-D and AFM were used to monitor the integration of PPCs compared to the one of bare protein molecules in LbL assemblies, and colorimetric assays were performed to determine the protein amount in the thin films. Layers built with PPCs show higher protein contents and hydration levels. Very importantly, the results also show that LbL construction with PPCs mainly relies on standard PE-PE interactions, independently of the charge state of the protein, in contrast to classical bare protein assembly with PEs.

vander Straeten, A.; Bratek-Skicki, A.; Jonas, A. M.; Fustin, C.-A.; Dupont-Gillain, C. Integrating Proteins in Layer-by-Layer Assemblies Independently of Their Electrical Charge. *ACS Nano* 2018, 12 (8), 8372–8381.

2.1. PPCs² properties and formation mechanism

Lyz was complexed with PSS_I at pH 3.0, 5.0, 7.5 or 9.6 and $I \approx 0, 10, 200$ or 800 mM (Figure IV.8a) starting from solutions with a (-)/(+net) charge ratio of 2. The charge ratio was computed as the ratio of PSS_I charge, *i.e.* -36, to the net structural charge of Lyz as a function of pH, *i.e.* +6.0, +8.0, +9.5 and +17 respectively at pH 9.6, 7.5, 5.0 and 3.0.¹⁸¹ When pH and I conditions are mentioned, they are valid for the building of PPCs as well as for the measurement condition.

The turbidity of the solution was first measured to confirm the presence of PPCs² (see Figure IV.8b). The turbidity of the PPCs² suspension highly depends on pH and I . At $I \approx 0$ mM, no turbidity was measured, *i.e.* $A_{450\text{ nm}} = 0.00$, in all pH conditions except at pH 7.5. When I increases, a non-monotonic dependence of $A_{450\text{ nm}}$ to $I^{1/2}$ is observed, peaking at midrange values (*i.e.*, $I = 200$ mM). The effect of pH on A_{450} is also non-monotonic with a maximum found at pH 7.5. The pH of a PPCs² suspension at $I \approx 10$ mM was also decreased or increased by NaOH or HCl addition. The turbidity was measured as a function of pH (Figure IV.8c). It is worth mentioning that the same values were obtained whatever the titration direction. Except at pH values above 10, the solution was turbid, with the highest turbidity recorded in a pH range between 6.0 and 9.5. These results suggest that PPCs² were formed in all conditions above $I \approx 0$ mM and that PPCs² with a wide variety of size and shape were obtained. At pH 3.0, 5.0 and 9.6, and $I \approx 0$ mM, no turbidity was measured, suggesting either that PPCs² are not formed, or remain soluble, or do not scatter enough light to be detected.

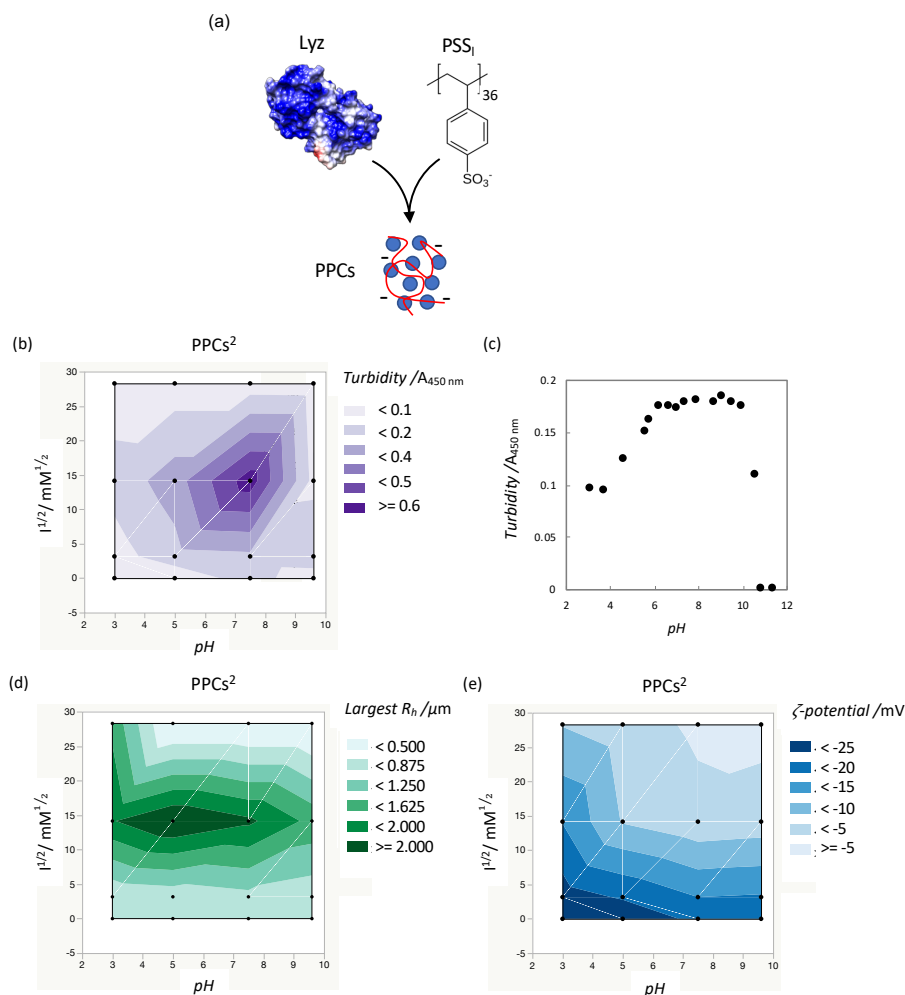


Figure IV.8 – (a) Schematic representation of PPCs² formation. Blue circles represent Lyz molecules while red lines represent PSS₁ chains. (b) Isoresponse map of the turbidity as a function of pH and I^{1/2}. The turbidity is defined as the A₄₅₀ of PPCs² suspensions (n=1). (c) Turbidity of a PPCs² suspension as a function of pH at I ≈ 10 mM (n=1). (d) R_h of the largest population of PPCs² measured by DLS (n=3). (e) ζ-potential of PPCs² (n=3). In (b), (d) and (e), results are presented as isoresponse maps depending on pH and I^{1/2}. I^{1/2}, used for the y-axis, is inversely proportional to κ⁻¹. Black dots show mean values of size and ζ-potential measurements (n=3).

DLS was used to assess the size of PPCs² at different pH and I. R_h of the largest detected population is presented in Figure IV.8d depending on pH and on I^{1/2}. The κ⁻¹, which indicates the distance over which electrostatic interactions are significant, is proportional to the inverse of I^{1/2}. Maps and charts built with

$I^{1/2}$ as a scale can thus be interpreted in terms of κ^{-1} . The pH has not much influence on the size of the largest PPCs². At high pH, PPC² size tends to decrease, while it tends to increase at medium pH. A non-monotonic dependence on $I^{1/2}$ is observed, with R_h values increasing, up to more than 2 μm , from $I \approx 0$ to 200 mM (*i.e.*, from 0 to 14.1 $\text{mM}^{1/2}$) and then decreasing from $I=200$ to 800 mM (*i.e.* from 14.1 to 28.3 $\text{mM}^{1/2}$).

ζ -potential measurements were performed on PPCs² depending on pH and I (Figure IV.8e). The PPCs² are negatively charged over the explored ranges of pH and I . Nevertheless, the surface electrical potential is more negative when the complexes are formed at low pH and low I . For example, the ζ -potential measured for PPCs² at pH 9.6 and $I=800$ mM is only -3.4 ± 0.4 mV, while at pH 3 and $I \approx 0$ mM, it is -29.1 ± 0.9 mV. PPCs² made one week before measurement showed exactly the same charge as when prepared just prior to measurement (see Figure IV.9). This indicates that the PPCs² are stable for at least one week. As PPCs² are negative for all tested pH and I conditions, they can thus be alternately adsorbed with PAH, a polycation, in order to build PPCs²-based multilayers.

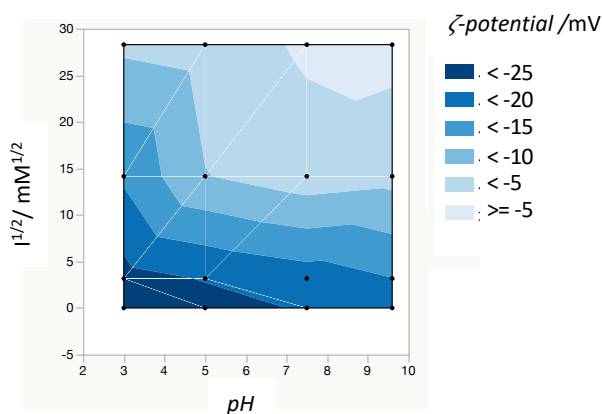


Figure IV.9 – Isoresponse map of PPCs² charge evaluated by zeta potential measurements one week after PPCs formation, as a function of pH and $I^{1/2}$. Black dots show mean values of experimental data ($n=3$).

Understanding the molecular forces driving complexation is crucial to shed light on PPCs² morphology, and therefore on the way they can interact with PEs to form a multilayer. Therefore, PSS₁ flexibility and Lyz electrical surface

potential were computed as a function of pH and I, to enable the interpretation of the data presented in Figure 1 and understand the way PPCs² interact with PAH to form a multilayer.

PSS_I is a strong polyanion and its degree of charge does not depend on pH. Hence, its conformation is not influenced by pH either, except for the contribution of protons and their counterions on I. The effect of I on PSS_I was evaluated by computing the l_p and the average root-mean-square distance between chain ends (R_E) based on the Odijk-Skolnick-Fixman theory. PSS_I conformation can be computed by analytical models that predict a power-law dependence on κ^{-1} of the distance over which a correlation of orientation exists between monomers units, *i.e.*, l_p . The Odijk-Skolnick-Fixman theory, which is based on the Debye-Hückel approximation (Equation (IV.1)), decomposes l_p into a bare l_p^0 (persistence length of a chain without electrostatic interactions) and an electrostatic persistence length l_p^e according to Equation (IV.1),^{193–196}

$$l_p = l_p^0 + l_p^e = l_p^0 + \frac{l_B \tau^2}{4\kappa^2} \quad (\text{IV.1})$$

where $l_B \approx 0.71$ nm in water at 20°C measures the distance between two unscreened ionized charges for which the electrostatic energy equals kT , τ is the linear charge density of the polymer chain and κ is the reciprocal Debye screening length. Due to counter-ion condensation, $\tau = 1/l_B$. The κ^{-1} can be computed considering the added NaCl, the Na⁺ released from PSS (35% of PSS is ionized as the distance between ionizable functions on the polymer (0.25 nm) over the average distance between charges l_B (0.71 nm) gives 0.35), sulfonate, proton activity and ions brought by pH adjustment with a strong monovalent acid or base. At pH 7.5, l_p is 73.9, 4.3, 1.2 and 1.0 nm for, respectively, I $\approx$ 0, 10, 200 and 800 mM. The R_E distance between chain ends can be found according to Equation (IV.2),¹⁹⁷

$$R_E = \left[2l_p \left(L - l_p \left(1 - e^{-\frac{L}{l_p}} \right) \right) \right]^{0.5} \quad (\text{IV.2})$$

where L is the total contour length of PSS which is equal to the length of a monomer in the direction of the polymer backbone multiplied by the average number of monomers. Computed values of R_E and κ^{-1} as a function of are presented in Figure 2a together with the protein size (dashed blue line) and l_B (dashed black line). It appears that PSS_i is in the rod-like conformation at low I , *i.e.*, $I \approx 0$ mM and 10 mM. Indeed, the obtained R_E are respectively 8.8 nm and 6.7 nm in these conditions, which is close to the total PSS_i contour length of 9 nm. At higher I , PSS_i chains form random coils and R_E decreases.

The computed values of l_p , R_E and κ^{-1} at pH 7.5 are presented for PSS_i as a function of $I^{1/2}$ in Figure IV.10a, together with the Lyz diameter (dashed blue line) and l_B (dashed black line). PSS_i is in a rod-like conformation at low I , *i.e.*, $I \approx 0$ mM and 10 mM, since the obtained R_E values are 8.8 nm and 6.7 nm, respectively, which is close to the total PSS_i contour length of 9 nm. At higher I , the PSS_i chains form random coils, with R_E decreasing to 4.3 nm and 4.1 nm. Values of l_p are similarly very much increased at low I ($l_p = 70$ nm at $I \approx 0$ mM), but are close to l_B at $I \geq 200$ mM. The Debye screening length κ^{-1} , which is proportional to the reciprocal of $I^{1/2}$ and is presented in Figure IV.10a at pH 7.5, reaches values lower than the Lyz diameter and l_B at, respectively, $I = 25$ mM (*i.e.*, $5 \text{ mM}^{1/2}$) and $I = 200$ mM (*i.e.*, $14.1 \text{ mM}^{1/2}$).

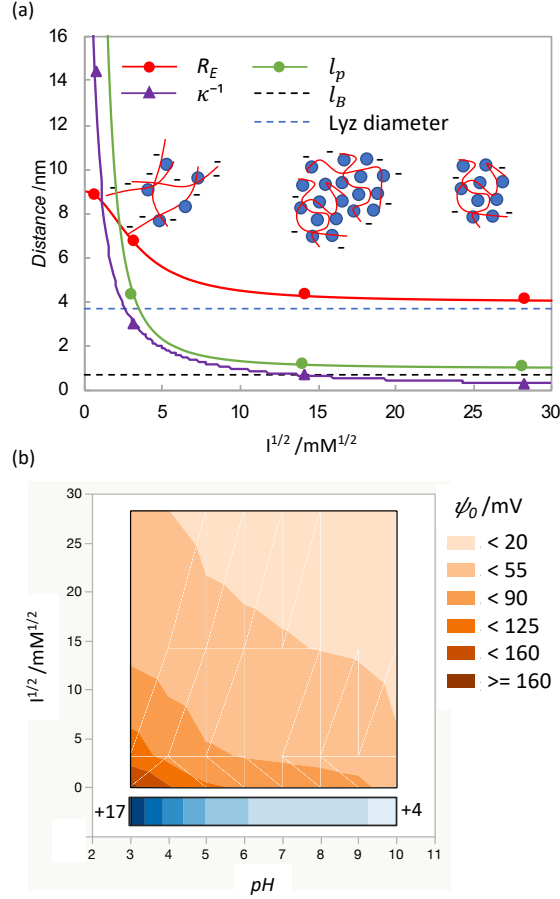


Figure IV.10 – (a) Schematic representation of the PPCs² as a function of I . Blue circles represent Lyz molecules while red lines represent PSS_I chains. Computed κ^{-1} at pH 7.5 (purple line) and R_E and l_p of PSS_I (red and green lines respectively) as a function of $I^{1/2}$ are presented together with the diameter of Lyz (dashed blue line) and l_B (dashed black line). (b) Isoresponse maps as a function of pH and $I^{1/2}$ of Lyz electrical surface potential (ψ_0). The Lyz total net charge between pH 3 and 10 is presented in the blue gradient scale.

Proteins are polyampholytes and the number of charges per Lyz molecule (q_l) was computed as a function of pH based on its a.a. sequence and structure (PDB file: 1HEL) using PDB2PQR.¹⁹⁸ Results are presented in blue as a gradient scale (Figure IV.10b). The Lyz surface potential (ψ_0 ; Equation (IV.3)) was estimated as a function of I assuming that Lyz is a sphere with a radius of 1.85 nm (R_s), using.^{199,200}

$$\psi_0 = \frac{q_l}{4\pi\epsilon_0\epsilon_r R_s(1 + \kappa R_s)} \quad (\text{IV.3})$$

where ϵ_0 is the vacuum permittivity, ϵ_r is the relative static permittivity of water and κ is the reciprocal of the κ^{-1} . ψ_0 is presented in Figure 2b as an isoresponse map of pH and $I^{1/2}$, showing that ψ_0 decreases as pH and I increase. For instance, $\psi_0=169$ mV at pH 3 and $I^{1/2}\simeq 0$ mM^{1/2}, while $\psi_0=6$ mV at pH 10 and $I^{1/2}=14.1$ mM^{1/2}, *i.e.* $I=200$ mM. The IEP was experimentally found to be reached at pH 11.35, in line with the low ψ_0 values predicted at high pH.³¹

The size variation of PPCs² with pH can be explained by the increasingly positive Lyz charge as pH decreases. At low pH, the high coulombic attraction between the highly positively charged Lyz molecules and negatively charged PSS_I chains is favorable to PPC² formation. Inversely, at a pH close to the Lyz IEP (pH 11.35), the lower net charge of the protein is less favorable to PPC² formation. This is confirmed by simulations that show that at fixed I , *i.e.* at fixed l_p since PSS_I is a strong polyacid, the probability of binding Lyz to PSS monomers increases when the net protein charge increases.¹⁹⁹ It should be noted that, although a low concentration of the different buffers was used to obtain different pH values, it results in the presence of different counter ions that may also influence complexation.

The non-monotonic PPCs² size measured as a function of I (Figure IV.8d) can be explained by a balance between the increasingly compliant conformation of PSS_I and less effective electrostatic interactions as I rises. When R_E decreases, *i.e.* PSS_I flexibility increases, with increasing I (Figure IV.10a), it leads to a possible better accommodation of PSS_I to Lyz surface charges with therefore a higher number of PSS_I monomers per chain interacting with each protein molecule, as sketched in Figure 2a.¹⁹⁹ On the other hand, electrostatic interactions decrease with increasing I due to charge screening (see κ^{-1} in Figure IV.10a and ψ_0 in Figure IV.10b).

When κ^{-1} reaches a value lower than l_B , *i.e.* at $I>200$ mM (Figure IV.10a), one can expect that the electrostatic contribution to complexation becomes

negligible and that other forces such as van der Waals forces and hydrophobic effects govern protein-PE interactions.²⁰¹ The Debye-Hückel approximation is actually not valid at high I and other models than Odijk-Skolnick-Fixman must then be developed. The high PPCs² size measured at $I=800$ mM and low pH might be due to favorable hydrophobic interactions between PSS_i and Lyz.²⁰² It is worth mentioning that stiffening of PE at high I has been reported.²⁰³ This led to a non-monotonic dependence of l_p on I , which could further explain a non-monotonic protein-PE binding constant and PPCs² size as a function of I .

Comparison of the two maps describing the surface electrical properties of PPCs² (Figure IV.8e) and of Lyz (Figure IV.10b) shows very similar trends, with negative and positive values for PPCs² and Lyz respectively. When Lyz surface potential increases, the resulting PPCs² take an increasingly negative charge. The PSS_i stiffening at low I leads to a higher overcompensation of Lyz charges since PSS_i is less likely to accommodate to the protein surface charge distribution. This explains the higher PPCs² negative charge at lower I (Figure IV.8e). The higher overcompensation of charges at low I is represented by the PPCs² sketch in Figure IV.10a. The accommodation of PSS_i to Lyz can also be interpreted in terms of l_p and assuming that Lyz is a sphere with a diameter that gives realistic charge-charge interactions, *i.e.* 3.7 nm (see Figure IV.10a).¹⁹⁹ l_p is higher than the Lyz diameter at $I \approx 0$ mM and 10 mM ($l_p=73.8$ and 4.26 nm respectively), while it is lower than the Lyz diameter at $I=200$ mM and 800 mM ($l_p=1.16$ and 1.04 nm respectively). This is further supported by Monte-Carlo simulations that showed a decreasing density of PE in close vicinity of Lyz as I decreases.¹⁹⁹ This is sketched in Figure IV.10a where Lyz density in PPCs² is lower at low I .

The interplay between electrostatic interactions and polymer flexibility results in PPCs² with very different surface charge and morphology as a function of I . Based on our data and on the existing literature, it appears that PPCs² with very different morphologies can be obtained as a function of pH and, to a larger extent, of I . As sketched (Figure IV.10a), negative PPCs² were always obtained, albeit with a variable protein density and polymer conformation. This will enable us to understand, in the following, how these

different PPCs² can be alternately adsorbed with PAH in order to construct a multilayered system.

2.2. PPCs² LbL assembly mechanism

The PPCs² constructed at pH 3.0, 5.0, 7.5 or 9.6 and $I \simeq 0, 10, 200$ or 800 mM were alternately adsorbed with PAH on a previously constructed [PAH-PSS_h]₂ cushion (as represented in Figure IV.17b). It is worth to mention that conditions of PPCs² formation were kept for multilayer building. For the sake of comparison to the common way used to immobilize proteins using the LbL method, bare Lyz molecules were alternately adsorbed with PSS_h (as represented in Figure IV.17a). The Lyz content of the multilayers was quantified by a BCA assay after five protein adsorption steps in all pH and I conditions for both [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ systems (Figure IV.11a and c). Multilayer construction at pH 3 or 7.5 and $I \simeq 0$ or 200 mM was moreover monitored by QCM-D and the masses deposited at each step were computed based on the measured frequency shift and the Sauerbrey equation (Figure IV.12).²⁰⁴ Finally, topographic images of silicon wafers coated with [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ constructed at pH 3 or 7.5 and $I \simeq 0$ mM were obtained by AFM in tapping mode (Figure IV.13) and the root-mean-square roughness (R_{RMS}) was extracted from these images (Figure IV.14).

For [Lyz-PSS_h]_{4.5}, both the amount of Lyz (Figure IV.11a) and the total hydrated film mass (Figure IV.12a and b) increase when pH decreases. This result is explained by an increasing Lyz net charge with decreasing pH, *i.e.*, respectively +6.0, +8.0, +9.5, +17.0 at pH 9.6, 7.5, 5.0 and 3.0 (blue gradient scale in Figure IV.11b and Figure IV.10b), which thus favors electrostatic interactions.¹⁸¹ The same effect of pH on protein immobilization was already reported for BSA and PEI multilayers.⁵¹ Note that PSS is a strong PE whose charge density is not altered by pH. Higher Lyz amount is found at midrange of I values, *i.e.* 200 mM. In line with DLS results, the non-monotonic dependence on I can be explained by the balance between l_p , κ^{-1} , van der Waals forces and hydrophobic effect.²⁰⁵ l_p has indeed been shown to be a critical parameter for LbL assembly on nanoparticles.²⁰⁶ As represented in the scheme in Figure IV.17a, at high I value, the electrostatic screening of intrapolymeric charges favors more loopy conformations of the PE (PSS_h, in

red). These loops favor charge overcompensation upon build-up, which improves protein integration into multilayers. At low pH, Lyz is uniformly positively charged (see far left molecular structure in Figure IV.11b) while negatively charged patches are present at high pH (see far right molecular structure in Figure IV.11b). These negative patches are unfavorable for LbL assembly with a strong polyanion, especially at low I due to electrostatic repulsion. This is confirmed by QCM-D data that show almost no film mass increase between the first and the last protein adsorption steps carried out at pH 7.5 and I=0 mM (Figure IV.12b). Topographic images acquired by AFM (Figure IV.13a) show that [Lyz-PSS_h]_{4.5} multilayers are smooth. This is confirmed in Figure IV.14, with R_{RMS} values of the order of magnitude of Lyz radius (≈ 1.85 nm).

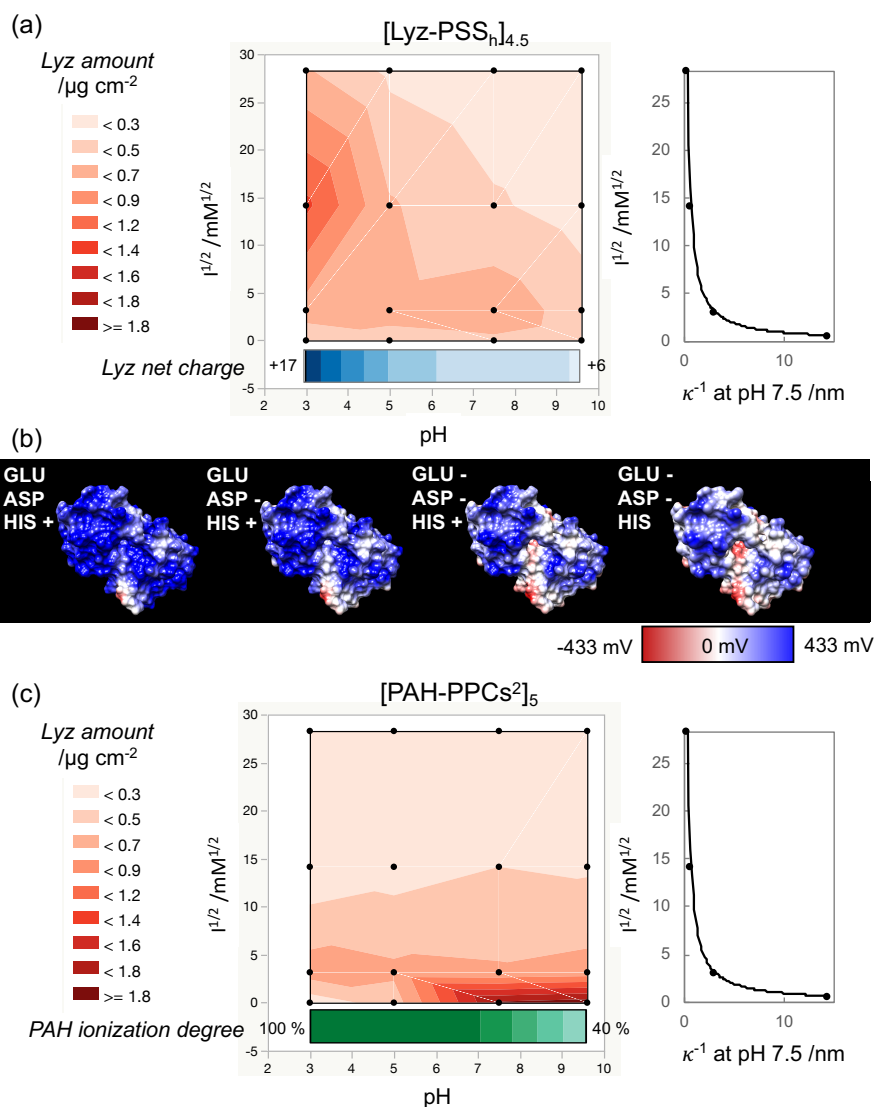


Figure IV.11 – Lyz amount within (a) [Lyz-PSS_n]_{4.5} or (c) [PAH-PPCs²]₅ multilayers presented under the form of isoresponse maps as a function of pH and $I^{1/2}$. Black dots show mean values of experimental data ($n=3$). Lyz net charge (a) and PAH ionization degree¹⁶⁵ (c) are represented respectively in blue and green as a function of pH as a gradient scale. κ^{-1} as a function of $I^{1/2}$ is also presented at pH 7.5 for the ease of data interpretation. (b) Molecular structure of Lyz with coulombic surface coloring. From left to right: aspartic acid, glutamic acid and histidine residues were successively deprotonated. The electrostatic potential scale ranges from -433 mV (red) to 433 mV (blue). These molecular structures were produced using the UCSF Chimera package and Lyz structure (P00698).^{207,208}

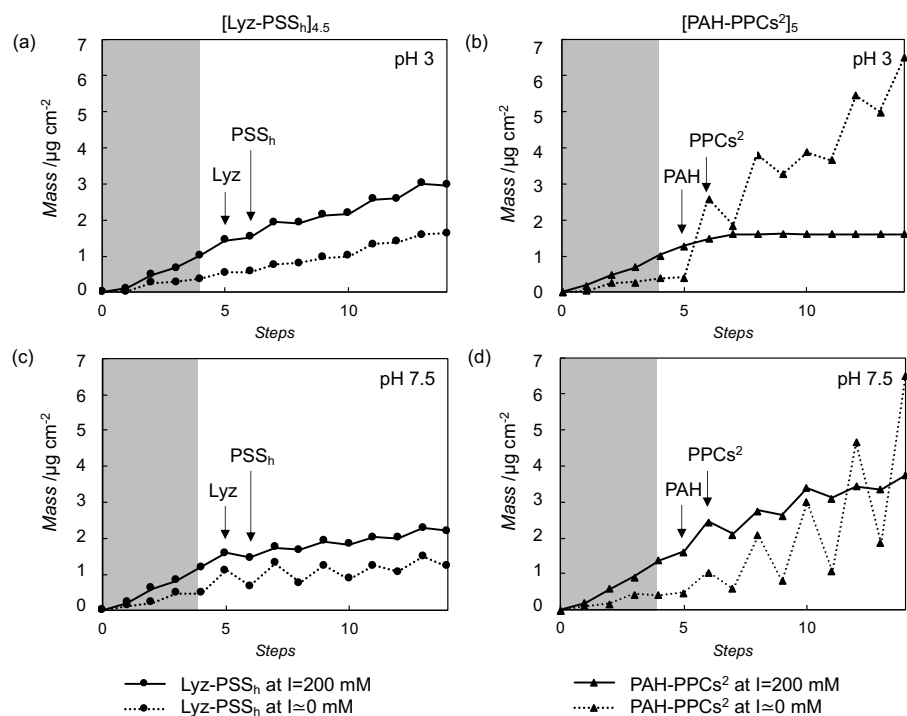


Figure IV.12 – Total hydrated mass measured by QCM-D after adsorption of a cushion of two PAH-PSS_h bilayers $[\text{PAH-PSS}_h]_2$ (shaded area) followed by either (a and b) $[\text{Lyz-PSS}_h]_5$ or (c and d) $[\text{PAH-PPCs}^2]_5$. Multilayers were constructed at pH 3.0 (a and c) or 7.5 (b and d) and I≈0 mM (dotted line) or 200 mM (solid line).

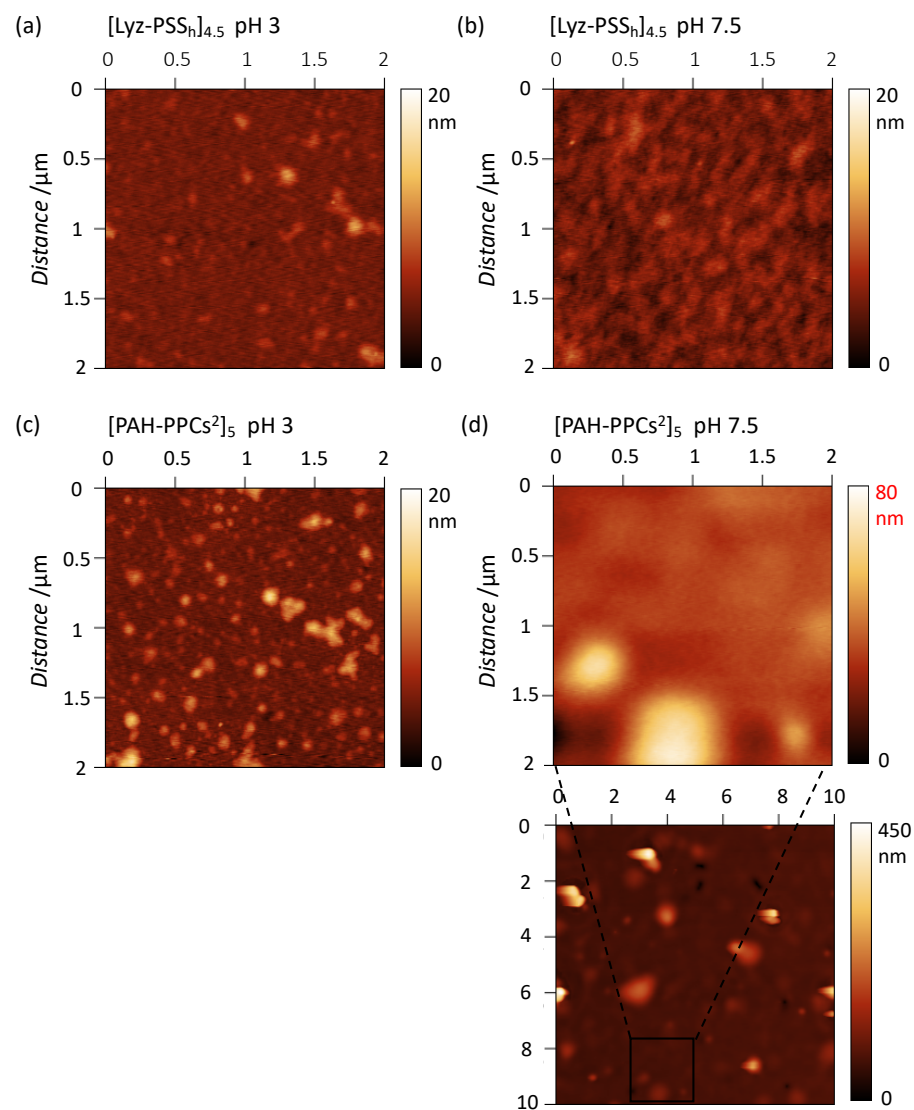


Figure IV.13 – Topographic images (2 μm width and Z=20 nm except for b.2: 2 μm width and Z=80 nm) of silicon wafers coated with a [PAH-PSS_n]₂ cushion followed by either (a and b) [Lyz-PSS_n]_{4.5} or (c and d) [PAH-PPCs²]₅. Images were obtained by AFM in tapping mode. Multilayer building was performed at I=0 mM and pH 3.0 (Figure IV.12a and c) and 7.5 (Figure IV.12b and d).

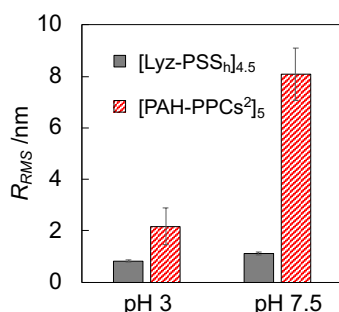


Figure IV.14 – R_{RMS} extracted from AFM images ($n>3$) and presented as a function of pH for [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ constructed at $I \approx 0$ mM on a silicon wafer (error bars show the standard deviation $n=3$).

For multilayers integrating PPCs², *i.e.* [PAH-PPCs²]₅, the amount of immobilized Lyz is close to zero when the multilayer is constructed at $I \geq 200$ mM. It does moreover not depend on pH at $I=10$ mM, and it depends on pH at $I=0$ mM (Figure IV.11c). This result suggests that I is the main parameter that drives LbL assembly. In multilayers constructed at $I \geq 200$ mM, the low immobilized Lyz amount and the absence of LbL growth of [PAH-PPCs²]₅ (Figure IV.11c and Figure IV.12c and d) can be attributed to the screening of PE charges at high I . This is in contrast with the [Lyz-PSS_h]_{4.5} system that benefits from the better PSS_h adaptation to Lyz surface charge distribution at $I=200$ mM (Figure IV.17a). In multilayers constructed at $I=10$ mM, the PSS_i shell of the complexes regulates the interaction with PAH, as supported by the negative ζ -potential measured for PPCs² (Figure IV.8e). Indeed, if LbL assembly was driven by protein-PE interactions, it would lead to a variation of the immobilized Lyz amount with pH (as witnessed with the [Lyz-PSS_h]_{4.5} system), since the Lyz charge varies with pH (Figure IV.11b).

When multilayers are constructed at $I=0$ mM with PPCs², a low Lyz amount is immobilized between pH 3.0 et 5.0, while it reaches $1.8 \mu g cm^{-2}$ of Lyz between pH 7.5 and 9.6, which is significantly higher than the amount reached for any condition with the [Lyz-PSS_h]_{4.5} system (Figure IV.11c). Unlike the Lyz amount, the hydrated film mass is not influenced by pH and the film weighs *ca.* $6.5 \mu g cm^{-2}$ when $I=0$ mM at both pH 3.0 and 7.5 (Figure IV.12c and d). The ratio of Lyz mass to the total hydrated film mass is thus 0.016 and

0.277 for [PAH-PPCs²]₅ constructed at $I=0$ mM and pH 3.0 or 7.5, respectively, showing that very different layer compositions are obtained.

The multilayer constructed at $I \approx 0$ mM and pH 3.0 is smooth (Figure IV.13c) and allows the thickness to be measured by ellipsometry. A thickness of only 5 nm was obtained, measured with a Nanofilm EP4 Accurion ellipsometer). Assuming a dry film density of 1.2 g cm^{-3} , the dry mass is only $0.6 \text{ } \mu\text{g cm}^{-2}$, indicating that the multilayer constructed at $I \approx 0$ mM and pH 3 contains around 90% of water before drying. This is further confirmed by the high D measured by QCM-D, *i.e.* $6.6 \cdot 10^{-5}$. This is the opposite of what is expected for a LbL construction carried out in the same conditions but only with PE. Indeed, the PE stiffening at low I causes lower charge overcompensation, creating usually thinner multilayers,²⁰⁹ as confirmed by the very low hydrated mass of a [PAH-PSS_i]₅ control multilayer, also constructed on a [PAH-PSS_h]₂ cushion at $I \approx 0$ mM and pH 3 (Figure IV.15). At low pH and low I , PAH is fully ionized and electrostatic interactions are maximal and effective at long distance (see the green gradient scale for PAH ionization state and κ^{-1} as a function of $I^{1/2}$ for the electrostatic screening in Figure IV.11c). Therefore, when PAH is adsorbed on a PPCs² layer, the strong PSS_i-PAH interactions may displace the more weakly-complexed Lyz and remove it from the layer, resulting in the observed low Lyz amount included in the layers. Additionally, the saw-tooth pattern observed by QCM-D upon construction (Figure IV.12c) supports this interpretation: the addition of PAH would induce the release of previously deposited components (and/or the loss of water). This result can also be interpreted as a low PPCs² stability in these conditions of LbL construction. Lyz release could be facilitated by both its low charge density compared to PAH, and the low PSS_i accommodation to Lyz charge that would leave many free negative charges as evidenced in PPCs² constructed in these conditions (Figure IV.8a). Since DLS has shown that PPCs² are 3D objects much larger than a monolayer of PE, as sketched in Figure IV.17b at low pH, their use may artificially create loops of PSS_i at the interface. The substitution of Lyz by the highly charged PAH may keep this 3D structure intact, resulting in a thick film that has a high water content and a very low protein content, *i.e.* only $0.1 \text{ } \mu\text{g cm}^{-2}$. Such a competitive electrostatic binding in multilayers as already been observed between M31 virus particles, PAA and linear PEI.²¹⁰

This is an interesting feature as PPCs² could be used to obtain thick films at $I \approx 0$ mM with a very high water content and made of almost only PE. In such case, the protein could play the role of a sacrificial compound, that would be released as the assembly progresses.

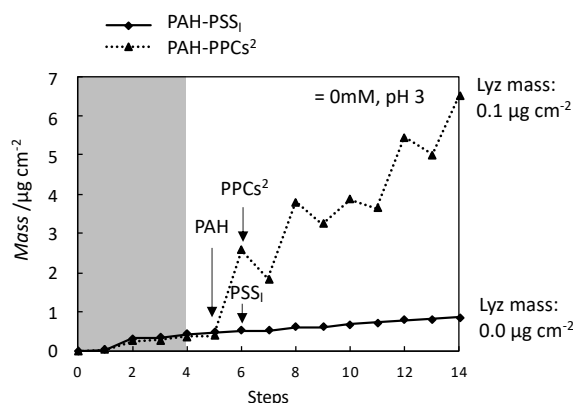


Figure IV.15 – Total hydrated mass measured by QCM-D after adsorption of a cushion of two PAH-PSS_h bilayers ([PAH-PSS_h]₂ (shaded area) followed by either PAH-PSS_I or PAH-PPCs² constructed at $I \approx 0$ mM and pH 3. The total Lyz mass measured after the last adsorption step is also presented.

In contrast, for multilayers constructed at $I \approx 0$ mM and pH 7.5 and 9.6, a very high Lyz content was found. The PAH ionization degree is of 70% and 40% at pH 7.5 and 9.6, respectively (green gradient scale in Figure IV.11c), which results in a larger population of loops and tails that favors charge overcompensation, and a lower ability to displace proteins from the multilayer.^{205,209,211} PAH low ionization degree thus increases PPCs stability when integrated in multilayers in these conditions. This is represented in Figure IV.17b by the increased number of loops made by PAH (in green) when pH increases. Similarly, higher thicknesses were predicted and experimentally witnessed in PE-PE LbL assemblies grown at low I , when one PE is fully ionized and the other one is less than 70% to 90% ionized.^{205,209,211} Moreover, an increased R_{RMS} was also observed under such circumstances. This same effect is actually observed in Figure IV.14, showing an increased R_{RMS} value with pH. Interestingly, the multilayer growth measured at pH 7.5 and $I \approx 0$ mM by QCM-D (Figure IV.12d) shows an exponential step-by-step mass increase. Additionally, high D were measured for [PAH-PPCs]₅

constructed at pH 7.5 and $I \approx 0$ mM, *i.e.*, $7.4 \cdot 10^{-5}$. This is five times higher than a $[\text{Lyz-PSS}_h]_{4.5}$ multilayer, suggesting that $[\text{PAH-PPCs}]_5$ multilayers constructed at pH 7.5 and $I \approx 0$ mM are more hydrated than similarly-constructed films based on bare Lyz molecule integration.

Interestingly, if the map of PPCs² size is compared to the one of Lyz mass immobilized in the multilayer, it appears that bigger PPCs² do not allow to immobilize a higher protein amount. As it was suggested in Chapter 1, this result suggests that either PPCs² reorganize upon LbL assembly, or that an undetected minor fraction, with a smaller R_h , is assembling.

In order to confirm that PE-PE interactions drive the LbL assembly, PPCs with a (-)/(+net) charge ratio of 0.5 (PPCs^{0.5}) were also assembled with PAH at pH 3, 5, 7.5 or 9.6 and $I \approx 0, 10, 200$ or 800 mM. Unlike all other PPCs used in this paper, a lower concentration of PSS_i, thus of negative charges, was used to form the PPCs^{0.5}. This results in the PPCs^{0.5} being positively charged, and in an increased limitation of PSS_i to complex with another PE as is it is already fully complexed with Lyz.²¹² Lyz content was quantified for multilayers constructed with PPCs^{0.5} in all pH and I conditions. The results are presented in Figure IV.16. Whatever the pH and I used for the LbL construction, no Lyz is immobilized in the multilayers. This confirms that PE-PE interactions drive the LbL assembly only for PPCs having a core-shell morphology, consisting of a negatively-charged PE corona shielding a core made of a protein/PSS complex. Such a core-shell morphology is therefore required for the successful integration of proteins in multilayers. When combined with a counter-PE of proper charge density, it allows to circumvent issues related to the charge distribution of protein molecules.

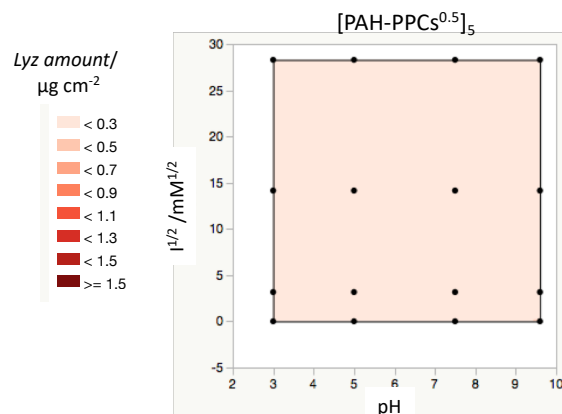


Figure IV.16 – Lyz amount within [PAH-PPCs^{0.5}]₅ multilayers presented under the form of an isoresponse map as a function of pH and I^{1/2} (n=3).

Taken together, the results thus show that [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ systems have completely different growth patterns, which enables different LbL coatings with well-controlled architecture and properties to be obtained. PPCs offer a wide range of deposition conditions to the LbL method. On the one hand, when the multilayer is constructed at I=10 mM, it allows a construction that is independent of the protein charge state over a very broad range of pH. On the other hand, when the multilayer is constructed at I≈0 mM, two distinct regimes are obtained, both depending on the counter polycation ionization degree: (1) multilayers made of almost only PE and with a high water content are obtained if the counter polycation is fully ionized (even if constructed at I≈0 mM) and (2) a large protein amount is immobilized in highly hydrated films when the counter polycation is less than 70% charged, whatever the protein charge. Immobilizing a high protein amount in hydrated films is much wanted for applications requiring high bioactivity such as drug delivery, biocatalysis and biosensing.

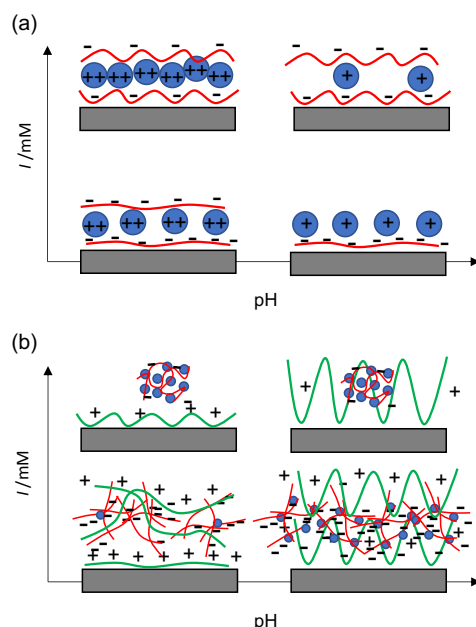


Figure IV.17 – Schematic representation of the deposition of three layers as a function of pH and I: (a) [PSS_h-Lyz] or (b) [PAH-PPCs]. When no LbL construction was observed, only two layers are represented. PSS_l and PSS_h are in red, PAH is in green and Lyz is in blue. The substrate and the [PAH-PSS_h]₂ cushion are in grey. Sizes are not at scale.

2.3. Conclusion

PPCs were obtained by complexation of Lyz with PSS. The effect of pH and I on PPCs formation was investigated by DLS and ζ -potential measurements. A model of PPCs morphology that coincides with obtained data, Lyz surface potential, PSS conformation and literature was proposed. It appears that the balance between intramolecular electrostatic repulsion within PSS and electrostatic attraction between PSS and Lyz drives PPCs formation. This results in a non-monotonic dependence of PPCs size to I, while their charge is negative over the whole pH and I range. PPCs were then integrated in multilayers by LbL assembly with PAH at different pH and I ([PAH-PPCs]₅). Bare Lyz molecules were also integrated in multilayers with PSS ([PSS-Lyz]_{4.5}). Lyz content and total hydrated film mass were measured, and topography was assessed in both cases for a wide range of pH and I values.

The protein charge highly determines LbL build-up between bare Lyz molecules and PSS. A higher protein amount is found when the protein

charge increases and at mid-range I values. Similarities with the driving forces that regulate PPCs formation were evidenced and it was shown that a high protein amount is immobilized when in conditions where a sufficiently favorable PSS conformation coexists with sufficiently attractive PSS-Lyz electrostatic interactions.

When PPCs were used to immobilize Lyz molecules in LbL assemblies, multilayers with a higher hydration level and PE amount were obtained. Importantly, the driving force for assembly was evidenced to be solely based on PE-PE interactions. Higher protein contents were obtained at low I when one of the two PEs was less than 70% charged. This observation is consistent with other data reported for protein-free LbL films. This represents a major achievement since the low conformational entropy, heterogeneous spatial distribution of charge and polyampholyte nature of proteins usually complicates the case of proteins in LbL assembly. These results give to PPCs a major importance for the development of functional biointerphases since they enable to obtain a higher protein content and a higher hydration level compared to bare protein molecules, and since LbL assembly becomes independent of protein charge, provided that PPCs morphology is carefully selected.

2.4. Materials and methods

2.4.1 PPCs preparation

All solutions were buffered with 10 mM citrate, acetate, HEPES or carbonate and pH was respectively adjusted at pH 3.0, 5.0, 7.5 or 9.6 with NaOH or HCl. The molar I was computed using²⁰⁰ $I = \frac{1}{2} \sum_{i=1}^n c_i z_i^2$, where c_i and z_i are respectively the molar concentration and the charge of ion i . I was adjusted to 10, 200 or 800 mM by adding NaCl (Merck, Darmstadt, Germany) to the buffer. The contributions of both the buffer and NaCl to I were considered. Solutions at $I \approx 0$ mM were obtained by only adding NaOH or HCl to ultrapure water until the desired pH was reached. PPCs at a (-)/(+net) charge ratio of 2 were prepared by mixing 2 volumes of a low molar mass sodium poly(styrene 4-sulfonate) (PSS_i, Mw=7,540 g mol⁻¹, Mw/Mn=1.08, Scientific Polymer,

Ontario, USA) solution (0.5 mM negative charge) with 5 volumes of a Lyz (Sigma-Aldrich, Steinheim, Germany, IEP=11.35) solution (0.1 mM net positive charge). The amount of positive charges in the system was kept constant at different pH values by increasing Lyz amount with increasing pH. Lyz charge was computed as a function of pH based on its a.a. sequence and structure (PDB file: 1HEL) using PDB2PQR.¹⁹⁸

2.4.2 ζ -potential

A Malvern Zetasizer Nano ZS instrument (Worcestershire, UK) was used to determine the electrophoretic mobility of PPCs² constructed in different pH and I conditions using the Laser Doppler Velocimetry technique. PPCs² formation conditions were kept for each measurement. The ζ -potential was calculated using Henry's equation.¹⁹² Each measurement was performed in triplicate and results are presented together under the form of an isoresponse map of ζ -potential as a function of pH and I.

2.4.3 Dynamic light scattering

Measurements were performed with a Malvern CGS-3 (Langen, Germany) apparatus equipped with a He-Ne laser with a wavelength of 632.8 nm, at 25°C and at an angle of 90°. The experimental intensity autocorrelation function was measured and analyzed by the CONTIN routine. Each DLS measurement was repeated at least ten times for 3 replicates and the results are presented as an isoresponse map of the size of the largest population detected at each pH and I. The size is expressed under the form of the mean apparent R_h calculated for each population in the CONTIN histogram. Solutions were also filtered with a 0.25 μm filter to check the disappearance of populations with a diameter higher than 0.25 μm .

2.4.4 Multilayer construction

Multilayers were constructed in 96-well polystyrene plates (well surface, including bottom and walls, is 1.5 cm² – Greiner Bio-One®) by successive adsorption steps. For all experiments, two bilayers of PAH (Mw=17,500 g mol⁻¹, Sigma-Aldrich, Steinheim, Germany) alternately adsorbed with high molar mass poly(styrene 4-sulfonate) (PSS_h, Mw=70,000 g mol⁻¹, Sigma-

Aldrich, Steinheim, Germany) were constructed from PAH and PSS_h solutions (0.5 g L⁻¹). Then, two different multilayers were built on this anchoring layer (cushion): bare Lyz molecules assembled with PSS_h, or PPCs assembled with PAH. The following solutions were used: PSS_h (0.5 g L⁻¹), Lyz (0.07 mM positive net charge), PAH (0.5 g L⁻¹) and PPCs prepared as described in *PPCs preparation* (2.4.1, p116). These solutions were buffered as described in *PPCs preparation*. Each adsorption step was carried out with 200 µL of solution. Adsorption lasted for 10 min for the construction of the cushion made of PAH and PSS_h, and 40 min for all subsequent layers. Each adsorption step was followed by three consecutive rinsing steps with the corresponding buffer.

2.4.5 Protein quantification using BCA assay

Protein amount within LbL assemblies was assessed by BCA assay (Thermo scientific, Rockford, USA). 20 µL of SDS (1%) was added to solubilize protein molecules from multilayers before quantification. The working reagent (200 µL) was then added and incubated 60 min at 60 °C before A₅₆₂ was measured. Lyz amount was determined based on a calibration curve obtained with known Lyz amounts. The high pH of the BCA buffer causes complete Lyz and PAH protonation, which results in the dissolution of the film. This is supported by the complete dissolution of PPCs measured above pH 10.8 (Figure I.8c). SDS was added to further ensure complete film dissolution.

2.4.6 Quartz crystal microbalance with dissipation monitoring

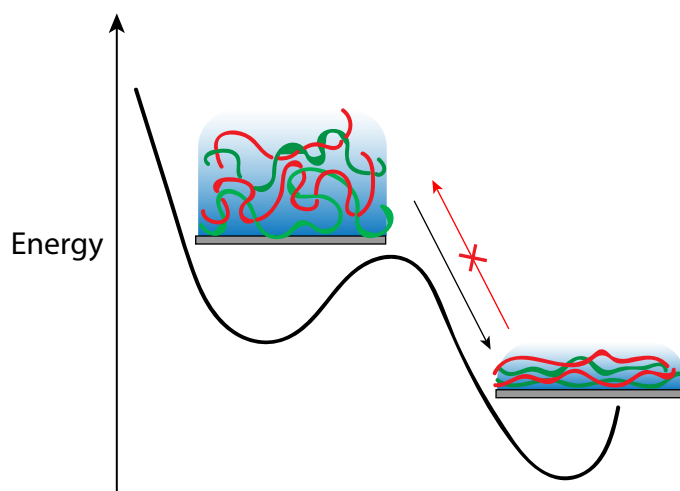
Commercially available 4.95 MHz AT-cut gold-coated quartz sensors (purchased from Q-Sense, Stockholm, Sweden) were used. These sensors were washed with H₂O₂/H₂SO₄ 1:3 (v:v) solution, successively rinsed with water and ethanol, then exposed to UV-ozone prior to measurement. Layer growth on gold-coated sensors was monitored using a QCM-D system (Q-Sense E4 system, Q-Sense, Stockholm, Sweden). The solution flow rate and temperature were respectively set at 20 µL min⁻¹ and 20 °C. Layers were constructed by successive adsorption steps from the same solutions as for multilayer construction in 96-well plate. The only difference was that each adsorption step was followed by rinsing for 20 min with pure buffer. The changes in frequency and *D* were recorded. Mass adsorbed on QCM-D

crystals was assessed by the Sauerbrey equation for the 7th overtone ; $\Delta m = -C \Delta f_7$, where Δm is the shift of adsorbed mass per surface area ($\mu\text{g cm}^{-2}$), Δf_7 the frequency shift measured for the 7th overtone (Hz), and C a constant equal to $17.7 \text{ ng.cm}^{-2}.\text{Hz}^{-1}$ for a 5 MHz crystal.²⁰⁴

2.4.7 Atomic force microscopy

Pieces of silicon wafers ($\sim 1\text{cm} \times 1\text{cm}$) were placed in 12-well polystyrene plates (Greiner Bio-One®) and multilayers were constructed by successive adsorption steps from 1 mL solutions in the conditions described in the *multilayer construction* section. Each adsorption step was followed by rinsing three times with the corresponding medium at $I \simeq 0$ mM. The coated wafers were finally left to dry at 4°C. AFM images were acquired on dry films with a Dimensions Icon AFM from Bruker (Santa Barbara, CA, USA) operating in tapping mode in air. Silicon probes (PPP-NCHR-50; resonance frequency=204 – 497 Hz) from NANOSENSORS™ were used. Topographic images were obtained by scanning a $2 \times 2 \mu\text{m}^2$ area at a scan rate of 1 Hz. R_{RMS} was extracted from the obtained images.

3. Out-of-equilibrium interfacial self-assembly of polyelectrolytes irreversibly loses water



In the previous Chapter, it was shown that Lyz could be used as a sacrificial element during LbL assembly to create highly hydrated PEMs. Using QCM-D mounted with a humidity module, we show that these PEMs are trapped in a non-equilibrium state. Their dehydration is irreversible as it causes their collapse into denser PEMs. After dehydration, the resulting PEMs share all common features with the ones constructed without the use of the Lyz as a templating agent. Interestingly, it suggests that the use of sacrificial elements in self-assemblies allows to create completely new structures that are on the edge of stability.

3.1. Introduction

As introduced in Part I, LbLs are out-of-equilibrium self-assemblies.³⁵ “Out-of-equilibrium” means that the path followed for build-up dictates the final state that is reached. If the very same PEs were tentatively adsorbed together instead of one layer at a time, a PEM would not be created. Interestingly, the layered nanoarchitecture of PEMs displays functional properties such as a high sensitivity to ambient moisture, the ability to strongly interact with light, or to release trapped molecules via an external stimulus.^{213–215} These properties are attributed to the layered structure of PEMs and therefore emerge from the path that has been followed to self-assemble the macromolecules.

The response to ambient moisture that originates in the nanolayered structure of PEMs has been under intensive investigation for the two last decades. Indeed, PEMs were shown to exhibit a measurable swelling and plasticization upon exposure to an increasing water vapor pressure.^{216,217} This swelling/deswelling behavior allowed to study the PEMs internal structure, but it also makes of them particularly well suited for humidity-sensing devices.^{213,214,218} In particular, PEMs constructed via the alternate adsorption of PAH and PSS have been intensively investigated. Lösche *et al.* demonstrated that [PAH-PSS] PEMs contain more than 40% of water when the air is saturated with water vapor.²¹⁹ The number of water molecules per PE chain can be notably high. For instance, it has been reported for [PAH-PSS] that eight water molecules are present per anion-cation complex. With respect to the swelling kinetics, Kügler *et al.* reported that hours were needed to reach equilibrium in [PAH-PSS] PEMs.²²⁰ In contrast, Wong *et al.* reported that only a few minutes were needed for the same PEM.²²¹ This difference might be due to the different salt concentration used to construct the PEMs. The swelling has been shown to be split into distinct modes. When going from low to high relative humidity (RH), a swelling transition occurs from a state where water and PEs are homogeneously distributed, to a state where water and PE are phase-separated. For instance, this transition was observed at a RH of 60% and a volume increase of 20% for PEMs made with PSS- poly(diallyldimethylammonium chloride)-N-methyl-N-

vinylacetamide.²²² By measuring the thickness as a function of RH, Secrist *et al.* demonstrated a swelling/deswelling hysteresis in PEMs made of [PAH-PAA]. Later, Lee *et al.* have shown for [PAH-PAA] PEMs that, depending on the building conditions, different degrees of hysteresis could be obtained. It was suggested that different water transport mechanisms were at play upon swelling and deswelling, which caused hysteresis in the swelling behavior.^{213,218} In all reported cases, the hydration/rehydration cycles are completely reversible and the PEMs response to ambient moisture was highly dependent on both the PE nature and assembly conditions.^{222–224}

In the previous Chapter (Chapter 2), PEMs that are only made of PE (no protein) were constructed via more complex adsorption sequences. It has been found that Lyz can be used as a sacrificial material to create highly hydrated PEMs. When PPCs² are LbL assembled with PAH at pH 3 and $I \simeq 0$ mM, *i.e.* PAH is fully ionized and long-range electrostatic forces are prevalent, PAH substitutes Lyz from the PPCs² upon assembly, as represented in Figure IV.18a. This results in a PEM that is almost only made of PAH and PSSi, and has a very high water content. With PPCs², it is thus possible to obtain PEMs that are made with the same constituent as in [PAH-PSS] PEMs (constructed as shown in Figure IV.18b), but with a hydration level that is completely different. Therefore, their internal structure must also be very different.

As introduced hereabove, structure correlates with functionality, and therefore, finding new structures could pave the way to new functionalities. For instance, the comparison of PEMs grown in different pH or salt conditions, *i.e.* PEMs with different structures, has been one of the major drivers of innovation in the field of LbL assembly (the very first papers published on the subject studied the effect of salt on the PEMs formation).²²⁵ It is thus essential to investigate how the use of PPCs² has affected the properties of the resulting PEMs, especially their swelling/deswelling behavior.

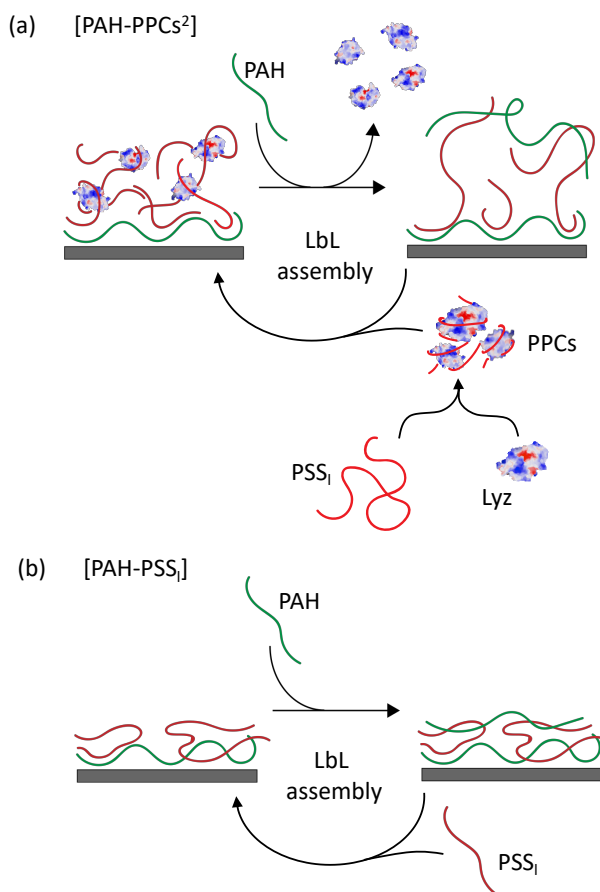


Figure IV.18 – (a) Schematic of the formation of PPCs² and subsequent LbL assembly with PAH. When constructed at low pH and low salt concentration, this results in the release of Lyz molecules upon assembly. (b) Schematic of the alternate adsorption of PAH and PSS_i and the resulting PEM.

In this Chapter, PEMs were constructed on a QCM-D sensor in the regular flow module by alternately adsorbing PAH with PSS_i [PAH-PSS_i], or PAH with PPCs² [PAH-PPCs²] at pH 3 and $I \approx 0$ mM. A convenient way to measure water sorption/desorption in PEMs is the use of QCM-D mounted with the humidity module. In this module, the coated QCM-D sensor is separated from a solution by a membrane that is only permeable to water (see Figure IV.19 for a schematic of the QCM-D humidity module).^{226,227} The principle is that a saturated LiCl solution is pumped through the humidity module, which gives a RH of 11% in the air above the sensor. In the meantime, the LiCl solution is continuously diluted by water that is pumped-in at the same volumetric rate.

Depending on the LiCl molality in the flowing solution, different water activities are obtained (a_w), resulting in a gradient of RH of the air in contact with the sensor (see Figure IV.19 for the experimental set up). This allows to control the RH from 11% to 100%, *i.e.* 100% being when LiCl is highly diluted. The water and LiCl vessels can be inverted to obtain a decrease in RH. In the latter case, the water solution is mixed with a concentrated LiCl solution. The mass variation of PEMs upon RH variation can be measured to construct water sorption/desorption isotherms.

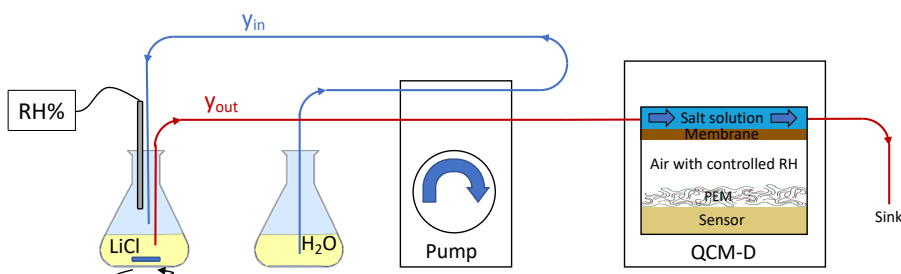


Figure IV.19 – Schematic of the experimental set up used to vary the RH of the air in contact with PEMs, in a QCM-D cell equipped with a humidity module. The QCM-D sensor is in direct contact with a small air volume, itself separated by a membrane from a circulating solution. A saturated LiCl solution is continuously diluted with water (y_{in}) and pumped through the QCM-D humidity module (y_{out}). The decrease in LiCl concentration in the flowing solution causes an increase in RH in the QCM-D cell. The water and LiCl vessels can be inverted to obtain a decrease in RH. In the latter case, the water solution is mixed with a concentrated LiCl solution.

3.2. Materials and methods

3.2.1 PEMs formation

[PAH-PSS]₅ and [PAH-PPCs²⁻]₅ were constructed at pH 3 and $I \approx 0$ mM as described in Chapter 2. Results of the PEMs construction are presented in Figure IV.20. Since it takes more than one day to build such systems, the films were rinsed with buffer and left for a night in the QCM-D flow module after the construction of the first bilayer. This is represented by the blue dashed box in Figure IV.20 (between 6 and 20 hours of construction). After construction, the coated sensors were stored one night in a desiccator and dried with a nitrogen flux before being mounted in the humidity module of the QCM-D. This storage step is represented by the yellow box in Figure IV.20.

3.2.2 Water sorption/desorption isotherms measurement

The method proposed by Graf *et al.* was used to continuously change the RH of the air in contact with the coated sensor between 11% and 100%. To do so, the setup presented in Figure IV.19 was constructed. A saturated LiCl solution was prepared by dissolving 45 g of dry LiCl in 50 mL water. Solutions were left under agitation three days for stabilization and centrifugated at 2000 rpm for 2 min (Heraeus multifuge X1, Thermofisher Scientific). The supernatant was filtrated first on a 5 μm cellulose filter, then on a 0.45 μm polyethersulfone filter. 25 ml of the obtained saturated LiCl solution were added to a dry Erlenmeyer with a RH probe (Novasina ms1, Defensor, Lachen, Swiss). The setup was closed hermetically and left to stabilize with a magnetic stirrer. After a few minutes, the air in the setup reached around 11% of RH. In the meantime, tubes going from the stock of water to the LiCl solution were filled with water while those going from the LiCl stock solution to the QCM-D humidity module were filled with dry N_2 . The temperature of the QCM-D humidity chamber was set at 20°C. The experiment was started by applying a flow of 0.1 $\text{cm}^3 \text{s}^{-1}$ in both the water-to-LiCl and LiCl-to-QCM-D humidity module tubes. In this way, the LiCl solution was pumped through the QCM-D humidity module while being diluted by water at the same volumetric rate. In the mean time, the crystal resonant frequencies were acquired and the QCM-D started to record the frequency and D shifts due to water sorption/desorption from PEMs previously deposited on the sensor as described above. The mass variation (Δm_w) due to the water sorption/desorption was estimated using the Sauerbrey equation for the 7th overtone (see above). After 22.5 h, the Erlenmeyer that initially contained the saturated LiCl solution contained highly diluted LiCl, *i.e.* $a_w \approx 1$. At this point, the setup was inverted and this almost pure water was mixed with a LiCl-saturated solution. After 8 h, this Erlenmeyer contained again 25 mL of a LiCl solution that is close to saturation, and consequently a gas phase with RH=11%.

In order to construct water sorption/desorption isotherms, it is critical to access the RH of the air in contact with the PEMs, *i.e.* directly in the humidity chamber. RH is given by a_w , which depends on LiCl molality in the flowing

solution. a_w has been experimentally measured for different LiCl molality by Robinson *et al.*²²⁸ A 6-order interpolation of these data can be used to determine a_w as a function of LiCl molality.²²⁷ In order to find the RH of the air in contact with the sensor, the LiCl molality variation as a function of time ($c_s(t)$) has thus to be computed. To do so, the LiCl and water mass transfer equations were solved (see supporting information p138 for details).

Results of the calculated a_w are presented in Figure IV.21a. These are in good agreement with literature.²²⁷ Moreover, the RH measured in the Erlenmeyer that initially contained LiCl is perfectly consistent with the computed a_w variation (see Figure IV.25a, p). In order to further confirm the accuracy of the modeling, both the initial and final water and LiCl masses were measured and compared to the modeling. This was achieved by weighting solutions and evaporating their content at 150°C. The mass difference allows to estimate the evaporated water mass and the remaining mass is attributed to LiCl. Results are presented in Figure IV.25a (p138) and show a perfect match between the modeling and the measurements. Finally, the absence of uncontrolled water condensation on an uncoated gold sensor was verified. To to so, a_w was increased from 0.11 to 1 and the mass variation of the uncoated sensor was recorded. Results are presented in Figure IV.26b (p138) and confirm the absence of any measurable water sorption on an uncoated sensor.

To measure water sorption and desorption with fast RH variations, the LiCl saturated solution that goes through the humidity module was simply replaced by water. As a consequence, RH abruptly goes from 11% to 100%. In order to go from 100% to 11%, the water was simply replaced by LiCl saturated solution. As for the slow a_w increase, the water condensation was checked on an uncoated sensor. Results are presented in Figure IV.27. It appears that contrarily to the gradual a_w increase, the fast switch from low to high RH causes water uptake on the gold surface. This water sorption results in a thickness increment of around 2 nm which largely surpasses that of a water monolayer. The measured Δm_w is thus due to water condensation on the sensor surface. The reason might be that a temperature gradient is present in the cell due to the abrupt water evaporation upon RH change.

Water could thus condense on the sensor. The condensation could not be avoided by varying the time of exposure to a_w of 0.11 or 1 as shown in Figure IV.27a and b. However, the amount of condensed water is between five and three fold lower than the amount absorbed in PEMs. Results obtained with large RH variations were thus considered significant.

3.3. Results and discussion

3.3.1 PEMs construction

Results of the construction of [PAH-PPCs²]₅ and [PAH-PSSi]₅ are respectively presented in Figure IV.20a and b. In both cases, a stepwise mass increase of the PEM is observed, which confirms that the LbL assemblies grow. For [PAH-PPCs²]₅, a “sawtooth” profile is observed and the total PEM hydrated mass decreases upon each PAH adsorption steps. As described in the previous Chapter, this can be attributed to the substitution of Lyz by PAH when the latter is adsorbed, and to a change in the water content. After the deposition of 5 bilayers, the [PAH-PPCs²]₅ multilayer reached a total hydrated mass of 8 $\mu\text{g cm}^{-2}$, while [PAH-PSSi]₅ reached only 1 $\mu\text{g cm}^{-2}$. After the construction of [PAH-PPCs²]₅ and [PAH-PSSi]₅ in the flow module of the QCM-D, they were

stored one night in a desiccator and dried with N_2 (represented as the yellow box in Figure IV.20).

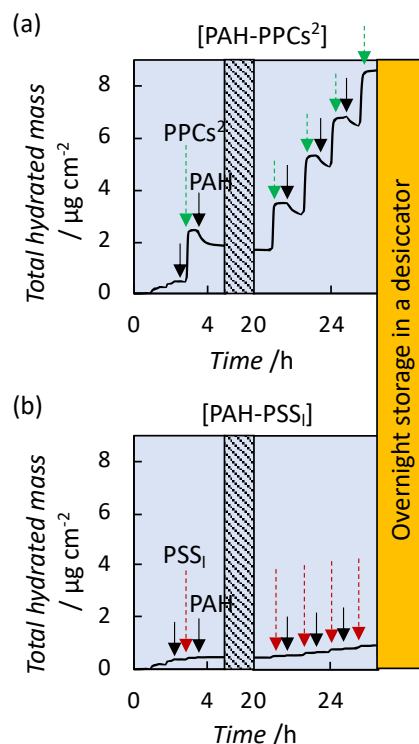


Figure IV.20 – (a) [PAH-PPCs²] and (b) [PAH-PSS_i] are constructed in the QCM-D flow module (in both cases, the first two hours correspond to a [PAH-PSS_n]₂ anchoring layer; the dashed box corresponds to an overnight storage in buffer). After the construction of five bilayers, PEMs are dehydrated via an overnight storage in a desiccator followed by exposure to a nitrogen flux (yellow box).

3.3.2 PEM hydration/dehydration cycles

After the PEM construction and the overnight storage in a desiccator, the coated sensors were mounted in the humidity module. The a_w of the solution flowing in the QCM-D humidity module was first brought to 0.11 – the air in contact with the PEMs thus has a RH of 11% – and the resonant frequencies were searched. The [PAH-PPCs²]₅ and [PAH-PSS_i]₅ PEMs were then exposed to a cycle of hydration and dehydration. To do so, a_w was increased from 0.11 to 1 in 22.5 h, then brought back from 1 to 0.11 during 8 h (see Figure IV.21a for the profile of a_w variation as a function of time). Since a_w varies at a low

rate, we assume that water sorption equilibrium is reached during the experiment. During the whole cycle of a_w variation, Δm_w and D were recorded. Results are presented in Figure IV.21b for [PAH-PPCs²]₅ and Figure IV.21c for [PAH-PSSi]₅. During the first 10h of a_w increase, Δm_w increases without any viscoelastic change (constant D). Both PEMs are thus swelling at similar rates. Then, when a_w reaches values close to 1, *i.e.* 0.977 (after 10h), D begins to increase together with Δm_w . This change in D suggests that a plasticization of both PEMs occurs. When a_w reaches a value that is very close to 1, *i.e.* 0.999, the [PAH-PPCs²]₅ and [PAH-PSSi]₅ masses have increased by respectively 1.45 and 0.88 $\mu\text{g cm}^{-2}$ due to water sorption. Since the PEM growth was measured in a regular QCM-D flow module while water sorption/desorption was measured after transfer in the QCM-D humidity module, there is a discrepancy regarding the recorded resonant frequency shifts. As a consequence, the PEMs dry masses cannot be accurately determined.

Yet, these results interestingly show that, at $a_w \approx 1$, [PAH-PSSi]₅ uptakes a mass of water that is equivalent to its initial hydrated mass, while [PAH-PPCs²]₅ uptakes only 20% of its initial hydrated total mass. Two scenarios are possible for [PAH-PPCs²]₅: upon storage in the desiccator, it loses an amount of water that is equivalent to 20% of its initial mass, then recovers its full hydration state (full initial mass) in the humidity chamber (1), or it loses a high proportion of water (>20% of its initial mass), then recovers a mass that is lower to its initial water content, *i.e.* it irreversibly dehydrate (2). In the previous Chapter, [PAH-PPCs²]₅ PEMs were shown to be made of up to 90% of water, which was lost by simply drying it at 4°C. By measuring the thickness of [PAH-PPCs²]₅ and comparing it to the total hydrated mass obtained from QCM-D it was found that the dry mass is only around 0.6 $\mu\text{g cm}^{-2}$. The overnight storage in a desiccator presented here has thus certainly led to a dehydration of [PAH-PPCs²]₅ that is of the same magnitude, *i.e.* around 90% of the mass is lost. Since the [PAH-PPCs²]₅ rehydrate by only 20% in the humidity chamber, it suggests that the latter PEM was unable to recover its initial hydration level. [PAH-PPCs²]₅ thus has irreversibly lost its water content.

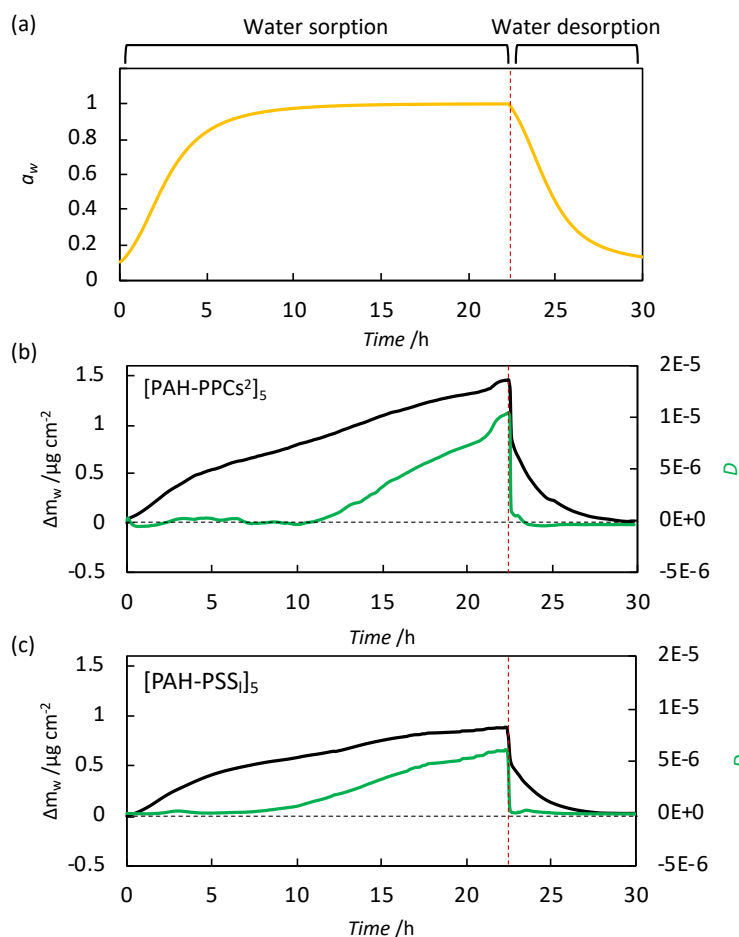


Figure IV.21 – (a) a_w variation as a function of time in the QCM-D humidity chamber, *i.e.* RH variation of the air in contact with the PEMs. Water sorption and desorption (Δm_w ; black curve) from [PAH-PPCS₂]₅ (b) and [PAH-PSSi]₅ (c) is measured as a_w increases from 0.11 to 1 and decreases from 1 to 0.11. Viscoelastic properties of the PEMs are also measured *via* D (green curve).

After their rehydration in the humidity chamber, PEMs were exposed to a low RH by bringing a_w back from 1 to 0.11 in the humidity chamber (between 22.5h and 30h in Figure IV.21). When a_w decreases, both PEMs readily lose their water content as indicated by the decreases of Δm_w . This water desorption is accompanied by a decrease in D . After 30h, *i.e.* when a_w reaches 0.11, both PEMs show the same Δm_w and D values as before the complete hydration/dehydration cycle that was carried-out in the humidity chamber.

This result suggests that both PEMS recovered the same hydrated state as after the overnight storage in the desiccator (at $t=0$ hour).

Since a_w variation is not linear, interpretation of D and Δm_w variation is not straightforward. In order to clearly identify PEMs swelling/deswelling transitions, which are key characteristics, Δm_w and D were expressed as a function of a_w . Results are presented in Figure IV.22a. When a_w increases from 0.11 to 0.9, both PEMs uptake water at linear rate without any change in the viscoelastic properties (as indicated by the constant D). When a_w reaches 0.9, the water sorption rate increases, which is accompanied with an important plasticization. These results suggest that, as reported for other PEMs, the water sorption and desorption can be divided in two regimes.^{213,218} For both [PAH-PPCs²]₅ and [PAH-PSS]₅ the transition occurs around $a_w=0.9$. A lower Δm_w increase rate is obtained below this value while a higher rate of water sorption is obtained at higher RH. A difference in the water transport mechanism could explain this transition. At higher RH, PE chain mobility increases, leading to a better diffusion of water molecules in the PEM. It was proposed that this transition is accompanied by a phase separation at high a_w .²²² This lead to PE rich phases separated by phases of only water. This hypothesis is supported by the massive increase in PEM softness observed for both PEMs at high RH (as indicated by the increase of D in Figure IV.22c and d). Another explanation is that the multilayers are porous. The water uptake at high RH could be due to the filling of the multilayer porosity. The increase in Δm_w and D at high RH were already reported. The RH at which the transition occurs was reported highly dependent of the building conditions and the PEs that are used.^{213,222} However, the PEM thickness, mass or viscoelastic properties were always measured using discrete RH values. This could lead to an underestimation of the RH at which the transition occurs. Especially, such sharp transition at very high RH were never reported, and, usually, an exponential growth starting from lower RH values was assumed.²²² The continuous RH variation that was achieved in this Chapter thus reveal much better the dynamic properties of PEMs.

Swelling/deswelling hysteresis were sometimes observed depending on the conditions and PEs that are used to construct the PEMs.^{218,224} Here, there is

no marked hysteresis effect, and Δm_w upon sorption is very similar to the water loss. This is consistent with data reported for [PAH-PAA] PEMs.²¹⁸ When [PAH-PAA] multilayers are constructed at intermediate pH values, PE chain mobility is high because both PE are not fully ionized. This is believed to cause the swelling/deswelling hysteresis. In contrast, [PAH-PAA] PEMs constructed at low pH, *i.e.* with fully ionized PAH, display a very small swelling/deswelling hysteresis due to lower PE chain mobility.²¹⁸ For [PAH-PPCs²]₅ and [PAH-PSS]₅, the PEMs were constructed at pH 3, both PEs are thus fully ionized and chain mobility is at its lowest. This causes the observed absence of swelling/deswelling hysteresis.

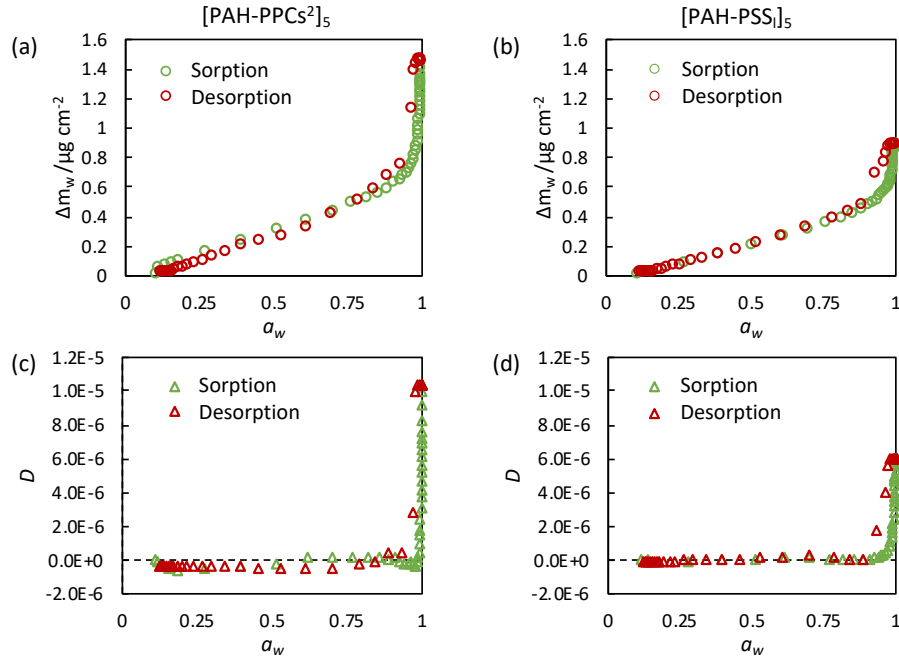


Figure IV.22 – Water sorption/desorption isotherm – Δm_w as a function of a_w – for (a and c) [PAH-PPCs²]₅ or (b and d) [PAH-PSS]₅. D variation as a function of a_w for (c) [PAH-PPCs]₅ or (d) [PAH-PSS]₅ ($n=1$).

The last parameter that was investigated is the reversibility of the sorption/desorption with repeated cycles of fast RH variation. Both [PAH-PPCs²]₅ and [PAH-PSS]₅ were sequentially exposed four times to an abrupt RH shift from 11% to 100%. W_u was measured and results are presented in Figure IV.23, together with the corresponding profile of a_w variation. It

appears that, for each hydration cycle, both $[\text{PAH-PPCs}^2]_5$ and $[\text{PAH-PSSi}]_5$ uptake the same water amount. Similarly, after each dehydration, the PEMs recover their initial hydration level. These results show that $[\text{PAH-PPCs}^2]_5$ and $[\text{PAH-PSSi}]_5$ hydration/dehydration is completely reversible as both PEMs uptake the same water mass during each of the four cycles. Interestingly, the Δm_w reached at each hydration cycle is exactly the same as the one measured after 22h of slow a_w increase. However, as presented in the, 200 ng cm⁻² of water condenses on the sensor when RH is abruptly changed. It is thus difficult to know whether the PEM presence avoided the water condensation on the sensor, or the measured mass is biased by 200 ng cm⁻².

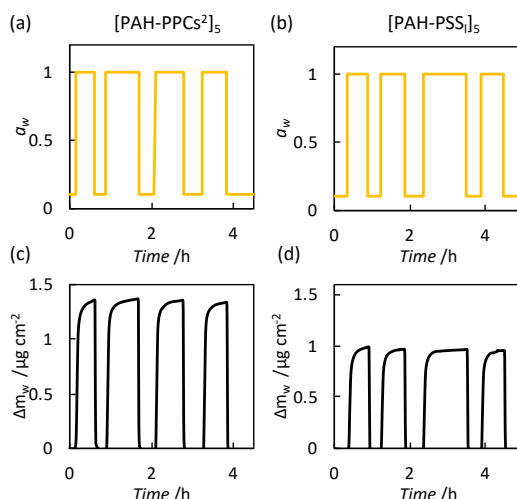


Figure IV.23 – Cycles of water sorption and desorption measured in the QCM-D humidity chamber for $[\text{PAH-PPCs}^2]_5$ multilayer and $[\text{PAH-PSSi}]_5$ multilayers. Top panels (a and b) shows the a_w variation as a function of time that was used to measure Δm_w in respectively (c) $[\text{PAH-PPCs}^2]_5$ and (d) $[\text{PAH-PSSi}]_5$ ($n=1$).

3.3.3 Discussion

When a $[\text{PAH-PPCs}^2]_5$ multilayer is constructed, it has a hydrated mass that is drastically different from $[\text{PAH-PSSi}]_5$, *i.e.* it is eight-fold heavier (see Figure IV.20). In the last Chapter, it was shown that $[\text{PAH-PPCs}^2]_5$ PEMs contain up to 90% of water while $[\text{PAH-PSSi}]_5$ are reported to be made of up to 40% of water.²¹⁹ The difference in mass is thus attributed to a higher water content when PPCs² are used as building blocks to build the PEMs. LbLs were already classified as nanogel by Chacko *et al.* due to their high water content and the

use of soluble polymers.²²⁹ [PAH-PPCs²] films definitely match the definition given their very high volume fraction of water. As already pointed in the previous Chapter, this feature is unseen for PEMs constructed in conditions where Debye screening is at its lowest and all PEs used are at their highest ionization degree. Indeed, thin LbLs are expected due to intramolecular electrostatic repulsion.

Both PEMs were constructed under the same conditions (pH 3, low I) and resulted in films that are made with similar constituents (water, PAH and PSS; only 100 ng cm⁻² of lysozyme are detected in [PAH-PPCs²], which represents approximately 1% of the initial mass). Interestingly, it means that the content of the LbL does not reflect the constituents that were used to grow it. Even if this is sensible, especially for out-of-equilibrium systems, it stresses the need for a complete analysis of the PEM when constructed. Indeed, the review of LbLs presented in the introduction has clearly shown that the constituents of a LbL are frequently assumed based solely on the observation of a stepwise mass increase or a charge inversion, or, even worse, based only on the constituents that were used to grow it.

After complete dehydration (storage during one night in a desiccator), both multilayers become indistinguishable in terms of their swelling behavior. They indeed absorb a similar water amount when RH is increased, display the same swelling/deswelling dynamics, show a transition in the swelling behavior at the same RH value, become soft at very high RH, show no hysteresis upon swelling/deswelling (which is typical of systems with very low PE mobility), and the swelling/deswelling is completely reversible over 4 cycles of hydration/dehydration. As illustrated in Figure IV.24, these observations taken together suggest that, after dehydration, both PEMs are identical. This implies that [PAH-PPCs²]₅ has irreversibly lost water and reorganized into a system that is more similar to [PAH-PSS]₅. When dehydrated, the [PAH-PPCs²]₅ collapses and loses its structure that had been acquired via the sacrificial use of Lyz. The idea that [PAH-PPCs²]₅ could collapse and lose its structure is consistent with a model where the latter is highly hydrated. After this collapse, [PAH-PPCs²] multilayers share the same swelling behavior as the LbL that has been constructed by directly assembling

PAH with PSS_I. The new structure that was obtained illustrates the potential of exploring new paths in the self-assembly of macromolecules. The use of sacrificial elements may allow to construct structures that are at a higher energy state and potentially possess new functionalities.

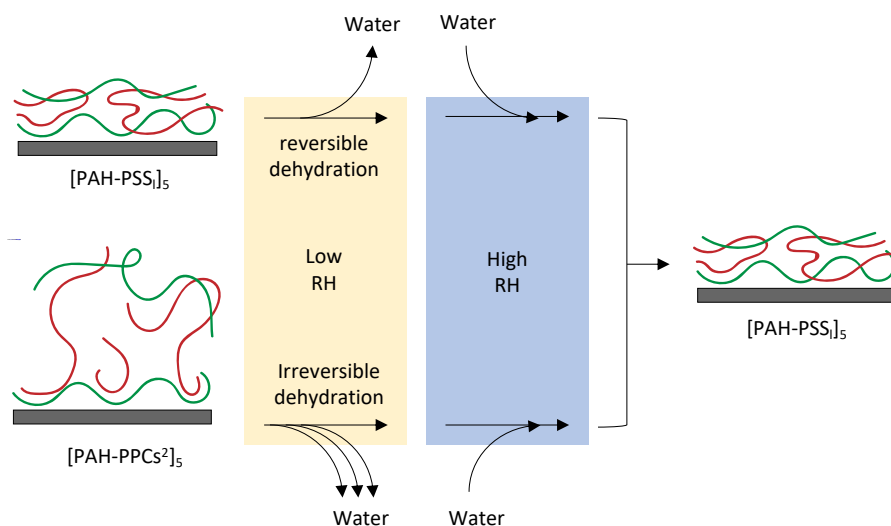


Figure IV.24 – Schematic of the fabrication of $[\text{PAH-PPCs}^2]_5$ and $[\text{PAH-PSS}]_5$ systems. The use of lysozyme as a sacrificial element leads to PEMs that are highly hydrated. When dehydrated, these PEMs irreversibly lose water and become undistinguishable from $[\text{PAH-PSS}]_5$ PEMs in terms of their swelling behavior.

3.4. Conclusion

In this Chapter, $[\text{PAH-PPCs}^2]_5$ and $[\text{PAH-PSS}]_5$ PEMs were constructed. These are similar in composition, *i.e.* made of PAH and PSS_I, but display drastically different hydration states. In particular, $[\text{PAH-PPCs}^2]_5$ resembles more a nanogel given its very high water content. The latter feature is attributed to the presence of Lyz in PPCs, which is used as a templating agent upon PEM formation. Lyz could thus be used as a sacrificial element during LbL assembly to create highly hydrated PEMs. Using QCM-D mounted with a humidity module, we show that when $[\text{PAH-PPCs}^2]_5$ is dehydrated, it collapses and irreversibly loses water. Further rehydration only brings a Δm_w similar to the one observed with $[\text{PAH-PSS}]_5$ PEMs, as well as a similar general behavior regarding swelling/deswelling. The results therefore suggest that, after dehydration, the $[\text{PAH-PPCs}^2]_5$ multilayers have reorganized into a structure

similar to the one obtained when constructing [PAH-PSSi]₅ multilayers. This result shows that the use of a sacrificial element in interfacial self-assemblies allows the construction of nanosystems featuring a complex architecture that cannot be reached when assembly is performed according to a more classical pathway, even though this nanosystem may ultimately rearrange into a more conventional structure.

3.5. Supporting information

3.5.1 LiCl molality as a function of time in the QCM-D humidity chamber

a_w as a function of time can be found by computing the LiCl molality as a function of time in the solution that flows through the QCM-D humidity module. The initial parameters that are known are initial water and LiCl masses, solution density and flow rate. The LiCl mass (m_s in g) and the water mass (m_w in g) variations are expressed as solving the mass transfer equations (Equations (IV.4) and (IV.5)):

$$\frac{dm_s}{dt} = c_{s,in}y_{in} - c_s(t)y_{out} = -\frac{m_s(t)}{V_{sol}(t)}y_{out} \quad (IV.4)$$

$$\frac{dm_w}{dt} = c_{w,in}y_{in} - c_w(t)y_{out} = c_{w,in}y_{in} - \frac{m_w(t)}{V_{sol}(t)}y_{out} \quad (IV.5)$$

where y_{in} is the water influx diluting the LiCl solution (in $\text{cm}^3 \text{s}^{-1}$), y_{out} is the solution flux pumped out of the LiCl solution (in $\text{cm}^3 \text{s}^{-1}$), $c_{s,in}$ is the LiCl concentration in y_{in} ($c_{s,in} = 0 \text{ g cm}^{-3}$ in the setup presented in Figure IV.19), $c_{w,in}$ the water concentration in y_{in} and V_{sol} is the volume of the LiCl solution (in cm^3). Contrarily to Graf *et al.*, the volumetric pump was assumed accurate and thus y_{in} equal $y_{out} = 0.1 \text{ cm}^3 \text{s}^{-1}$. V_{sol} is found according to Equation (IV.6):

$$V_{sol}(t) = \frac{m_{sol}(t)}{\rho_{sol}(t)} = \frac{m_s(t) + m_w(t)}{\rho_{sol}(t)} \quad (IV.6)$$

where ρ_{sol} is found using the 5-order interpolation for density as a function of LiCl molality used by Graf *et al.*²²⁶ By solving the differential equation, the

LiCl and water masses can be calculated as a function of time. Results obtained are presented in Figure IV.25. a_w as a function of LiCl molality is then found *via* a 6-order interpolation of data reported by Robinson *et al.*^{227,228}

The water and LiCl gravimetric masses were also experimentally measured before and after the experiment to verify the validity of the computed values. Results are also presented in Figure IV.25 and it appears that the LiCl solution is well saturated and initially reaches the values used for computation. A LiCl amount lower than the detection limit of the scale in y_{out} after 22h of experiment is also confirmed. Finally, the RH that was measured in the atmosphere of the LiCl solution is presented in Figure IV.25 and shows a good correspondence between calculated values and experimental values. Taken together, these results suggest that a_w could be accurately varied for the duration of the experiment. The computed value of a_w as a function of time will thus be used in further experiment to measure the mass of a PEM as a function of RH.

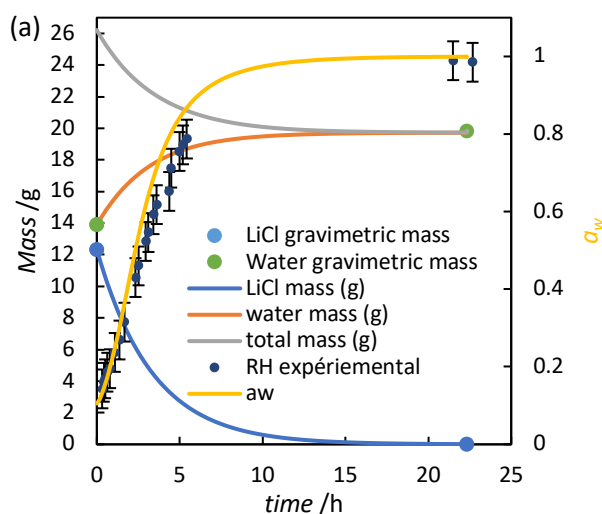


Figure IV.25 – Modelling of the LiCl and water masses transfer as a function of time, and the resulting a_w . In order to confirm the computed values, LiCl and water gravimetric masses are measured in initial and final solutions, and RH is measured in the air of the vessel as shown in Figure IV.19. Error bars show the error of the instrument (given by the manufacturer).

3.5.2 Water uptake on an uncoated QCM-D sensor

An experiment was carried out to verify that no water condensation occurred at the uncoated sensor surface. Indeed, the whole humidity module is thermostated and no temperature gradient should be present, especially by slowly increasing a_w which leaves time for the water sorption/desorption equilibrium to be reached. Results of mass detected on the sensor as a function of time are presented together with the calculated a_w value in Figure IV.26. It appears that no significant mass uptake could be measured. This confirms that the system can be used to measure mass variations in hydrated films without measurement artifacts by slowly varying a_w . Then, the absence of water condensation with rapid RH variations was also verified. Uncoated sensors were exposed four times to an abrupt RH shift from 11% to 100%. Δm_w was measured and results are presented in Figure IV.27 together with a_w variation. It appears that 200 ng cm^{-2} of water are condensed on the naked sensor surface. This is much lower than the amount absorbed in PEMs. Therefore, the results presented in Figure IV.23 were considered significant.

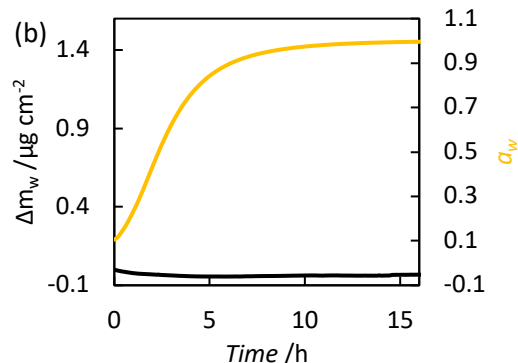


Figure IV.26 – Δm_w with increasing a_w measured by QCM-D on a naked gold sensor ($n=1$).

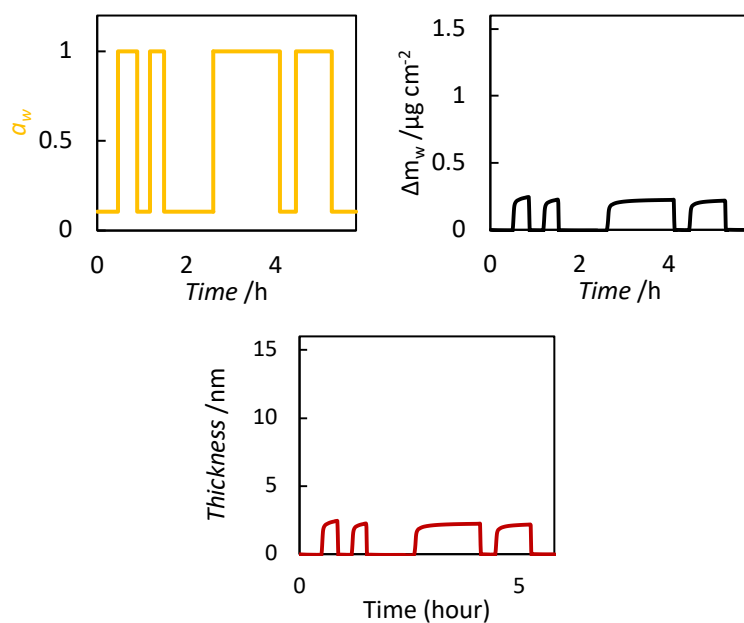
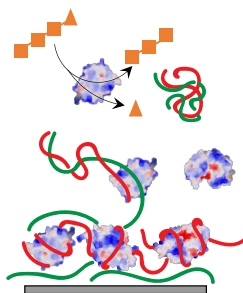


Figure IV.27 – Cycles of water sorption and desorption are measured by QCM-D with the humidity module for an uncoated gold sensor. The thickness variation is measured based on the water density at 20°C ($n=1$).

4. Protein–polyelectrolyte complexes for the delivery of active proteins from self-assemblies



In this Chapter, the enzymatic activity of Lyz is thoroughly investigated and a strategy that is similar to the one used in Chapter 2 is used. The Lyz enzymatic activity is first studied in solution, when it is free or in PPCs², over a wide range of pH and I conditions. This allows to get hints on how the presence of PE can affect the protein activity. It appears that the presence of PSS_i around Lyz creates a pH microenvironment, which improves up to six-fold its enzymatic activity in basic conditions. In Chapter 2, it was evidenced that the conditions of LbL assembly that immobilize the most Lyz are for [Lyz-PSS_h]_{4.5} and [PAH-PPCs]₅ respectively at pH 3 – I=200 mM and pH 7.5 – I≈0 mM. [Lyz-PSS_h]_{4.5} and [PAH-PPCs]₅ were thus constructed in both conditions and efforts were put in studying their stability upon incubation in the reaction medium that is used to measure the enzymatic activity. Interestingly, all multilayers constructed at pH 3 – I=200 mM are stable, while those constructed at pH 7.5 – I≈0 mM release between 75 and 100% of their Lyz content. A comprehensive analysis of Lyz activity shows that immobilized Lyz retains only 10% of its activity. Upon its release from [Lyz-PSS_h]_{4.5} or [PAH-PPCs]₅, it retains respectively between 19% and 35%, or between 13% and 58% of its activity. Data suggest that Lyz released from [Lyz-PSS_h]_{4.5} is still complexed with PSS_h, while a significant fraction of Lyz released from [PAH-PPCs]₅ is in its native naked form. This is attributed to the presence of PAH that can competitively substitute Lyz in the multilayer. This substitution combined to the presence of more Lyz in [PAH-PPCs]₅ films leads to a total enzymatic activity that is three-fold higher when compared to the one of [Lyz-PSS_h]_{4.5} films. This system is thus valuable for drug delivery devices as it allows the release of large amounts of active protein molecules.

4.1. Introduction

The delivery of active molecules from coated materials is a cornerstone in modern medicine.⁵⁸ Especially, the vast array of functions achieved by proteins has made of them a valuable class of molecules to be delivered. So far, their surface immobilization and subsequent release has brought innovations in fields such as vaccination and tissue engineering.^{18,230,231} Many different ways exist to functionalize surfaces with proteins. Yet, in terms of responsiveness and universality, self-assemblies are becoming topical and the last decades have seen different systems flourishing.^{8,232}

With the aim of immobilizing any protein on any surface for its delivery, LbL holds the best promises as it is conducted in soft conditions on any surface geometry or chemistry, and as it enables a precise control of the immobilized amount. The vast majority of multilayers has found applications in biosensing.^{77–79} In a few studies, the release of active proteins was reported.⁷⁶ Especially, coated polymer microneedles are used for the transdermal delivery of proteins, and implantable materials coated with growth factors improve both osteointegration and cell response.^{76,80–86} In most cases, the activity of the released protein was reported through the evaluation of cell response.⁷⁶ While this method qualitatively confirms that a fraction of the proteins has kept its bioactivity, it does not quantitatively evaluate the loss of activity subsequent to their immobilization.

The release of active components used to construct multilayers can be achieved by disruption of layer interactions, chemical degradation of polymer layers, film destruction, phase transitions and polymer rearrangements.^{76,233} With respect to protein, the stimulus that are mostly used to trigger their release are pH and/or temperature variations, enzymatic degradation or light exposure.^{76,233} pH variation causes a change of the PE or the protein ionization degree. This leads to the degradation of the multilayer *via* a decreased coupling strength between the PE and the protein.⁷⁶ For example, insulin undergoes a negative-to-positive (decreasing pH) or positive-to-negative (increasing pH) charge shift at a pH close to physiological condition. This was used to trigger its release by either acidification or alkalinization.²³⁴ Besides, to simulate a system immobilizing brain-derived

neurotrophic factor (BDNF), S. Mehrotra *et al.* immobilized Lyz in H-bond poly(ethylene glycol)(PEG)/PAA hybrid LbL thin films.²³⁵ They achieved a month-long sustained release of active Lyz. The H-bond multilayer degradation was attributed to PAA deprotonation at physiological condition. However, only half of the released protein molecules had kept their activity. This diminished activity was attributed to the interaction between the released PE and Lyz in solution. A way to circumvent this issue is the use of a degradable PE. For example, ovalbumin (Ova) was assembled LbL with poly(β -amino ester) and native free nonaggregated/nondegraded Ova was released.²³⁶ Unfortunately this method is restricted to the use of a few PEs. Similarly, light-responsive polymers have also been used to trigger the disassembly of LbL films by self-organization of the PE in aggregates. This resulted in the weakening of intermolecular forces and subsequent capsule disruption.²³⁷ Lastly, competitive substitution in the presence of chemical moieties is a promising method to destabilize and release the intact content of a multilayer.²³³ For instance, multilayers made of boronic-acid functionalized PAA and a polyol (mannan or poly(vinyl alcohol) (PVA)) are disassembled in the presence of glucose, as glucose binding to the boronic acid-modified polymer is more favorable than the binding of the latter with the polyol.²³⁸

For now, the disassembly of the multilayers and subsequent release of intact proteins requires either an alteration of PE-protein (from attractive to repulsive mode) response to an environmental change or particular PE properties (degradability, responsiveness, differential affinity). The former is highly challenging to engineer as it requires a charge shift as well as the absence of other favorable interactions taking the lead over electrostatics/ion pairing, *e.g.* hydrophobic interactions. The latter is limited to a few PEs. A universal method should allow the immobilization and release of a protein that does not especially present a charge inversion (usually in the range of pH physiological conditions for biological applications), *i.e.* any protein, with a PE that does not have any particular feature (charge inversion, degradability, responsiveness, etc.).

In the two previous Chapters, we successively have shown that the LbL assembly of PPCs² with an oppositely charged PE is independent of the protein electrical charge, and that the resulting LbL film possesses dynamic properties that allow the film to reorganize. Especially, the construction at pH 3 causes PAH to substitute Lyz upon assembly, while the very same assembly at pH 7.5 results in stable systems. Based on these results, we hypothesize that the transition between a stable and an unstable system, triggered by lowering the pH, could cause a rearrangement of the LbL film and the subsequent release of intact, *i.e.* naked non complexed Lyz molecules.

In this Chapter, we investigate the potential of PPCs-based multilayers ([PAH-PPCs²]₅) to release intact protein molecules upon pH, I and temperature variations. To do so, the latter multilayer is compared to the film prepared by the classical alternate adsorption of a protein with a PE, *i.e.* [Lyz-PSS_n]_{4,5}. In both cases, Lyz was immobilized in the multilayer, and its release and bioactivity were investigated. It is worth mentioning that Lyz is useful for medical applications since it is a practical analog to BDNF (similar size and high isoelectric point (IEP)).²³⁵ Due to its high IEP, a pH-induced charge inversion of the protein cannot be used to disassemble the multilayer. The counter PE that are used are PSS and PAH. These are nondegradable PEs which do not present any particular feature nor charge inversion.

4.2. Results and discussion

4.2.1 Activity of free and complexed lysozyme

In order to evaluate the enzymatic activity of Lyz immobilized in multilayers, it is essential to first extensively investigate Lyz activity when it is in presence of PEs in solution, *i.e.* in PPCs². The enzymatic activity of Lyz that is free or complexed with PSS_i was thus measured at pH 3.0, 5.0, 7.5, or 9.6 and I of 0, 10, 200, or 800 mM. Enzymatic activity was assessed using (GlcNAc)₃-MeU as the substrate of Lyz. For clarity, the (GlcNAc)₃-MeU solution which is in different pH and I conditions will be referred to as the *reaction medium*. It is worth mentioning that a DMSO volume fraction of 7.4% is present in the reaction medium. Glycosidic bond hydrolysis and subsequent release of MeU

were detected by means of fluorescence spectrometry (see Figure IV.28).²³⁹ The calibration curve of MeU fluorescence and the Lyz mass were used to compute the turnover number, *i.e.* K_{cat} (see Figure IV.37 for the MeU calibration curve). As in previous Chapters, Lyz was complexed with PSS_I in such a way that the ratio of negative charge from PSS_I to positive charges from Lyz equals 2, *i.e.* $(-)/(+net)=2$ (PPCs²). When pH and I conditions are mentioned, they are valid for the building of PPCs² as well as for the enzymatic activity measurement. Lyz enzymatic activity is presented in Figure IV.28b and c under the form of K_{cat} isoreponse maps as a function of pH and $I^{1/2}$. Note that Lyz activity in PPCs² will also be referred to as *in solution*, even though PPCs² colloidal suspensions were rather obtained in the majority of pH and I conditions. It was also checked that PSS_I used to build PPCs² does not interfere with the activity measurement (see Figure IV.38 of supporting information, p173).

The maximal enzymatic activity of free Lyz, *i.e.* not complexed, is around pH 5 and no activity is observed at pH 9.6 (see Figure IV.28b), which is consistent with previously reported data for (GlcNAc)₃-MeU hydrolysis by hen egg white Lyz.²³⁹ The decrease in enzymatic activity as I rises was also previously reported.²⁴⁰ The K_{cat} values that are obtained are in the range of the one reported for (GlcNAc)₃-MeU hydrolysis by hen egg white lysozyme (the maximal K_{cat} reported by Yang *et al.* is $2 \cdot 10^{-5} \text{ s}^{-1}$ at pH 5.1).²⁴¹ These results validate the enzymatic activity assay and reveal that the maximal sensitivity is achieved at pH 5.

With respect to the enzymatic activity of Lyz in PPCs², it is also maximal at pH 5 and minimal at pH 9.6 (see Figure IV.28c). In contrast with free Lyz, the enzymatic activity of Lyz in PPCs² however increases with I. In order to compare the enzymatic activity of free *versus* complexed Lyz, the ratio of PPCs² activity to free Lyz activity was computed and presented under the form of an isoresponse map in Figure IV.28d. No data is presented at pH 9.6 as enzymatic activities close to zero were measured in all conditions. Raw fluorescence data with the associated standard deviation are presented under the form of a bar chart in Figure IV.39 of the supplementary material (p173).

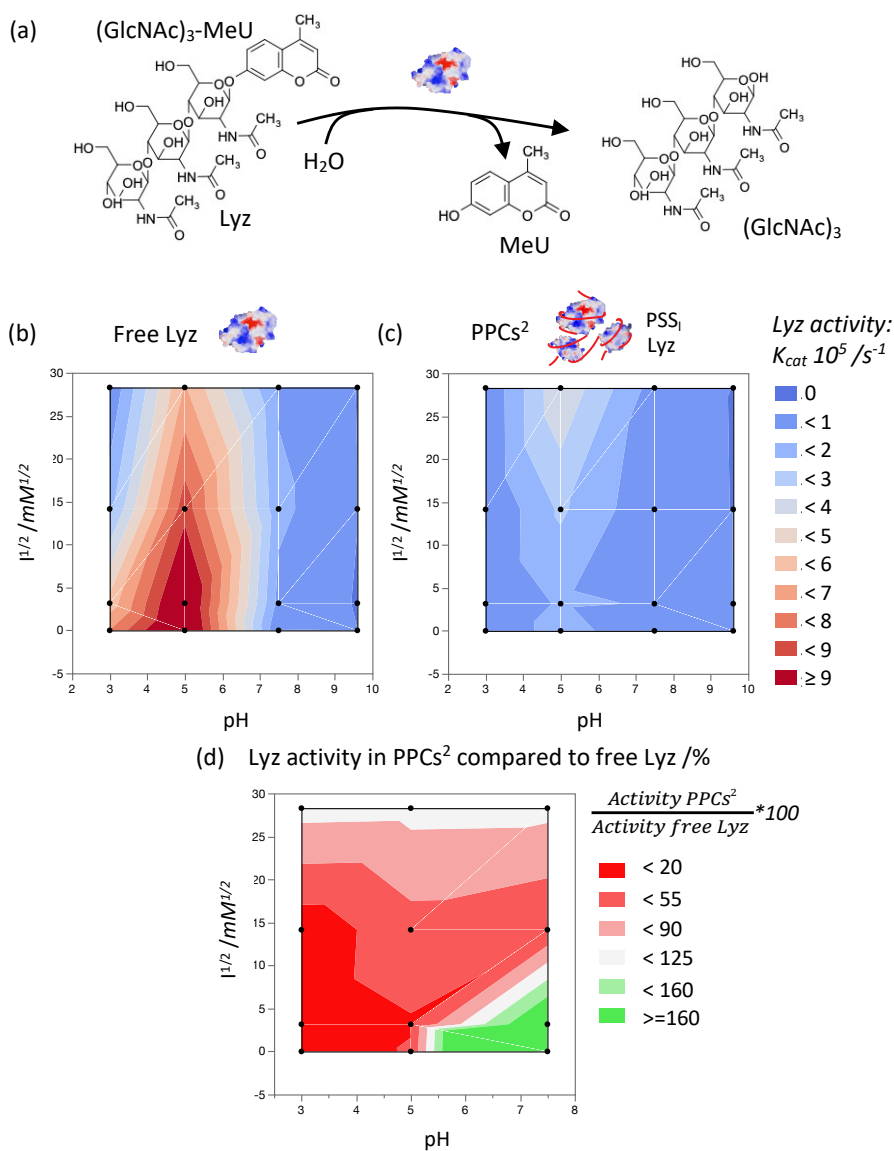


Figure IV.28 – (a) Schematic of the enzymatic reaction that is carried out. – Enzymatic activity measured for (b) free Lyz or (c) Lyz complexed with PSS_I (PPCs²) presented under the form of isoresponse maps as a function of pH and I^{1/2}. Black dots show mean values of experimental data (n=3). Activity is measured as the fluorescence of MeU that is released from hydrolysis of (GlcNAc)₃-MeU. The same Lyz concentration was used for all experiments. – (d) Lyz activity in PPCs² compared to free Lyz in different conditions of pH and I. – All data are presented with the associated standard deviation in Figure IV.39 (Supporting Information, p175).

It appears that the activity of free and complexed Lyz are close to each other for $I=800$ mM in all pH conditions. Data presented in Figure IV.40 of the supplementary material (p175) and in Chapter 2 suggest that PPCs² exist in these conditions, but represent a small fraction of the Lyz that is present. This is also supported by data presented in the Appendix on the Preliminary results on PPCs formation (p225), which show that more PSS_I has to be added at high I to fully complex Lyz. The similar enzymatic activity could thus originate from the high free Lyz fraction that is present in the solutions of PPCs² at $I=800$ mM.

At lower I , Lyz activity is lower in PPCs² compared to free Lyz, except at pH 7.5 and low I . This lower enzymatic activity is attributed to the limited diffusion of (GlcNAc)₃-MeU in PPCs², especially at $I=200$ mM for which PPCs² larger than $1\text{ }\mu\text{m}$ were found (see Chapter 2). The complexation of Lyz with PSS_I might also hinder (GlcNAc)₃-MeU access to Lyz catalytic site.

At pH 7.5 and $I\approx 0$ or 10 mM, the activity of complexed Lyz is respectively 6 and 2 times higher than the one of free Lyz. Data presented in Figure IV.40 of the supplementary material (p175) and in Chapter 2 show that PPCs² exist in both conditions. The enzymatic activity improvement that is observed thus originates in the effect of the complexation. This improvement might come from a decreased Lyz aggregation upon complexation with PSS_I at pH 7.5. Indeed, PPCs² were reported to prevent protein aggregation while Lyz aggregation was reported when pH increases.^{242,243} Besides, it appears that one product of the reaction, *i.e.* MeU, is negatively charged above pH 7 (MeU has a pK_a of 7.8 – the charge of (GlcNAc)₃-MeU, (GlcNAc)₃ and MeU was computed as a function of pH using ChemAxon software, see Figure IV.29). Since PPCs² are negatively charged, the reaction equilibrium might be displaced by Le Chatelier's principle towards product formation by repulsion of the latter from PPCs².⁵² Especially, the improved enzymatic activity at low I is consistent with an electrostatic repulsion of the product. Indeed, long range electrostatic forces are at play at $I\approx 0$ or 10 mM.

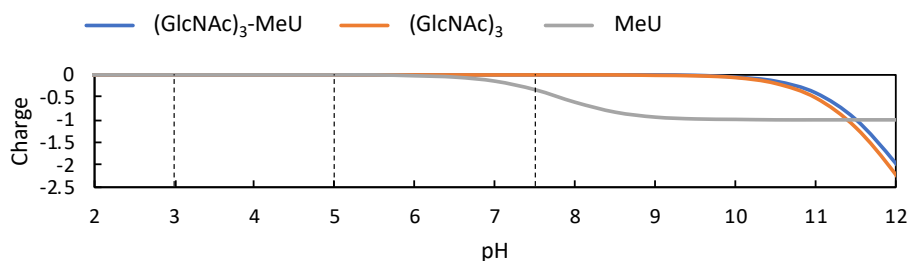


Figure IV.29– Charge of the substrate and products of the enzymatic reaction as a function of pH (computed using ChemAxon software). The dotted lines represent the pH values that were investigated in this Chapter.

Importantly, the pH microenvironment that is created by charged polymers can affect nearby enzyme bioactivity.^{244–247} In this way it has been proved that the enzymatic activity of glucose oxidase and horse radish peroxidase anchored to a polyanionic carrier increases by 1.2- and 4-fold respectively. This effect was attributed to a pH decrease in the local carrier environment. Indeed, they computed that the pH shifts could show values up to 2 pH units.²⁴⁴ The local acidification was recently used to improve cytochrome C activity by covalent link with a polyanion.^{244,246} Interestingly, the pioneering work of Goldstein *et al.* has also shown that protease-PE conjugates lead to an improved activity due to a shift of local pH that modifies the activity.²⁴⁶ With respect to self-assembled conjugates, *i.e.* PPCs, it was recently shown that PE interaction with key amino acids of the protease Ca^{2+} binding site boosts its activity.²⁴⁸ The local acidification has also been shown to cause a shift of the optimal pH activity to more alkaline values for Lyz complexation with polysaccharides or potassium poly(vinyl alcohol sulfate) with various degrees of esterification.²⁴⁷ The PE presence thus has a critical effect on the local proton activity and amino acids pKa. In our case, it is possible to compute the pH as a function of the distance using PPCs² surface charge potential that was measured by streaming potential in Chapter 2.²⁴⁴

The pH near a charged surface can indeed be computed according to Equation (IV.7):²⁴⁹

$$pH_s(d) = pH_b + \frac{F\psi_s(d)}{2.3 RT} \quad (\text{IV.7})$$

Where F is the Faraday constant, R is the perfect gas constant, T the temperature, $pH_s(d)$ the pH at a distance d from the surface, pH_b the bulk pH and $\psi_s(d)$ the surface potential. In order to calculate the pH as a function of d , ψ_s dependence on d has to be calculated. This can be achieved by solving the Grahame equation and using the Poisson-Boltzmann equation. An infinite plane surface was assumed and the boundary conditions were considered as shown in Equation (IV.8) and (IV.9). The solution is given in Equation (IV.10).²⁴⁴

$$\psi_s(d = 0) = \xi \quad (IV.8)$$

$$\psi_s(d = \infty) = 0 \quad (IV.9)$$

$$\psi_s(d) = \frac{2RT}{F} \log \left(\frac{1 + \exp(-\kappa d) \tanh(\alpha)}{1 + \exp(-\kappa d) \tanh(\alpha)} \right) \quad (IV.10)$$

$$\alpha = \frac{F\xi}{4RT} \quad (IV.11)$$

$$\kappa = \sqrt{\frac{2F^2 C_0}{\varepsilon RT}} \quad (IV.12)$$

Where ξ is the electrical potential that was measured by streaming potential in Chapter 2, *i.e.* ψ at the shear plane. κ is the Debye length reciprocal (, Equation (IV.11)). C_0 is the ion concentration in the bulk, *i.e.* H^+ , OH^- , buffer, added NaCl and PE released counter-ions are taken into account. ε is the relative permittivity.

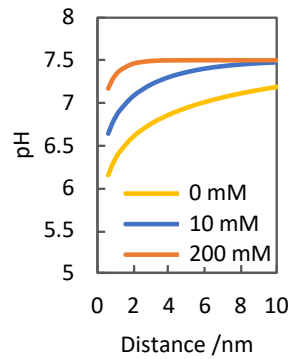


Figure IV.30 - pH-distance profile near a charged surface at different I , for a bulk pH of 7.5.

The pH-distance profile is presented for pH 7.5 and $I \approx 0$, 10 and 200 mM in Figure IV.30e. It appears that the local pH is significantly lower for $I \approx 0$ and 10 mM compared to its bulk value. For $I \approx 0$ mM, local pH is still a half unit lower than its bulk value at 9 nm from the surface. This is a rough approximation as Lyz is distributed in the bulk of the PPCs², while the pH variation was evaluated near the surface (the surface potential was used to compute pH variation). Inside PPCs², the electrical potential, and consequently the pH microenvironment, will be influenced by the distance between uncompensated charges. It is highly challenging to access this information, which depends on the conditions of PPCs² formation.⁵² Nevertheless, the approximation of the surface-pH variation gives a good idea of the pH likelihood to deviate from its bulk value, given the measured experimental surface charge and I that is used.²⁴⁴ The improved enzymatic activity in these conditions may thus come from a slightly more acid microenvironment, *i.e.* shift closer to pH 5 which is more favorable for Lyz (see Figure IV.28b). Importantly, this confirms that the catalytic activity of enzymes can be improved by simply mixing them with PEs.

Finally, the lower enzymatic activity observed in PPCs² constructed at $I=10$ mM compared to 0 mM might also originate from the lower (GlcNAc)₃-MeU diffusion. Indeed, larger PPCs² that could be centrifugated are present at $I=10$ mM, while soluble PPCs² are obtained at $I \approx 0$ mM (see Figure IV.40b of the supporting information). This difference was not evidenced in Chapter 2 using dynamic light scattering. Indeed, only the radius of the larger population was reported in Chapter 2, which might then account only for a small fraction of the objects.

4.2.2 Immobilization of lysozyme

Lyz was immobilized in a multiwell plate by two methods that were already used throughout this thesis: the classical LbL that is the alternate adsorption of single Lyz molecules with PSS_n [Lyz-PSS_n] (see Figure IV.31a), or the PPCs-based method that is the alternate adsorption of PPCs² with PAH [PAH-PPCs²] (see Figure IV.31b).

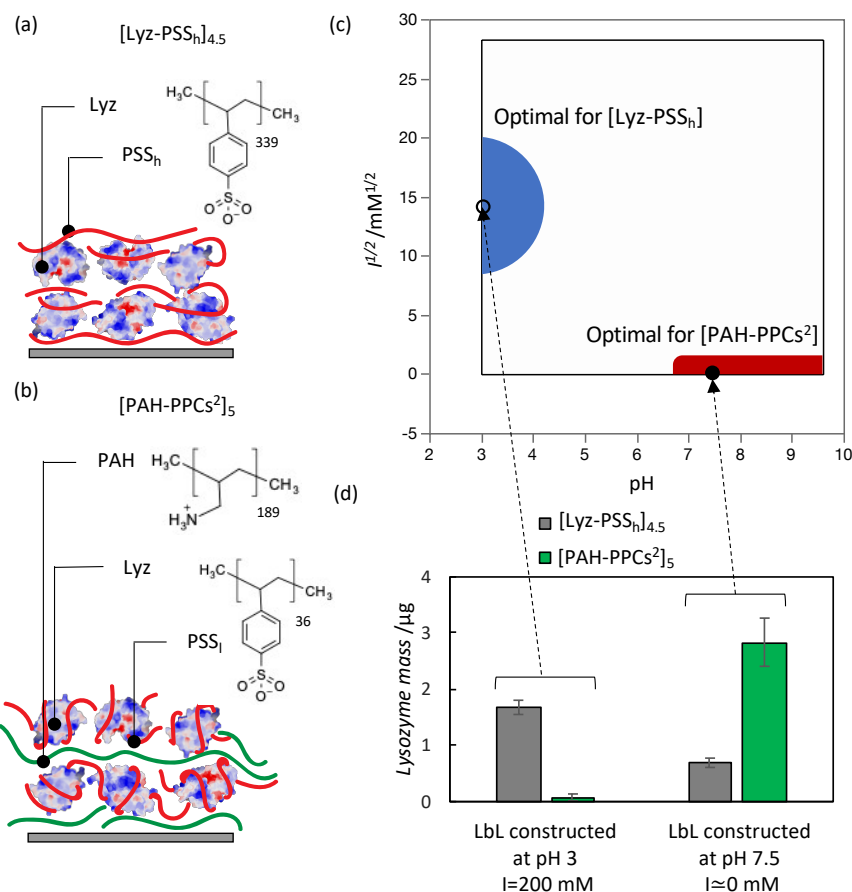


Figure IV.31 – Schematic representation of the LbL assembly of (a) Lyz with PSS_h [Lyz-PSS_h] or (b) PPCs² with PAH [PAH-PPCs²] – (c) Landscape of the different pH and I conditions that were used in this study. The blue half circle represents the optimal conditions for [Lyz-PSS_h] LbL growth and the red rectangle represents the optimal conditions for [PAH-PPCs²] LbL growth. The construction of both LbL types was carried out at pH 3 and I=200 mM (open circle), or at pH 7.5 and I≈0mM (closed circle). – (d) Lyz mass immobilized through LbL assembly of 4.5 bilayers of free Lyz with PSS_l ([Lyz-PSS_l]_{4.5}) or through LbL assembly of 5 bilayers of PAH with PPCs² ([PAH-PPCs²]₅). Error bars show the standard deviation n=3.

In both cases, 5 bilayers were constructed on a bilayer of [PAH-PSS_h]. These results are thus a partial repetition of those presented in Chapter 2. However, we find it crucial for the comprehension of the following to remind these. The landscape of pH and I conditions that were used in this Chapter is represented in Figure IV.31c to guide reading. On this Figure, the conditions that immobilize the highest protein amount for either [Lyz-PSS_h] or [PAH-PPCs²] are respectively represented as the blue half circle and the red

rectangle. The conditions that were used in this Chapter to immobilize Lyz are represented as the open (pH 3 and $I=200\text{mM}$) and the closed black circles (pH 7.5 and $I\approx 0\text{mM}$). Note that the same condition was used for PPCs² formation and for multilayer building. The Lyz content of the multilayers was quantified by a BCA assay after five protein adsorption steps for both [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ systems. The Lyz mass is presented in Figure IV.31d. It shows that 2.8 μg of Lyz per well can be immobilized using PPCs-based assembly at low I and neutral pH. This is significantly higher than using the classical LbL assembly that immobilizes 1.7 μg per well at best, as demonstrated in Chapter 2. It is worth to mention that very small Lyz amounts were immobilized from [PAH-PPCs²]₅ systems at pH 5 and $I=10\text{mM}$. It was thus decided not to further investigate the enzymatic activity of Lyz immobilized in this condition.

4.2.3 Release of lysozyme

After Lyz was immobilized in the multiwell plate, its release triggered by a 24h incubation in the reaction medium was investigated at 40°C or 4°C for different pH and I condition. Note that the reaction medium is the same as the one used for the enzymatic activity measurement, *i.e.* a (GlcNAc)₃-MeU solution which is in different pH and I conditions. In a view to further detect Lyz activity after its release from multilayers, conditions that lead to high Lyz activities in solution were selected. Based on data obtained in solution (Figure IV.28b), it was chosen to evaluate Lyz release at pH 5 and different I values ($I\approx 0$, 10 or 200 mM; orange ellipses in Figure IV.32a and b). When possible, it was also decided to measure the release of Lyz in the same condition as for the building of the multilayer. The amount of released Lyz was computed by difference between the initial Lyz amount in the multilayer and the one left on the walls of the well after incubation at 40°C or 4°C with the corresponding reaction medium and removal of that medium.

The [Lyz-PSS_h]_{4.5} multilayer constructed at pH 3 and $I=200\text{ mM}$ was thus incubated either in the same pH and I condition as for the construction, at higher pH and same I condition (pH 5 and $I=200\text{ mM}$), or at higher pH and lower I (pH 5 and $I=10\text{mM}$ – black crosses in Figure IV.32a). The percentage of Lyz that is released upon incubation in the reaction medium at 40°C or 4°C

is respectively shown in Figure IV.32c and e. It appears that 13% to 25% of the Lyz content is released in all conditions after incubation at 40°C or 4°C. [Lyz-PSS_h]_{4.5} multilayers are thus relatively stable upon pH, I or temperature changes.

The [Lyz-PSS_h]_{4.5} and [PAH-PPCs²] multilayers constructed at pH 7.5 and I≈0mM were incubated either at lower pH and same I as for the construction (pH 5 and I≈0 mM), or lower pH and higher I (pH 5 and I=10 or 200 mM – black crosses in Figure IV.32b). Unfortunately, it appears that it is not useful to investigate Lyz release in the same conditions as for the construction, since the Lyz enzymatic activity is too low to be detected (data presented in Figure IV.28 were obtained with 28 µg of Lyz and resulted in enzymatic activities hardly higher than the LOQ, while maximum 2.8 µg of Lyz are immobilized in multilayers).

The percentage of Lyz that is released upon incubation with the corresponding reaction medium at 40°C or 4°C is respectively shown in Figure IV.32d and f. Results show that the Lyz released from [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ is the same in all conditions. After incubation at 40°C, up to 100% of the Lyz content is released. At 4°C, the same trend is observed and between 75% and 100% of Lys is released from both [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ systems (see Figure IV.32f). These multilayers are thus highly sensitive to condition changes and Lyz release can be triggered upon reaction medium incubation in any condition (between 75% and 100% can be released). Heat was already reported to induce multilayer degradation, which explains the increased Lyz release as temperature rises.⁷⁶

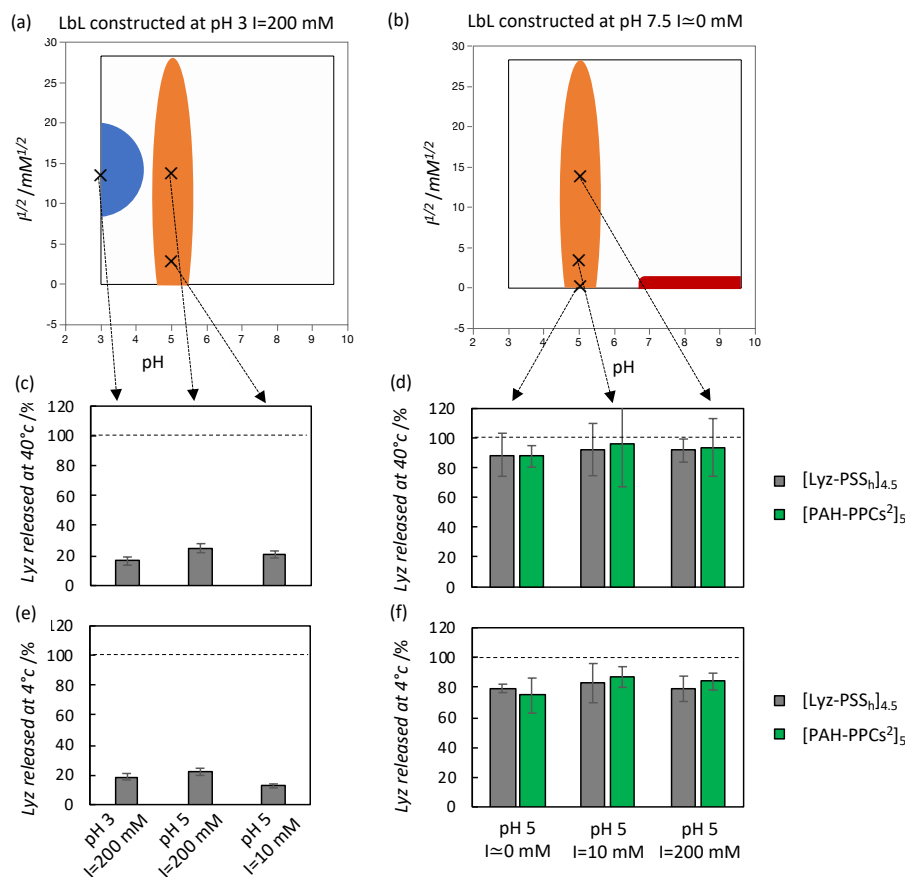


Figure IV.32 – Landscape of the different pH and I conditions to which LbLs were exposed. The blue half circle represents the optimal conditions for [Lyz-PSSi]_{4.5} LbL growth and the red rectangle represents the optimal conditions for [PAH-PPCs₂]₅ LbL growth. The orange ellipse represents the optimal conditions for Lyz activity. The landscape is presented for LbL films constructed at pH 3 and I=200mM (a) and at pH 7.5 and I≈0 mM (b) – Lyz released from [Lyz-PSSi]_{4.5} constructed at pH 3 and I=200 mM, and incubated in a reaction medium at pH 3 and I=200 mM, pH 5 and I=200 mM, or pH 5 and I=10 mM at (c) 40°C or (e) 4°C. – Lyz released from [Lyz-PSSi]_{4.5} or [PAH-PPCs₂]₅ constructed at pH 7.5 and I≈0 mM, and incubated at pH 5 and I=0, 10 or 200 mM, or pH 7.5 and I≈0 mM at (d) 40°C or (f) 4°C. Error bars show the standard deviation n=3. The amount of released Lyz is assessed by measuring the residual mass using a BCA assay.

4.2.4 Activity of LbL-assembled lysozyme

The enzymatic activity of Lyz was first assessed by simply incubating the reaction medium in the coated multiwell at 40°C. The enzymatic activity was expressed as K_{cat} , which was computed based on the MeU calibration curve and the total Lyz amount that was immobilized in each multilayer.

With respect to multilayers constructed at pH 3 and $I=200$ mM (Figure IV.33b), the only enzymatic activity that is significantly different from zero is obtained when the reaction medium is at pH 5 and $I=200$ mM. These results show the total enzymatic activity in the coated wells, without however distinguishing the contribution of immobilized Lyz from the one of released Lyz. As expected, it appears that Lyz is inactive at 4°C (see Figure IV.41, p176).²⁵⁰ By incubation at 4°C, it is thus possible to release inactive Lyz and to transfer it to a new plate that is subsequently incubated at 40°C. This procedure is represented in Figure IV.33d. In this way, only the enzymatic activity of Lyz that is released from the multilayer is measured. The obtained results are presented in Figure IV.33e. It appears that no activity is detected whatever the conditions. This strongly suggests that the Lyz enzymatic activity that was measured directly in the well (Figure IV.33b) originates from immobilized Lyz. Interestingly, no enzymatic activity is measured at pH 5 and $I=10$ mM whereas this condition shows maximal activity when Lyz is free in solution. The significant activity observed at $I=200$ mM might come from a LbL architecture that facilitates substrate diffusion. Indeed, the swelling of a LbL film was reported when the NaCl concentration was increased from 10 mM to 500 mM.²⁵¹

With respect to multilayers constructed at pH 7.5 and $I \approx 0$ mM, active Lyz is detected in all conditions. Interestingly, the activity of Lyz is independent of I in $[\text{Lyz-PSS}_h]_{4.5}$, while it increases with increasing I in $[\text{PAH-PPCs}]_5$. Moreover, Lyz activity is higher in $[\text{PAH-PPCs}]_5$ than in $[\text{Lyz-PSS}_h]_{4.5}$ for $I > 0$ mM (up to 3-fold higher – see Figure IV.33c). Since Lyz is almost fully released from the multilayers in these conditions (see Figure IV.32), the measured activity originates from released Lyz molecules solely. Still, the multilayers were incubated at 4°C, the medium was transferred in a new plate and incubated at 40°C in order to measure the enzymatic activity of the released Lyz (as it was done for multilayers constructed at pH 3 and $I=200$ mM). Results are presented in Figure IV.33f. High activities are detected, which confirms that these multilayers released their Lyz content and that the enzymatic activity originates from this released Lyz. The lower activities obtained after a release step performed at 4°C compared to 40°C could be partially attributed to a lower amount of Lyz released at 4°C compared to 40°C. However, at high I ,

the activity of Lyz is twice higher when the release occurs at 40°C compared to 4°C. This result cannot be explained by an increase in the released Lyz and will be further discussed later.

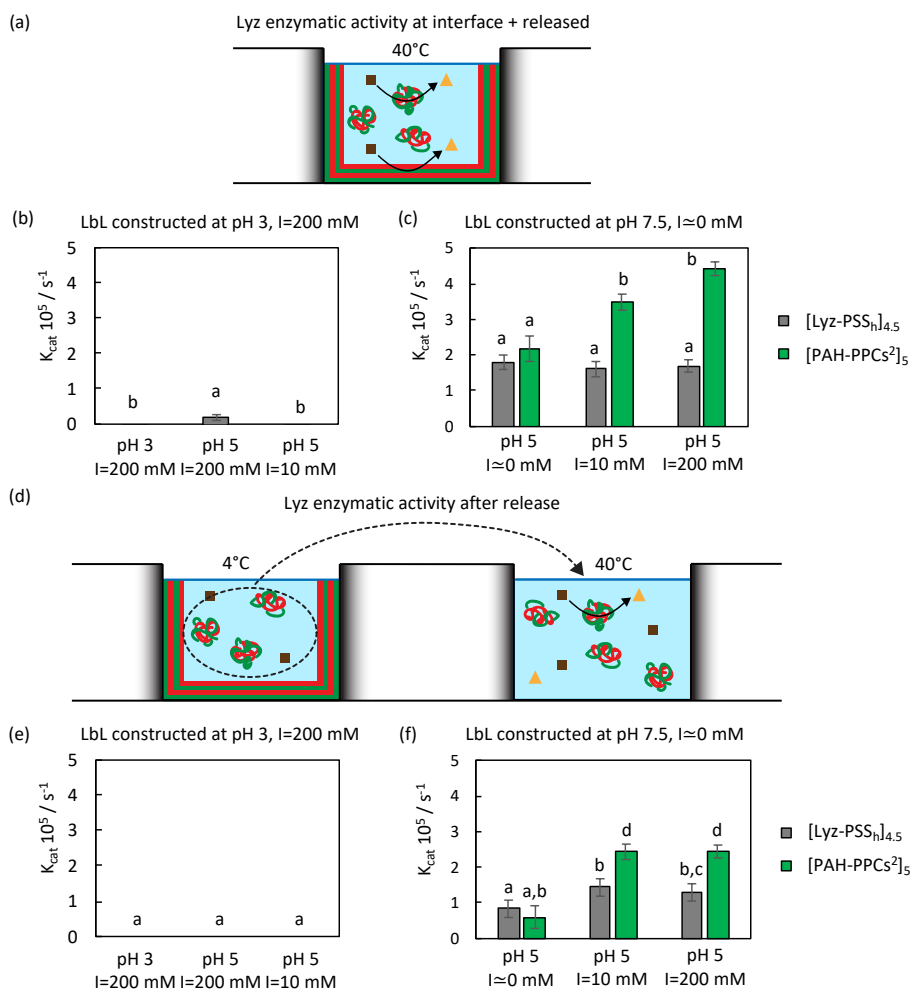


Figure IV.33 – (a) Lyz enzymatic activity at 40°C measured directly in the wells coated with [Lyz-PSS]_{4.5} or [PAH-PPCs]₅ at (b) pH 3 and I=200mM or (c) pH 7.5 and I=0mM. For each coating, enzymatic activity is measured in three different conditions. – (d) Wells coated with [Lyz-PSS]_{4.5} or [PAH-PPCs]₅ at (e) pH 3 and I=200mM or (f) pH 7.5 and I=0mM are incubated for 24h at 4°C with the reaction medium. The medium is then transferred into a new plate and incubated at 40°C before enzymatic activity is measured. All bars that share a common letter (within a given graph) are not statistically different ($\alpha=5\%$). Error bars show the standard deviation n=3.

Now that active Lyz has been located (in the multilayer or released in the solution), it is possible to evaluate the specific activity of Lyz that is kept after immobilization or release. This will be referred to as the Lyz residual activity. To do so, the activity of free Lyz molecules is compared to the one of Lyz immobilized in the multilayers or released from the multilayers.

As demonstrated hereabove, the enzymatic activity measured in [Lyz-PSS_h]_{4.5} constructed at pH 3 and I=200 mM originates from interfacial Lyz. The Lyz mass left in these multilayers after incubation of the reaction medium at 40°C is of 1.5 µg, 1.4 µg and 1.5 µg respectively at pH 3 and I=200 mM, pH 5 and I=200 mM, and pH 5 and I=10 mM (as deduced from results presented in percent of released Lyz in Figure IV.32c). The enzymatic activity of a corresponding free Lyz mass can be calculated via a linear fitting of the fluorescence as a function of Lyz mass (Figure IV.42a, p176). This calibration was repeated for all pH and I conditions. Moreover, note that the wells used for calibration were coated with PAH to prevent Lyz adsorption and subsequent activity loss (PAH and Lyz are both positively charged at pH 3 and 5). In this way, the enzymatic activity of 1.5 µg, 1.4 µg and 1.5 µg per well of free Lyz molecules are known and compared to the one measured after incubation of the reaction medium in a multiplate coated with [Lyz-PSS_h]_{4.5} (where only the immobilized Lyz participate to the measured activity, see the comparison between Figure IV.33b and Figure IV.33e). This is expressed as the residual Lyz activity in the multilayer (in % of the activity of free Lyz molecules) and results are presented in Figure IV.34a for all conditions. It appears that only 10% of the Lyz enzymatic activity has been retained in the only condition that has an activity significantly different from zero (pH 5, I=200 mM). The activity of enzymes that are immobilized in LbL is indeed usually much smaller compared to the one of free enzyme molecules.⁷³ This can be attributed to the limited diffusion of both the substrate and the product of the enzymatic reaction, and/or to a fraction of Lyz that is in an inactive conformations due to the presence of the PSS_h. The location of active Lyz is schematized in Figure IV.34a and shows immobilized enzymes hydrolyzing (GlcNAc)₃-MeU.

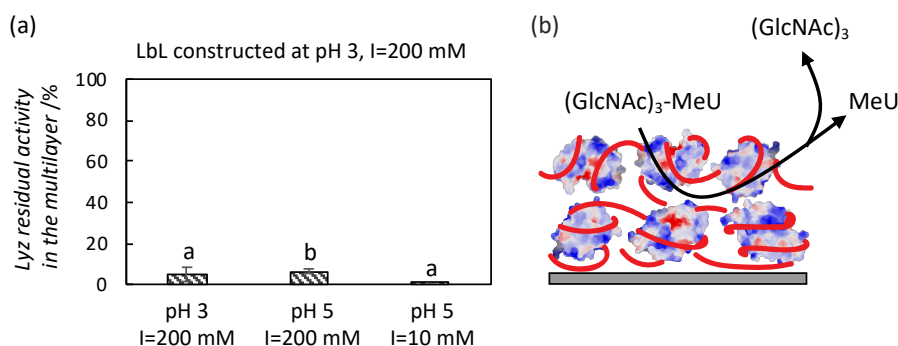


Figure IV.34 – (a) Activity of Lyz immobilized in [Lyz-PSS_h]_{4.5} constructed at pH 3 and I=200 mM compared to the activity of free Lyz in different conditions. (b) Schematic of the active Lyz in [Lyz-PSS_h]_{4.5}. Error bars show the standard deviation n=3.

With respect to [Lyz-PSS_h]_{4.5} and [PAH-PPCs]₅ multilayers constructed at pH 7.5 and I≈0 mM, it was demonstrated hereabove that the measured activity originates from released Lyz. Depending on the temperature, these multilayers indeed release between 75% and 100% of their Lyz content (Figure IV.32d and f), which corresponds to masses ranging from 1.7 to 3.5 µg of released Lyz per well. As it was done for [Lyz-PSS_h]_{4.5} constructed at pH 3 and I=200 mM, a calibration based on the recorded fluorescence as a function of free Lyz mass was used to compute the activity of a Lyz mass corresponding to the one that has been released from multilayers. The difference between multilayers constructed at pH 3 - I=200 mM, and pH 7.5 - I≈0 mM is thus that the interfacial Lyz was taken into account for the former, while the released Lyz was used for the latter. Moreover, two different protocols were used to measure the activity of Lyz. The first one is exactly the same as for multilayers constructed at pH 3 - I=200 mM: the reaction medium is directly incubated in the well at 40°C (Figure IV.33a). The fitting presented in Figure IV.42a (p176) can thus be used to compute the residual activity after release. Note that the consequence of the latter protocol is that the release of Lyz occurs at 40°C. For the second protocol, multilayers were incubated at 4°C, then transferred and incubated at 40°C to launch the enzymatic reaction (see Figure IV.33d). The fluorescence as a function of Lyz mass was thus measured for Lyz undergoing the same treatment (see Figure IV.42b, p176). Importantly it means that, with this second protocol, the release of Lyz has occurred at 4°C. In conclusion, the

residual Lyz activity after release was computed for Lyz released from [Lyz-PSS_h]_{4.5} or [PAH-PPCs] constructed at pH 7.5 and I $\approx$ 0 mM. The release was triggered by incubation at either 4°C or 40°C, and at either I $\approx$ 0, 10 or 200 mM. Results of the residual activity are presented in Figure IV.35a for [Lyz-PSS_h]_{4.5} (grey bars) and in Figure IV.35b for [PAH-PPCs]₅ (green bars). For the sake of discussion, the enzymatic activity measured in PPCs² (in solution) compared to free molecules is presented as the red bars in Figure IV.35a and b. These red bars are thus a partial reproduction of data presented in the isoreponse map shown in Figure IV.28d.

When Lyz is released from [Lyz-PSS_h]_{4.5} constructed at pH 7.5 and I $\approx$ 0 mM, it appears that between 19% and 35% of Lyz activity is recovered. It also appears that temperature and I have a very small effect on the Lyz residual activity after release (significance tested using the t-test function of JMP, all bars that share the same letter cannot be considered significantly different, $\alpha=5\%$). Indeed, except for Lyz released at 40°C and I $\approx$ 0 mM that is higher, all residual activities after release are equal if the temperatures are compared for a given I and the different I values are compared for a given temperature.

Two hypotheses can be invoked to explain a loss of enzymatic activity after release. The first is that Lyz molecules that are released are at least partly denatured and lost their enzymatic activity. This hypothesis is unlikely given the very high Lyz stability, especially in PE multilayers. For instance, we have shown in Chapter 1 that Lyz simply adsorbed on a polystyrene plate is still active. The second hypothesis that may explain the low residual activity after release is that, upon release from [Lyz-PSS_h]_{4.5}, Lyz is still complexed with PSS_h. This is schematized in Figure IV.35c. This is consistent with data reported on the activity of Lyz released from multilayers.²³⁵ Indeed, Lyz loses activity at pH 5 upon complexation with PSS_i (as demonstrated in the isoreponse map in Figure IV.28d). It might be argued that, if complexes were present, the same residual activity evolution as a function of I should be obtained when compared to PPCs² in solution (red bars in Figure IV.35a). However, PSS_i was used in PPCs² while PSS_h was used to immobilize Lyz in [Lyz-PSS_h]_{4.5}. Comparison is thus not straightforward. Yet, the same range of

residual activity is obtained when both are compared, *i.e.* from 16% to 43% for Lyz in PPCs² *versus* 19% to 35% for Lyz released from [Lyz-PSS_h]_{4.5}.

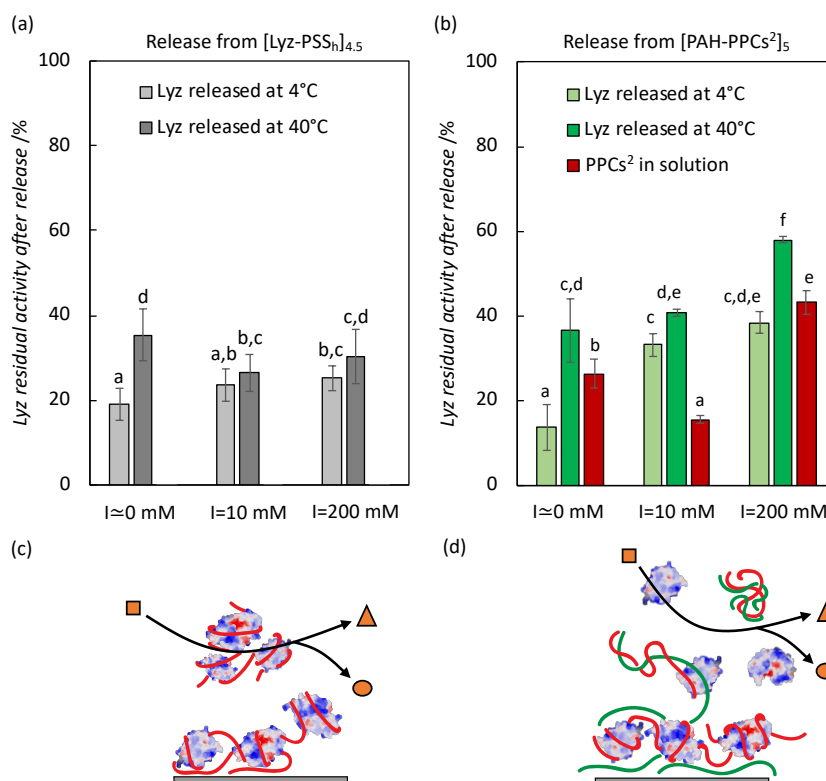


Figure IV.35 –Lyz residual activity at 40°C, pH 5 and I ≈ 0, 10 or 200 mM (0, 3.2, 14.1 mM^{1/2}) after release at 4°C or 40°C from (a) [Lyz-PSS_h]_{4.5} or (b) [PAH-PPCs²]₅ constructed at pH 7.5 and I ≈ 0 mM. (c) and (d) Schematic of the active Lyz in different conditions. (c) [Lyz-PSS_h]_{4.5} is unstable and releases complexes between PSS_h and Lyz. (d) [PAH-PPCs²]₅ is unstable and releases free Lyz (see discussion in text). Data that do not share the same letter are significantly different (p-value < 0.05). Error bars show the standard deviation n=3.

With respect to Lyz released from [PAH-PPCs]₅ constructed at pH 7.5 and I ≈ 0 mM, the residual activity after release ranges between 13% and 58%. Contrarily to Lyz released from [Lyz-PSS_h]_{4.5}, the residual activity increases with both the temperature and the I value. Indeed, almost all residual activities after release are significantly different if the temperatures are compared for a given I and the I values are compared for a given temperature (the only exceptions being for I values that are close to each other: the comparison of the release at 40°C and I ≈ 0 mM *versus* I = 10 mM, or at 4°C and

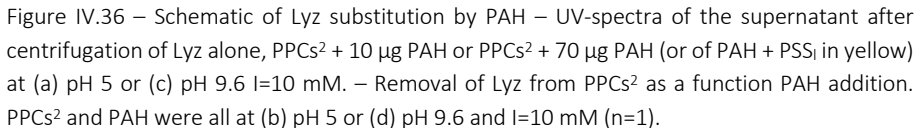
$I=10$ mM *versus* $I=200$ mM). Importantly, when compared to Lyz activity in PPCs² (red bars), Lyz that is released at 40°C recovers a higher activity for all I that were probed. This clearly suggests that in the above-mentioned conditions, a fraction of Lyz has been released from PPCs². This is attributed to the presence of PAH that interacts with PSS thereby being exchanged with Lyz in the multilayer, which releases a higher fraction of Lyz in a naked, non complexed, state, as represented in Figure IV.35d. The competitive binding has already been suggested in other protein-based systems and modeled for PECs reorganization upon addition of a third PE.^{52,210,252} Interestingly, it has been theoretically and experimentally shown that the substitution of two associated polyions by another polyion increases with salt and temperature, which is consistent with our data.

4.2.5 Competitive substitution of lysozyme by PAH

The potential of PAH to competitively substitute Lyz in PPCs² was investigated. To this end, increasing amounts of PAH were added to PPCs² constructed at pH 5 or pH 9.6 and $I=10$ mM. Mixed suspensions of PAH and PPCs² were then centrifuged and Lyz was quantified in the supernatant *via* UV molecular absorption. The salt concentration was chosen as PPCs² could not be centrifuged at $I\approx 0$ mM (see Figure IV.40 of the supporting information). The complete UV spectra of the supernatant is presented in Figure IV.36a for Lyz alone, PPCs² after the very first PAH addition, and after the addition of 70 μ g of PAH. A mixed solution of PSS_i and PAH was used as a control. For PPCs² constructed either at pH 5 or pH 9.6, the UV-spectra obtained after the addition of 70 μ g of PAH are the same as the ones obtained for Lyz alone. If soluble PPCs² were obtained, features of the PSS_i molecular absorption, *i.e.* maximum at around 260 nm, would be observed. (see Figure IV.40 of the supporting information). Since this is not observed, the presence of soluble PPCs² can be discarded, which confirms that Lyz molecules that are not complexed are obtained. Note that at pH 5, the absorbance is shifted to higher values (by around 0.028 at 280nm wavelength) when PAH is added to either PPCs² or PSS_i (see Figure IV.36a). This contribution, which is due to the slight turbidity of the solution, was subtracted in order to quantify Lyz. Results of the Lyz substitution as a

function of PAH addition are presented in Figure IV.36b and d. It appears that the Lyz fraction that is complexed gradually decreases upon PAH addition for PPCs² at pH 5 (Figure IV.36b) and pH 9.6 (Figure IV.36c). These results confirm that PAH is able to compete with Lyz for the association with PSS, which causes the release of Lyz in a non complexed, naked form. The competitive substitution is even possible in conditions where PAH has an ionization degree around 40%, *i.e.* at pH 9.6.¹⁶⁵ At lower pH, a lower PAH mass is enough to release Lyz from PPCs². This result confirms that by lowering the pH, the affinity of PAH for PSS_i increases which causes its favored substitution with Lyz. Moreover, the ability of PAH to displace Lyz and complex PSS is consistent with its reported very high binding strength with PSS.²⁵³ Interestingly, this suggests that it could be possible to control the substitution, and *in fine* the multilayer stability, by changing the PE-PE coupling strength. Such exchange of charged species have already been observed in poly(l-glutamic acid)-poly(l-lysine) [PGA-PLL] multilayers. When PSS was deposited on top of a [PGA-PLL] multilayer, PSS diffuses into the [PGA-PLL] film and exchanges partially with the PGA chains.²⁵⁴

With respect to the recovery of the enzymatic activity, Tomita *et al.* have shown in a similar experiment that the activities of ribonuclease A, Lyz, cellulase and α -amylase were lost upon complexation with PAA or PAH depending on their charge. Excepted for α -amylase, these could be fully restored by addition of an oppositely charged PE.²⁵⁵ Since then, such so-called complementary polymer pair system (CPPS) has been used numerous times as an on/off switch of enzyme activity.^{255–257}



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It is thus clear that either particular PE properties (light responsiveness, degradability) or added competitive molecules are needed to easily trigger the release of intact protein from/disassembly of polyelectrolyte multilayers (PEMs) (see introduction section).^{76,233} To our knowledge, reorganization of a simple PEM (no particular PE property) *via* an intrinsic stimulus (no added chemical moiety), *i.e.* self-reorganization, has never been achieved, neither in solution nor at interfaces. Intrinsic stimulus effects are usually restricted to a decreased strength of the coupling that drives the self-assembly.^{261,262}

4.3. Conclusion

In this Chapter, Lyz activity in the presence of PSS_I was first investigated over a large range of pH and I conditions in solution. It was evidenced that Lyz loses activity upon complexation with PSS_I, except over a wide range of pH at very high I, as well as at pH 7.5 and low I. It was demonstrated that at high I, an important fraction of Lyz is not complexed, which results in the measured maintain of Lyz activity in the presence of PSS_I. Interestingly, when Lyz was complexed with PSS_I at pH 7.5 and I $\approx$ 0 or 10 mM, it resulted in activities that are respectively two and six-fold higher compared to the one of free Lyz molecules. It was proposed that the PE presence creates a pH microenvironment that is more favorable for Lyz.

[Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ assemblies were then constructed using the pH and I condition that immobilize the most Lyz, *i.e.* respectively at pH 3 – I=200 mM and pH 7.5 – I $\approx$ 0 mM (as demonstrated in Chapter 2). Their stability upon incubation in the reaction medium that is used to measure the enzymatic activity was then investigated. It appears that [Lyz-PSS_h]_{4.5} constructed at pH 3 – I=200 mM is stable over a wide range of conditions. Contrarily, [Lyz-PSS_h]_{4.5} and [PAH-PPCs²]₅ constructed at pH 7.5 – I $\approx$ 0 mM release between 75 and 100% of their Lyz content in all conditions.

A comprehensive analysis of the Lyz activity was then presented. It was evidenced that the measured activity in [Lyz-PSS_h]_{4.5} constructed at pH 3 – I=200 mM originates from interfacial Lyz. The activity of Lyz immobilized in the latter multilayer was shown to represent only 10% of the activity of the same mass of free Lyz.

For [Lyz-PSS_n]_{4.5} and [PAH-PPCs²]₅ constructed at pH 7.5 – I≈0 mM, it was shown that the measured activity mostly originates from the released Lyz. Interestingly, when released from [Lyz-PSS_n]_{4.5}, Lyz retains up to 35% of its activity, while it retains up to 58% of its activity upon release from [PAH-PPCs²]₅. This difference was attributed to the release of Lyz-PSS_n complexes from [Lyz-PSS_n]_{4.5}, while a significant fraction of native, non complexed, Lyz was released from [PAH-PPCs²]₅. We hypothesized that the presence of PAH that substitutes Lyz in [PAH-PPCs²]₅ could explain the higher residual activity after release from the latter multilayer. Since both PAH, PSS_i and Lyz are present in the multilayer (no added chemical moiety), it is suggested that a self-reorganization of the multilayer occurs. The hypothesis of a self-reorganizing multilayer was thus supported by correlating the Lyz activity in solution (free molecules or in PPCs²) to the one obtained after release. In this way, it was shown that Lyz in PPCs² (created in solution) is less active than upon its release from [PAH-PPCs²]₅ (both are compared to the activity of free Lyz molecules). It was concluded that, given the very low Lyz tendency to unfold, a significant fraction of native Lyz has been released from [PAH-PPCs²]₅. Moreover, the temperature dependence that is observed is consistent with reported simulations for a similar system.

In order to bring more evidence to this, a competitive substitution was achieved in solution by adding PAH to PPCs². It was shown that by lowering the pH, less PAH had to be added to substitute Lyz in PPCs² (at a constant I). This clearly suggests that such a substitution could occur by decreasing the pH of a multilayer that contains all three charged species: PAH, Lyz and PSS_i.

In this Chapter, an intrinsic stimulus is proposed to cause a competitive substitution between three macromolecules self-assembled at interfaces, which results in the release of a higher fraction of intact protein molecules. The latter release is thus not triggered by a decreased strength of the protein-PE interaction, but rather by an active substitution of the protein by a PE. PPCs are thus highly promising in the design of systems that aim at delivering intact molecules. It also raises the importance of creating self-assembled systems by associating macromolecules using a particular sequence to obtain new properties.

4.4. Materials and methods

4.4.1 Solutions

All solutions were buffered with 10 mM citrate, acetate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or carbonate and pH was respectively adjusted at pH 3.0, 5.0, 7.5 or 9.6 with NaOH or HCl. I was adjusted to 10, 200 or 800 mM by adding NaCl (Merck, Darmstadt, Germany) to the buffer. Solutions at $I \approx 0$ mM were obtained by only adding NaOH or HCl to ultrapure water until the desired pH was reached.

4.4.2 Enzymatic activity of free Lysozyme and PPCs²

The solutions of free, *i.e.* not complexed, Lyz were realized by adding 2 volumes of buffer to 5 volumes of Lyz solution (0.2 g L^{-1}). All solutions were at pH 3, 5, 7.5 or 9.6, and $I \approx 0$ mM, 10 mM, 200 mM or 800 mM before mixing. PPCs² with a constant (-)/(+net) charge ratio of 2 were prepared by adding sodium poly(styrene 4-sulfonate) solution (PSS_i, $M_w=7,540 \text{ g mol}^{-1}$, Scientific Polymer, Ontario, USA) of different concentrations to Lyz solution (0.2 g L^{-1}). Indeed, Lyz charge changes with pH and the PSS_i concentration has to change accordingly. The number of charges per Lyz molecule was computed as a function of pH based on its amino acid sequence and structure (PDB file: 1HEL) using PDB2PQR.²⁶³ 0.2 g L^{-1} of Lyz at pH 3, 5, 7.5 or 9.6. gives the following charge concentration: 0.238 mM of negative charge at pH 3 (unfolded state considered), 0.133 mM of negative charge at pH 5, 0.112 mM of negative charge at pH 7.5 and 0.084 mM of negative charge at pH 9.6. PPCs² were thus formed by adding 2 volumes of PSS_i solution of respectively 0.247 g L^{-1} , 0.138 g L^{-1} , 0.116 g L^{-1} or 0.087 g L^{-1} , *i.e.* 1.199 mM, 0.669 mM, 0.565 mM or 0.423 mM of negative charge, to 5 volumes of Lyz solution of 0.2 g L^{-1} at pH 3, 5, 7.5 or 9.6. The pH and I of all solutions was adjusted before mixing Lyz with PSS_i.

For the enzymatic activity, 20 μL of 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside (GlcNAc)₃-MeU, 0.1 g L^{-1} , Steinheim, Germany) in DMSO/water 2:1 (v:v) stock solution were added to each well containing 140 μL of free Lyz or PPCs² (prepared as described hereabove) and 40 μL of the

corresponding buffer. (GlcNAc)₃-MeU was used since it is a soluble substrate and the MeU produced is fluorescent which enable its detection. The plate was kept under agitation (250 rpm) for 24h at 40 °C. 180 µL of reactional media were then transferred to a black 96-well plate and mixed with 50 µL of 200 mM glycine buffer at pH 10.6 (adjusted with NaOH). The fluorescence was measured with excitation and emission wavelengths respectively set at 360 nm and 450 nm (Tecan Infinite 200 Pro, Männedorf, Switzerland). The gain was set at 80 for the measurement of the activity in solution/suspension and at 100 for the measurement of the activity in multilayers. As a control, emission of only PSS_i (same concentration as in PPCs²) incubated at 40°C with (GlcNAc)₃-MeU was measured (Figure IV.38). K_{cat} was computed based on the amount of Lyz and the MeU produced by the reaction. The amount of MeU was found using its fluorescence as a function of concentration. The enzymatic activity in PPCs² was compared to the one of free Lyz molecules by computing the ratio of Lyz activity in PPCs² to free Lyz activity.

4.4.3 Multilayer construction

The PPCs² that were used to immobilize Lyz in LbL were also constructed such as $(-)/(+net)=2$. However, in that case the negative charges in the system were kept constant at different pH values by increasing Lyz amount with increasing pH. PPCs² were thus created by adding 2 volumes of a low molar mass sodium poly(styrene 4-sulfonate) (PSS_i, Mw=7,540 g mol⁻¹, Scientific Polymer, Ontario, USA) solution (0.5 mM negative charge) to 5 volumes of a Lyz (Sigma-Aldrich, Steinheim, Germany, isoelectric point (iep) = 11.35) solution (0.1 mM net positive charge).

Multilayers were constructed in 96-well polystyrene plates (Greiner Bio-One®) by successive adsorption steps. For all experiments, two bilayers of poly(allylamine hydrochloride) (PAH, Mw=17,500 g mol⁻¹, Sigma-Aldrich, Steinheim, Germany) alternately adsorbed with high molar mass poly(styrene 4-sulfonate) (PSS_h, Mw=70,000 g mol⁻¹, Sigma-Aldrich, Steinheim, Germany) were constructed from PAH and PSS_h solutions (0.5 g L⁻¹). Then, two different multilayers were built on this anchoring layer: bare Lyz molecules assembled with PSS_h, or PPCs² assembled with PAH. The following solutions were used: PSS_h (0.5 g L⁻¹), Lyz (0.07 mM positive net charge), PAH

(0.5 g L⁻¹) and PPCs² prepared as described in *PPCs² preparation*. These solutions were buffered as described in *PPCs² preparation*. Each adsorption step was carried out with 200 µL of solution. Adsorption lasted for 10 min for the construction of the anchoring layer made of PAH and PSS_h, and 40 min for all subsequent layers. Each adsorption step was followed by three consecutive rinsing steps with the corresponding buffer.

4.4.4 Protein quantification using BCA assay

Protein amount within LbL assemblies was assessed by bicinchoninic acid assay (BCA, Thermo scientific, Rockford, USA). 20 µL of sodium dodecyl sulfate (SDS, 1%) was added to ensure protein solubilization before quantification. The working reagent (200 µL, reagent A:reagent B, 50:1, v:v) was then added and incubated 60 min at 60 °C before absorbance was measured at 562 nm. Lyz amount was determined based on a calibration curve obtained with known Lyz amounts. The high pH of the BCA buffer causes complete Lyz and PAH protonation, which results in the dissolution of the film.⁵² SDS was added to further ensure complete film dissolution.

4.4.5 Enzymatic activity of after immobilization

20 µL of 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotrioside (GlcNAc)3-MeU, 0.1 g L⁻¹, Steinheim, Germany) in DMSO/water 2:1 (v:v) stock solution were mixed with 180 of different buffers prepared as described in *PPCs² preparation*, and added to each well containing multilayers.

For the enzymatic activity assay in the coated well, the plate was kept under agitation (250 rpm) for 24h at 40 °C. 180 µL of reactional media were then transferred to a black 96-well plate and mixed with 50 µL of 200 mM glycine buffer at pH 10.6. Fluorescence was then measured. As a control, emission of a multilayer incubated at 4°C was measured and presented in Figure IV.41.

For the enzymatic activity assay carried-out with released Lyz, the plate was kept under agitation (250 rpm) for 24h at 4 °C. Then 180 µL were transferred to an uncoated plate and incubated at 40°C for 24h. 180 µL of reactional media were then transferred to a black 96-well plate and mixed with 50 µL of 200 mM glycine buffer at pH 10.6. Fluorescence was then measured.

In order to assess for the fluorescence as a function of Lyz mass, a plate was coated with PAH 0.5 g L^{-1} for 20 min and washed three times with water before that Lyz was added. This allows to hinder Lyz surface adsorption and subsequent decreased enzymatic activity. The enzymatic activity was then measured as presented above, *i.e.* either by incubation at 40°C or by incubation at 4°C followed by a transfer into a new plate.

4.4.6 PAH competitive substitution to Lyz in PPCs²

1.4 ml of PPCs² were constructed the same way as for the enzymatic activity measurement at pH 7.5 or 9.6 ($I = 10 \text{ mM}$). To these PPCs², between 20 and $140 \mu\text{l}$ of PAH 0.5 g L^{-1} (same pH and I condition as PPCs²) were added. Then, the volume was brought to $1.540 \mu\text{l}$ with corresponding buffer for all samples, *i.e.* the same Lyz concentration was thus obtained. PPCs² were centrifugated at $22,830 \times g$ for 15 min and 1 ml of supernatant was transferred to a spectrophotometric cuvette. The full UV-spectra was recorded for the supernatant of PPCs² with 20 or $140 \mu\text{l}$ of PAH added and only Lyz. For the condition pH 7.5, $140 \mu\text{l}$ of PAH was added to PSS_I (same concentration as in PPCs²) and the UV-spectrum of the supernatant is also presented. The amount of Lyz in the supernatant was quantified using its atomic absorption at 280 nm (using a DU800 spectrophotometer (Beckman Coulter, USA)).

4.5. Supporting information

4.5.1 PSS_I fluorescence in the reaction medium and MeU calibration curve

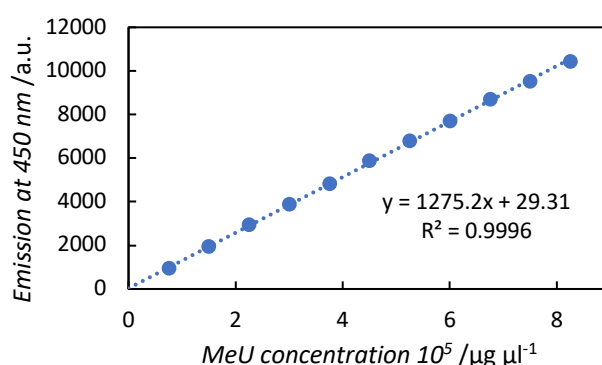


Figure IV.37 - Calibration curve of the MeU fluorescence (gain=80).

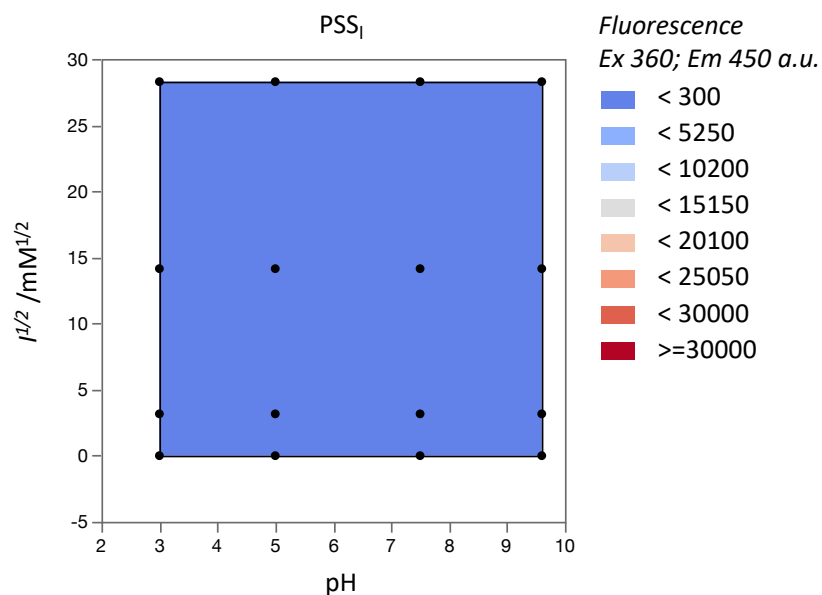


Figure IV.38 – Fluorescence of PSS_I incubated with the reaction medium used for enzymatic activity. The fluorescence is presented under the form of an isoresponse map as a function of pH and $I^{1/2}$. Black dots show mean values of experimental data ($n=3$).

4.5.2 Activity of Lyz in $PPCs^2$ compared to free Lyz molecules

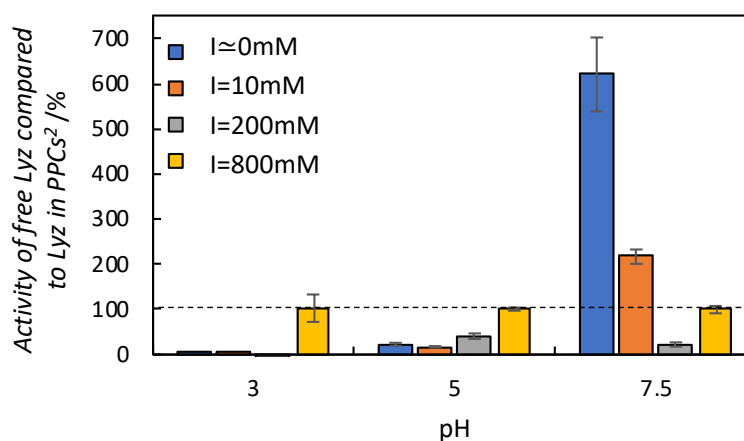


Figure IV.39 – Lyz activity in $PPCs^2$ compared to activity of free Lyz in different conditions of pH and I . The error bars show associated standard deviation. Error bars show the standard deviation $n=3$.

4.5.3 Evaluation of the Lyz fraction complexed at pH 7.5 and different I

The UV absorption spectra of PSS_i, Lyz and centrifuged PPCs² was recorded on a DU800 spectrophotometer (Beckman Coulter, USA). For Lyz, 2 mL of a 0.2 g L⁻¹ Lyz solution was mixed with 0.8 ml of buffer at pH 7.5 and $I=10$ mM. The PSS_i solution was obtained by mixing 0.8 ml of a 0.116 g L⁻¹ PSS_i solution with 2 ml of buffer at pH 7.5 and $I=10$ mM. PPCs² were obtained by mixing 0.8 ml of 0.116 g L⁻¹ PSS_i with 2 ml of a 0.2 g L⁻¹ Lyz solution, all solutions are at pH 7.5 and $I \approx 0, 10, 200$ or 800 mM. Lyz and PSS_i are thus at the same concentration in all conditions. PPCs² were then centrifuged at $22,830 \times g$ for 15 min and 1 ml of supernatant was transferred to a cuvette. The obtained spectra are presented in Figure IV.40a. It appears that both PSS_i and Lyz absorb light. For the supernatant of PPCs² prepared at $I=10, 200$ or 800 mM, spectra with the same shape as free Lyz were obtained (data shown only for $I=800$ mM). This suggests that PPCs² were formed and that all PSS_i was complexed with Lyz and thus eliminated from the supernatant after centrifugation. An accurate Lyz quantification could thus be performed using Lyz maximal atomic absorption at 280 nm. The Lyz fraction in PPCs² that was obtained is presented in Figure IV.40b. It appears that a substantial fraction of Lyz is complexed at $I=10$ mM and 200 mM, but a large fraction of Lyz is still not complexed at $I=800$ mM. However, no PSS_i is detected, which suggests that PPCs² are nevertheless formed and centrifuged at $I=800$ mM. This is supported by the reported small turbidity of this system and the presence of negatively charged objects before centrifugation.⁵² With respect to PPCs² constructed at $I \approx 0$ mM, it appears that PSS_i and Lyz are both present in the supernatant after centrifugation. The presence of PSS_i thus increases the UV absorption at 280 nm, resulting in an overestimation of Lyz content in this condition (see Figure IV.40b). Taken together with other data reported on this system, *i.e.* small charged objects are detected by dynamic light scattering and streaming potential, these results strongly suggest that soluble complexes are obtained in these conditions.⁵²

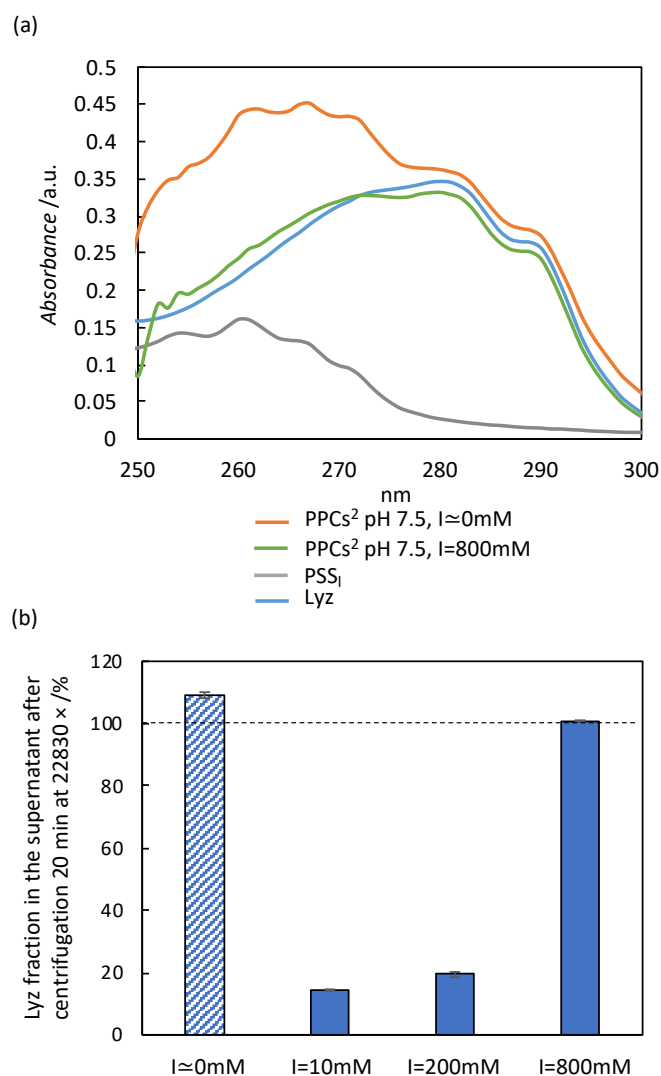


Figure IV.40 – (a) UV absorption spectra of PSS_I, Lyz and PPCs² at I≈0mM or 800mM (pH 7.5). (b) fraction of Lyz found in the supernatant after centrifugation at 22,830 × g for 15 min. Error bars show the standard deviation n=3.

4.5.4 Multilayers at 4°C

Enzymatic activity of immobilized Lyz at 4°C

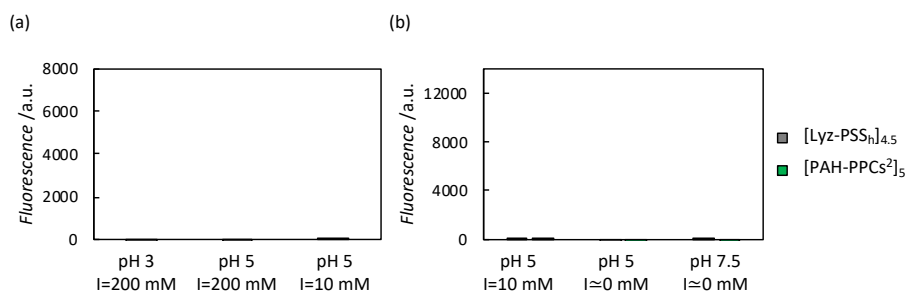


Figure IV.41 – Lyz enzymatic activity at 4°C measured directly in the wells coated with [Lyz-PSSh]_{4.5} or [PAH-PPCs₂]₅ at (a.1) pH 3 and I=200mM or (a.2) pH 7.5 and I=0mM. For each coating, enzymatic activity is measured in three different conditions. The scale is the same as for results presented in other sections, the purpose being to show that no signal is measured. Error bars show the standard deviation n=3.

4.5.5 Enzymatic activity as a function of the Lyz mass at 40°C for different pH and I conditions

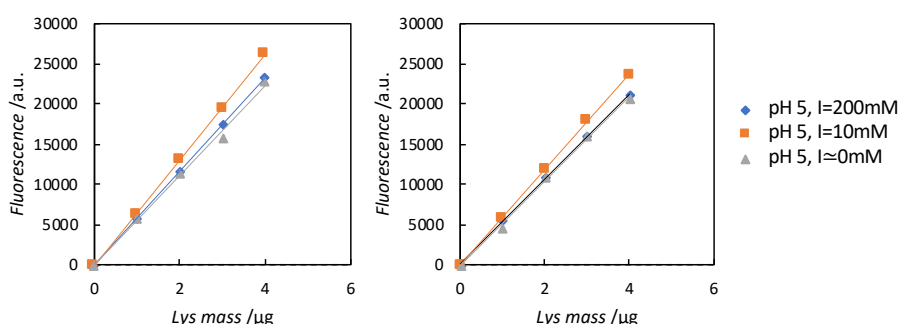
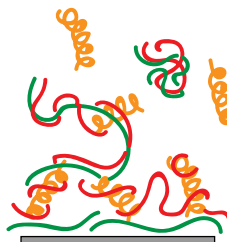


Figure IV.42 – Enzymatic activity as a function of Lyz mass at 40°C at pH and I=0, 10 or 200 mM. On the left panel Lyz is directly incubated with the reaction medium at 40°C. On the right panel Lyz is first incubated at 4°C with the reaction medium, then transferred to a new plate that is incubated at 40°C. Error bars show the standard deviation n=3.

5. Towards LL37-coated bandage to promote wound-healing



In this Chapter, the potential of the method that has been developed is tested for a biomedical application. The aim is to create a bioactive coating for dressings *via* the surface immobilization of LL37. This small AMP, which is produced by the human immune system, possess angiogenic and antimicrobial properties. LL37 was first complexed with alginate to form PPCs_{l/a} and the properties of the complex formed were investigated in solution. As with Lyz complexation with PSS_i, the charge of the PPCs_{l/a} could be easily tuned. Moreover, it appears that LL37 takes an α -helical conformation upon complexation with alginate. LL37 sensitivity to protease was also tested when it is alone or complexed. It appears that depending on the buffer, either LL37 sensitization or protection against trypsin activity is achieved through PPCs_{l/a} formation. This was attributed to the presence of alginate that either protect the LL37 against trypsin activity or get released from PPCs_{l/a} and forms a complex trypsin, which subsequently improve its hydrolytic activity. PPCs_{l/a} or bare LL37 molecules were then alternately adsorbed with respectively chitosan or alginate on a Tegaderm Contact dressing. We demonstrate that the classical bare protein LbL assembly fails to immobilize any LL37, while up to 5 μg of LL37 per cm^2 were immobilized using PPCs_{l/a}. This confirms the potential of PPCs-based LbL assembly. The LL37 release profile was investigated and it was decided the dressing should be changed every four days. Finally, the set-up was developed in order to test *in vivo* the effect of LL37-containing multilayers on wound healing.

5.1. Acknowledgements

This Chapter was highly collaborative and would not have been possible without the expert contributions of Prof. Pr  at, Bernard Ucakard and Kevin Vanvarenberg (LDRI, UCLouvain) for the *in vivo* assays, and of Prof. Mireille Dumoulin (ULg) for the circular dichroism measurements. This Chapter also benefited from Prof. Delcorte and Claude Poleunis (UCLouvain) for the ToF-SIMS images, and from Prof. Elias (UCLouvain) for the fluorescence spectroscopy measurements. Results presented were made possible by the comprehensive preliminary results obtained by Laure Lambrecht (UCLouvain). Finally, Anna Bratek-Skicki kindly did the streaming potential measurements in Krakow (Jerzy Haber Institute of Catalysis and Surface Chemistry, Poland).

5.2. Introduction

The last two decades have seen the number of bacteria that are resistant to common antibiotics rising.¹⁶⁶ In the meantime, it has been found that ancient components of the innate system, *i.e.* anti-microbial peptides (AMPs), can be used efficiently as antibiotics against bacterial and fungal pathogens.^{168–170} Besides their antibiotic activity, AMPs are able to modulate the immune response, making them good candidates as therapeutic agent.^{171,172} Many groups of AMPs are known, of which defensins and cathelicidins have been the most studied.¹⁷³

LL37 is a 37-amino-acid-long peptide encoded in hCAP18, and it is the only peptide belonging to the cathelicidin family that has been identified in humans.²⁶⁴ This small positive peptide may take an α -helical conformation, depending on its concentration, on pH and on salinity.²⁶⁵ It has a conformation-dependent antimicrobial activity since α -helices create toroidal pores in the membrane surface, which causes molecule efflux from the cell.¹⁷⁴ LL37 also exerts different immunomodulatory functions such as modulation of pro-inflammatory response, chemotaxis, influence on cell proliferation and differentiation, promotion of wound healing and angiogenesis, etc.¹⁷⁵

LL37 suffers from two disadvantages that makes it difficult to use as therapeutic agent. First, LL37 has a relative cytotoxicity towards mammalian cells, *i.e.* eukaryotic cytotoxicity was reported at only 13–25 μM of LL37.²⁶⁶ Since the minimal inhibitory concentration values were reported from 1 to 10 μM of LL37 for Gram-positive and Gram-negative bacteria, the workable LL37 concentration range (absence of eukaryotic cytotoxicity and antibacterial activity) appears to be small.²⁶⁵ The second issue is related to LL37 exposure to host or bacterial proteases that can inactivate it.¹⁷³

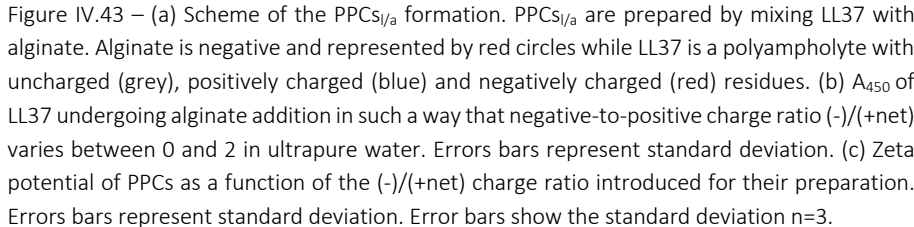
Many systems that aim at exploiting LL37 biological activity were already proposed. LL37 covalently linked to titanium surfaces was shown to retain its antibacterial activity.²⁶⁷ LL37 was also incorporated in mesoporous silica films for controlled release. Such films displayed potent bactericidal properties and no cytotoxic effect was reported on mammalian cells.²⁶⁸ More recently, it was shown that LL37 can be loaded in poly lactic-co-glycolic acid -nanoparticles and its release promoted wound healing while antibacterial activity was maintained.²⁶⁹ Collagen/hyaluronic acid PEMs were constructed and loaded with LL37 by physical adsorption or covalent binding. It resulted in less than 3% of the surface covered with bacteria and no cytotoxicity was observed for up to 16 μM LL37.²⁷⁰ As an alternative, Toppazzini *et al.* proposed to take inspiration from tuberculosis bacterial defense mechanism. These bacteria produce alginate that complexes LL37 and subsequently decreases its cytotoxic activity. Toppazzini *et al.* thus proposed to use this complexation to lower mammalian cytotoxic activity while maintaining a sufficient bactericidal effect on some bacteria strains. In this way, they have shown that, using alginate-LL37 complexes (PPCs_{1/a}), the cytotoxic activity on osteoblasts could be lowered while keeping a significant antibacterial activity against Gram-negative bacteria.²⁶⁶ Additionally, we anticipate that LL37 complexation with alginate could protect it from protease action the same way as it protect self-DNA from DNases degradation in self-DNA/LL37 complexes.²⁷¹

As demonstrated in Chapter 2 and 4, the multilayers constructed with PPCs are particularly suitable for drug delivery applications. Indeed, this method allows to extend the scope of the LbL assembly to new proteins and

immobilize high amount of active protein. Since LL37 forms complexes with alginate, which is reported to maintain its bactericidal activity, the method that has been developed in this thesis may prove particularly helpful with respect to LL37 immobilization.²⁶⁶ In this Chapter, PPCs_{I/a} will be first created. As it was done for Lyz-PSS_I in previous Chapters, the negatively charged alginate will be added to the positively charged LL37 to yield negatively charged PPCs_{I/a}. Circular dichroism (CD) will be used to monitor the formation of α -helices, which confer the observed bactericidal effect to LL37. Since LL37 is highly sensitive to protease hydrolysis, the effect of PPCs_{I/a} formation on chymotrypsin activity against LL37 will be tested.¹⁷³ Then, the obtained PPCs_{I/a} will be assembled LbL with chitosan, which is positively charged below pH 5. The LbL assembly will therefore be carried out below the latter pH value. The constructed multilayers will thus also benefit from the antimicrobial and wound healing properties of chitosan.²⁷² We thus anticipate that LL37 released from such a multilayer would possess a significant bioactivity. As with Lyz, which was studied in all other Chapters, LL37 will be immobilized using PPCs-based method or the classical method (bare LL37 assembled with alginate). Both methods will be compared in terms of LbL growth and immobilized amount. Finally, the wound healing properties will be tested *in vivo*.

5.3. LL37 complexation with alginate

PPCs_{I/a} were formed in water at pH 5 by adding alginate to LL37. The addition of alginate was expressed as a negative-to-positive charge ratio $[(-)/(+net)]$, which is defined as the structural charge ratio, where the LL37 charge is +6 and the alginate charge is equal to the number of monomers.²⁷³ The PPCs_{I/a} obtained by complexation are represented in Figure IV.43a. The turbidity of the obtained PPCs_{I/a} suspensions as a function of $(-)/(+net)$ is presented in Figure IV.43b. A_{450} increases with increasing $(-)/(+net)$, *i.e.* with alginate addition, until it reaches a plateau at $(-)/(+net)=1.25$. The surface charge of the PPCs_{I/a} as a function of $(-)/(+net)$ was investigated using zeta potential measurements (Figure IV.43c). It appears that the LL37 charge compensation by alginate occurs at $(-)/(+net)=1.25$, in agreement with the $(-)/(+net)$ value at the onset of the turbidity plateau. The results from turbidity and zeta



solution).²⁷⁵ With respect to LL37 in water, a very low ellipticity is measured above 210 nm, while a negative band is measured at 195 nm. This band is typical of disordered proteins, therefore suggesting that LL37 in water is in the random coil conformation.²⁷⁵ For PPCs_{I/a}, two negatives peaks are observed at 222 nm and 208 nm, and a positive band is obtained at 193 nm. Interestingly, these bands are typical of α -helices, which suggests that when LL37 is complexed by alginate, the peptide takes an α -helical conformation.²⁷⁵ This interesting feature has already been reported for LL37-alginate mixed solutions, as well as for poly(isobutylene-alt-maleic acid) or poly(1-tetradecene-alt-maleic acid) complexation with apocytochrome c.^{266,276} It was suggested that electrostatic and hydrophobic interactions between the protein and the PE exclude water molecules and therefore promote hydrogen bond formation to generate the α -helix structure. In order to confirm the α -helical conformation of LL37 in PPCs_{I/a}, the CD spectra of LL37 alone was also recorded in a solvent that promotes α -helix formation, *i.e.* ethanol.²⁷⁷ Results are presented in Figure IV.44b. Both the CD spectra of LL37 molecules dissolved in ethanol and LL37 molecules complexed with alginate to form PPCs_{I/a} suspended in water have the three bands (two negatives and 1 positive) that are typical of α -helix. This confirms the α -helical structure of LL37 in PPCs_{I/a}.

Then, the dependence of the α -helix formation on PPCs_{I/a} concentration was investigated by recording CD spectra for different PPCs_{I/a} concentrations. The different concentrations were obtained by successive dilution of concentrated PPCs_{I/a} suspensions. The molar ellipticity $[\Theta]$, *i.e.* the ellipticity corrected for concentration, was computed and presented as a function of wavelength in Figure IV.44c.²⁷⁵ It appears that, over the range of concentration that was investigated, free LL37 molecules are always in the random coil conformation, *i.e.* a negative band at 195 nm, while LL37 in PPCs_{I/a} always take an α -helical conformation *i.e.* two negatives bands at 208 nm and 222 nm and a positive band at 193 nm.²⁷⁵ It is worth mentioning that very high LL37 concentrations were tested because LL37 α -helix formation is reported to be concentration-dependent, with α -helices formation occurring only at high LL37 concentration.²⁷⁴ This was not observed in our case, which might be due to the use of pure water. Indeed, the concentration dependent

LL37 conformation were reported in saline buffer instead of pure water, which may favor α -helix formation.²⁷⁴

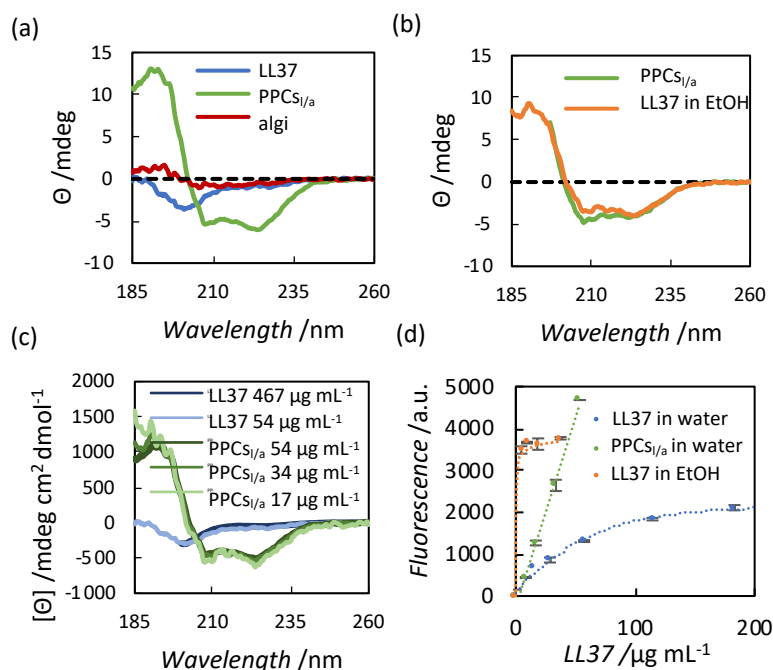


Figure IV.44 – (a) CD spectra obtained for LL37 alone, PPCS_{I/a} at (-)/(+net)=2 and alginate alone. (b) CD spectra obtained for PPCS_{I/a} at (-)/(+net)=2 suspended in water and for LL37 alone dissolved in ethanol (95%). (c) CD spectra obtained for different PPCS_{I/a} concentrations at (-)/(+net)=2. (d) Fluorescence of LL37 or PPCS_{I/a} in water, and LL37 in EtOH as a function of LL37 mass. Error bars show the standard deviation n=3.

Finally, the intrinsic fluorescence of LL37 was investigated as it gives information with respect to solvent-fluorophore proximity, which could in turn reveal structural details of PPCS_{I/a}. LL37 comprises 4 phenylalanines that were excited at 210 nm. The emission was integrated between 250 and 400 nm and results are presented as a function of LL37 concentration in Figure IV.44d for LL37 in water, PPCS_{I/a} in water, and LL37 in ethanol (EtOH). It appears that phenylalanine fluorescence is low when it is in water, while it is high in PPCS_{I/a} and in EtOH. First, the lower fluorescence when LL37 is in water can be attributed to the fluorescent quenching by water molecules. When LL37 is in PPCS_{I/a} or EtOH, the formation of α -helices protects the phenylalanine residues from quenching by the solvent. Interestingly, water

is known to quench fluorescence much more compared to EtOH, yet, LL37 in PPCs_{I/a} suspended in water shows a higher fluorescence compared to LL37 dissolved in EtOH. This result suggests a close proximity between alginate and the phenylalanine residues that would exclude water to a larger extent. It is worth mentioning that 5% water is present in EtOH, which could also participate to the fluorescent quenching that is observed in EtOH. Phenylalanine residues are well distributed in LL37 (amongst 37 a.a., with phenylalanine distributed between the 5th and the 26th a.a.), which suggests that a large fraction of LL37 is in close contact with alginate.

LL37 is known to be highly sensitive to proteolysis in the healing wound environment.²⁷⁸ In the following, LL37 was exposed to α -chymotrypsin (α -C) in pure water (pH 5) or in TRIS-CaCl₂ buffer (pH 7.8). The activity of α -C against LL37 or PPCs_{I/a} with a (-)/(+net) of 2 was evaluated using the procedure that is presented in Figure IV.45a. α -C was incubated with LL37 for 24h at 30°C and the reaction was stopped by bringing the solution to pH 3. Then, the solution was centrifuged through a protein concentrator that retains proteins with a M_w higher than 3000 Da. In this way, only the LL37 that is hydrolyzed is recovered after centrifugation. A BCA assay was performed to quantify the amount of hydrolyzed protein compared to the initial amount. Results of the LL37 mass fraction that has been hydrolyzed are presented in Figure IV.45b.

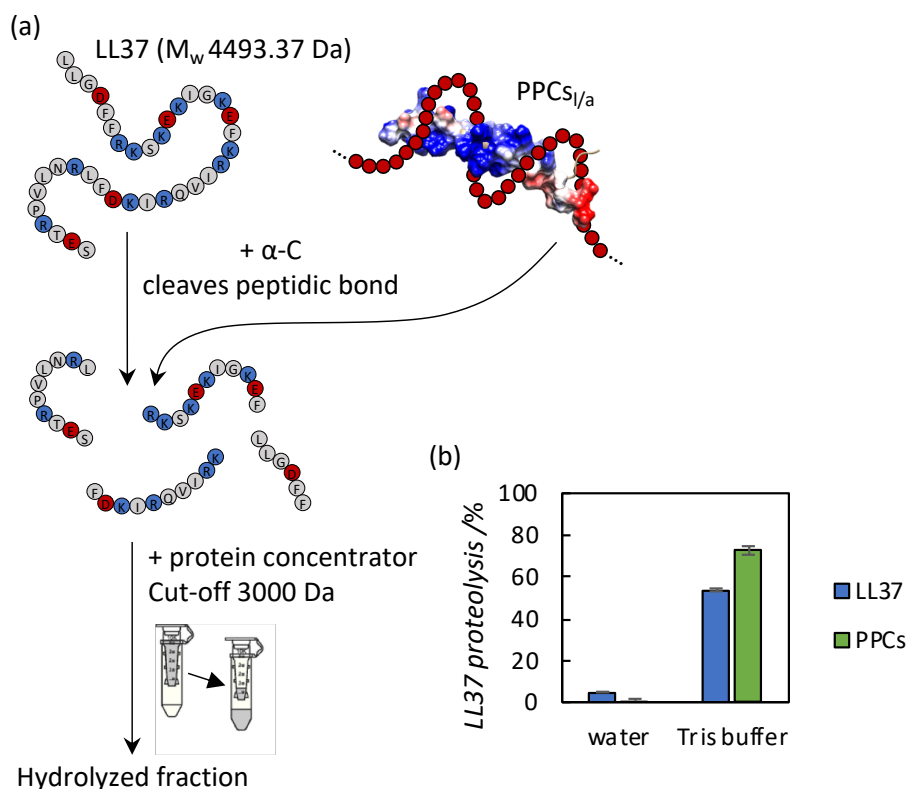


Figure IV.45 – (a) Schematic of the procedure that was used to assess for the degradation of LL37 by α -C. (b) LL37 proteolysis by α -C in water or in TRIS- CaCl_2 buffer. Error bars show the standard deviation $n=3$.

In water, around 5% of the initial LL37 (bare protein) mass has been hydrolyzed. This low proteolysis is expected as it is not the optimal pH for α -C and as CaCl_2 , which is essential for the catalytic activity of proteolytic enzymes, was not added.^{279,280} When LL37 is incorporated in PPCs_{I/a}, 0% of the initial LL37 mass has been hydrolyzed (see Figure IV.45b). The largest fragments that could be produced is half the LL37 M_w , which is smaller than the protein concentrator cut-off (3000 Da). Therefore, any hydrolysis would result in the detection of the fragment after centrifugation. This strongly suggests that PPCs_{I/a} has protected LL37 from proteolysis. However, the hydrolyzed fragments can be trapped in the PPCs_{I/a}, which could in turn lead to an underestimation of the hydrolyzed fraction.

In TRIS-CaCl₂ buffer, LL37 is more prone to hydrolysis when it is in PPCs_{I/a} compared to alone. This very unexpected result can be explained by the disassembly of PPCs_{I/a} and subsequent complexation of α -C with alginate in TRIS-CaCl₂ buffer. Indeed, it has been recently shown that protease complexation with PEs increases their performances. For example, subtilisin complexation with PAA or poly(L- γ -glutamic acid) results in a much higher enzymatic activity.²⁴⁸ The authors show that the enthalpy-driven interaction between the protease and PAA or poly(L- γ -glutamic acid) occurs via key a.a. residues close to the protease Ca²⁺ binding sites, which causes the improved catalytic activity.²⁴⁸ This result importantly raises the question of the potential detrimental side-effect of PEs when it comes to their use in combination with active proteins. Indeed, while enzyme hyperactivation has been reported numerous times in the field of engineered biocatalysis, it has, to our knowledge, never been reported as a negative side effect of protein delivery/carrier systems based on PEs.^{281–285} This important question should therefore be investigated to a much larger extent, especially given the tremendous number of delivery systems based on naturally occurring PEs that are currently under development.

5.4. LbL assembly of PPCs_{I/a} with chitosan

PPCs_{I/a} with a charge ratio above 1.5 are negatively charged (see Figure IV.43c). PPCs with a (-)/(+net) of 2 (PPCs_{I/a}) were thus assembled with chitosan, a polycation, by the LbL method. As a matter of comparison, free LL37 molecules were also assembled with alginate. The multilayer construction was monitored by QCM-D and frequency shifts were converted into mass by the Sauerbrey model. Results of the mass deposition on flat QCM-D sensors as a function of adsorption steps are presented in Figure IV.46. Two bilayers of chitosan assembled with alginate ([chito-algi]₂) were first constructed in all cases. When referring to multilayers, these two bilayers will systematically not be mentioned. A mass of 0.45 $\mu\text{g cm}^{-2}$ was recorded for these two bilayers. Then, five bilayers were constructed by alternating the adsorption of chitosan and PPCs_{I/a}. These [chito-PPCs_{I/a}]₅ layers led to a mass increase of 8.65 $\mu\text{g cm}^{-2}$. This mass is significantly higher than an equivalent number of [chito-algi] bilayers, *i.e.* [chito-algi]₅, which

reaches around $1.7 \mu\text{g cm}^{-2}$ (data not shown). The $7.4 \mu\text{g cm}^{-2}$ difference is thus due to the use of PPCs_{I/a} as building block and can be attributed to either LL37, a higher PE content and/or a higher water content. As the D reached for [chito-algi]₅ and [chito-PPCs_{I/a}]₅ is of respectively $1.7 \cdot 10^{-6}$ and $7.8 \cdot 10^{-5}$, a higher water content is expected in PPCs_{I/a}-based multilayers. Importantly, it appears that when free LL37 molecules are alternately adsorbed with alginate, the system fails to grow into a multilayer (see Figure 4). In line with Chapter 2, this result shows the potential of PPCs to immobilize new proteins using the LbL method.

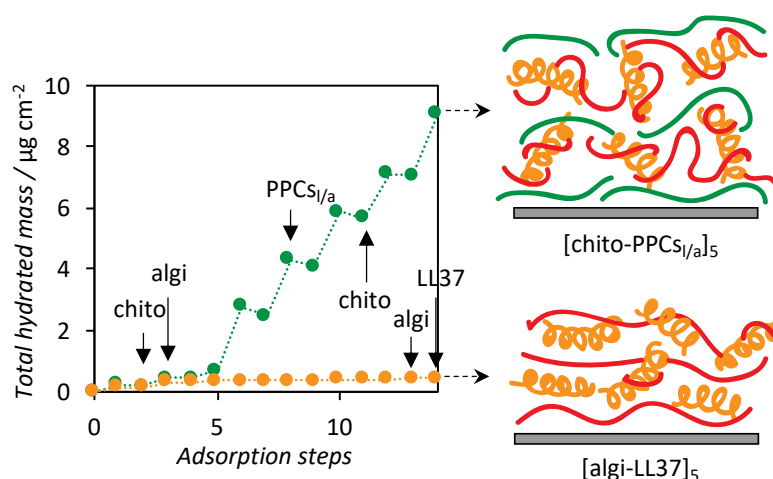


Figure IV.46 – Total hydrated mass measured by QCM-D after adsorption of a cushion of two chito-alginate bilayers followed by either chitosan-PPCs_{I/a} or LL37-alginate at pH 5 in water. Schemes of these LbL assemblies are presented on the right. LL37 is presented in orange, alginate in red and chitosan in green.

A BCA assay was used to quantify the LL37 content of the multilayer. Indeed, it has been recently shown that at least 5 μg of LL37 is needed on a 0.5 cm^2 wound to observe an improved wound healing.²⁸⁶ Unfortunately, the presence of an amide bond in the acetylated monomer of chitosan interferes with the BCA assay (see Figure IV.53a of the supporting information for chitosan structure). In order to measure the extent of this interference, the BCA assay was calibrated using pure LL37 or chitosan solutions as presented in Figure IV.47a. It appears that, even though only 5% of the chitosan monomers are acetylated, chitosan interferes significantly with the BCA

assay. The BCA assay selectivity for LL37 in presence of chitosan is 0.9 (the slope of the LL37 response divided by the slope of the chitosan response equals 10, which means that if there are ten times as many chitosan as LL37 in the multilayer, the same BCA assay response will be obtained for both). The BCA assay was thus combined with the *o*-phthalaldehyde (*o*-Ph) assay in order to quantify both the LL37 and chitosan contents of the multilayers.²⁸⁷ The development of the method is presented in supporting information (5.9.1, p204)

Briefly, the *o*-Ph assay was optimized so that it can be conducted in presence of LL37 and alginate, which both interfere. It resulted in the cancellation of the effect of alginate, and a selectivity for chitosan of 50% in presence of LL37 (the chitosan to LL37 calibration slope ratio is around 2).

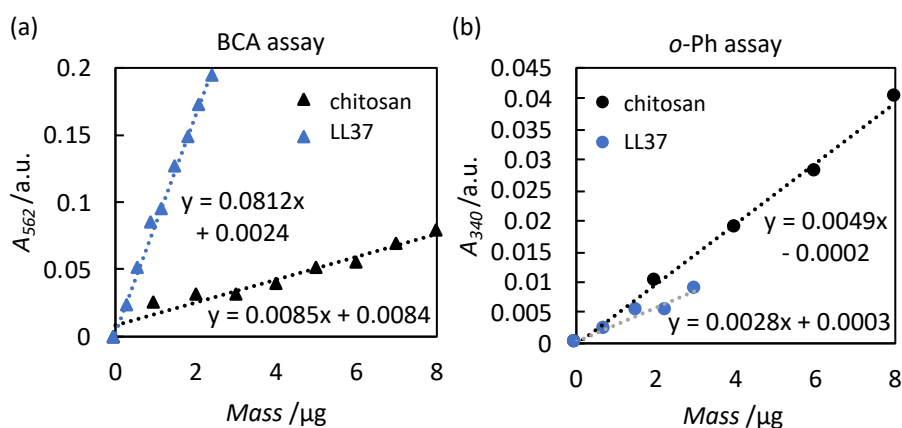


Figure IV.47 – (a) Calibration curve of the BCA assay for LL37 and interfering response of chitosan. (b) Calibration curve of the *o*-Ph assay for chitosan and interfering response of LL37.

LL37 and chitosan are thus both detected by the BCA and *o*-Ph assays, with however a higher sensitivity of the BCA assay for LL37 and of the *o*-Ph assay for chitosan. The equations of the four different linear calibration curves (for each assay, one equation for each compound) can be combined to write a system of two equations (Equations 1 and 2) with two unknowns as follows:

$$A^{BCA} = m_{LL37} S_{LL37}^{BCA} + I_{LL37}^{BCA} + m_{chito} S_{chito}^{BCA} + I_{chito}^{BCA} \quad (1)$$

$$A^{o-Ph} = m_{LL37} S_{LL37}^{o-Ph} + I_{LL37}^{o-Ph} + m_{chito} S_{chito}^{o-Ph} + I_{chito}^{o-Ph} \quad (2)$$

By solving this system, it is possible to extract the mass of LL37 and of chitosan in a mixed solution of these compounds, in presence or absence of alginate.

In order to use the *o*-Ph assay, multilayers have to be disassembled and recovered. This was achieved using H₂SO₄, which was then neutralized before *o*-Ph (details in the supporting information, p204). Results of the *o*-Ph assay on multilayers are presented in Figure IV.48a together with the calibration curves used. A very small A₃₄₀ value is obtained, *i.e.* 0.005 ± 0.001, which is higher than the detection limit, but lower than the LOQ. Therefore, equations 1 and 2 cannot be used. The information that it brings is nonetheless critical. Indeed, the chitosan mass in the well can be assumed to be lower than the LOQ of the *o*-Ph assay, *i.e.* < 2.5 µg. Since the BCA assay has a very small sensitivity to such a small chitosan mass, *i.e.* 2.5 µg of chitosan is lower than the LOQ for chitosan by the BCA assay, it can be used to assess the LL37 mass solely. In other words, the BCA assay is only sensitive to the LL37 and the interference of chitosan can be neglected. Therefore, using the BCA assay, 2.10 ± 0.15 µg cm⁻² of LL37 is found. For much higher masses of LL37 (deposition of more bilayers or use of a larger surface area), chitosan mass should increase proportionally. As a consequence, the BCA assay selectivity for LL37 can be used to assess for the error on the measure, *i.e.* 10% of the value obtained. Using data obtained from QCM-D, BCA assays and *o*-Ph assays, the composition of [chito-PPCs_{1/a}]₅ multilayers is reported in Figure IV.48b. The total hydrated mass and LL37 mass is reported with precision. The represented chitosan mass is its maximal estimated amount. The alginate and water amounts cannot be differentiated. Nevertheless, it can be estimated that, together, they represent around 4 µg cm⁻², *i.e.* half of the multilayer mass.

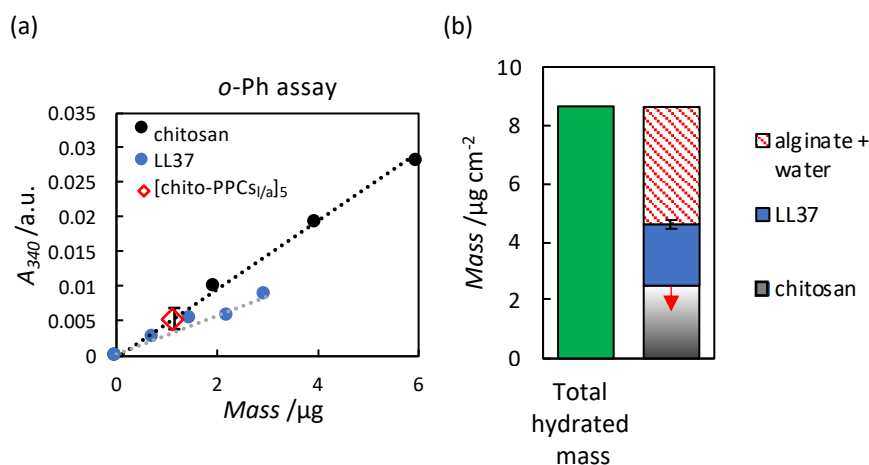


Figure IV.48 – (a) A_{340} obtained after that the *o*-Ph dosage is conducted on the solution recovered from [chito-PPCsI/a]₅ coated well. As a matter of comparison, the calibration curves are presented for chitosan and LL37. (b) Data obtained from QCM-D, BCA assay and *o*-Ph assay are synthesized to present the composition of [chito-PPCsI/a]₅. The maximal estimated chitosan mass is presented. Error bars show the standard deviation $n=3$.

5.5. LL37 coated dressing

Woven nylon dressings were coated with a [chito-*alg*]₂ anchoring layer. Then, 3, 5, 6, 9, 10, 12, 15, 20 or 25 [chito-PPCsI/a] bilayers were constructed. As a matter of comparison, the LbL assembly of LL37 with alginate was also performed. All multilayers were constructed under a laminar flow hood and using sterile or sterilized products. The amount of immobilized LL37 was quantified in each multilayer using a modified BCA assay (see materials and methods). As shown hereabove, the interference coming from chitosan could be neglected. The results are presented in terms of LL37 mass per cm^2 of dressing as a function of the number of bilayers in Figure IV.49a. As already observed on QCM-D sensors, it appears that a sustainable LbL growth is obtained for [chito-PPCsI/a] while [LL37-*alg*] fails to grow into a multilayer. Interestingly, around $5.1 \pm 0.6 \mu\text{g}$ of LL37 per cm^2 of dressing is immobilized after 9 PPCsI/a bilayers, which is enough to improve wound healing according to previously reported data.²⁶⁹ Unfortunately, the amount of LL37 saturates at around $5 \mu\text{g}$ of LL37 per cm^2 of dressing *i.e.* after the deposition of *c.a.* 10 bilayers. This limited LbL grow might be either due to the geometry of the dressing, or to an intrinsic property of the [chito-PPCsI/a] multilayer. In the latter case, the same behavior would be observed on planar surfaces. To

verify this hypothesis, the LL37 mass per bilayer per cm^2 of dressing was computed and compared to the data obtained on planar surfaces *i.e.* in 96-well plates. The geometry of the dressing is highly accurate and given by the manufacturer. The dressing was thus reconstructed in the software 123D design and a square of a 1 cm^2 of dressing was estimated to develop a surface of 1.8 cm^2 . From this, it can be computed that $0.32 \pm 0.15 \text{ }\mu\text{g}$ of LL37 per cm^2 of dressing are immobilized per bilayer, while a value of $0.42 \pm 0.07 \text{ }\mu\text{g}$ of LL37 per cm^2 is found on planar surfaces. These values are not significantly different (t-tested in JMP software, $\alpha=5\%$), which suggests that the geometry of the dressing has no effect on the LbL construction. The saturation after 9 bilayers might thus be an issue that is intrinsic to the [chito-PPCs_{I/a}] system.

In order to further confirm the presence of LL37 on the dressing, time of flight secondary ion mass spectrometry (ToF-SIMS) was used in imaging mode. Secondary ions emitted from phenylalanine (F), *i.e.* $\text{C}_8\text{H}_{10}\text{N}^+$, were chosen as the signature of LL37. Indeed, LL37 has four F among its 37 a.a. Results are presented in Figure IV.49b for an uncoated dressing and for a dressing coated with [chito-PPCs_{I/a}]₉. A very low signal is detected for $\text{C}_8\text{H}_{10}\text{N}^+$ ions on the uncoated dressing while $\text{C}_8\text{H}_{10}\text{N}^+$ ions are strongly detected and uniformly distributed on the [chito-PPCs_{I/a}]₉-coated one. The dressing geometry also appears very clearly on the latter image. This result ultimately confirms the presence of LL37, which is confined in the multilayer at the surface of the dressing.

From data presented hereabove, it appears that around $5 \text{ }\mu\text{g}$ of LL37 are immobilized on 1 cm^2 of dressing. The total multilayer mass is approximately four folds the one of LL37 (see Fig. 6d). This gives a total mass of around $20 \text{ }\mu\text{g}$ per cm^2 of dressing. Assuming a density of 1.2 g cm^{-3} and the surface area of the dressing that was computed above, it can be calculated that [chito-PPCs_{I/a}]₉ is around 100 nm thick. Multilayers that are in the same thickness range were already observed by SEM.²⁸⁸ [chito-PPCs_{I/a}]₉ deposited on the dressing were thus covered with a 8 nm -thick gold layer and imaged by SEM as presented in Figure IV.49c for an uncoated dressing and for a [chito-PPCs_{I/a}]₉-coated dressing. The size of SEM images comparatively to the one

obtained using ToF-SIMS is presented as the small white squares in Figure IV.49b. It is clear from these images that the [chito-PPCs_{l/a}]₉ coating is present and causes irregularities on the dressing surface. It is not straightforward to conclude whether these irregularities are inherent to the multilayer or caused by the dewetting of the multilayer upon drying.

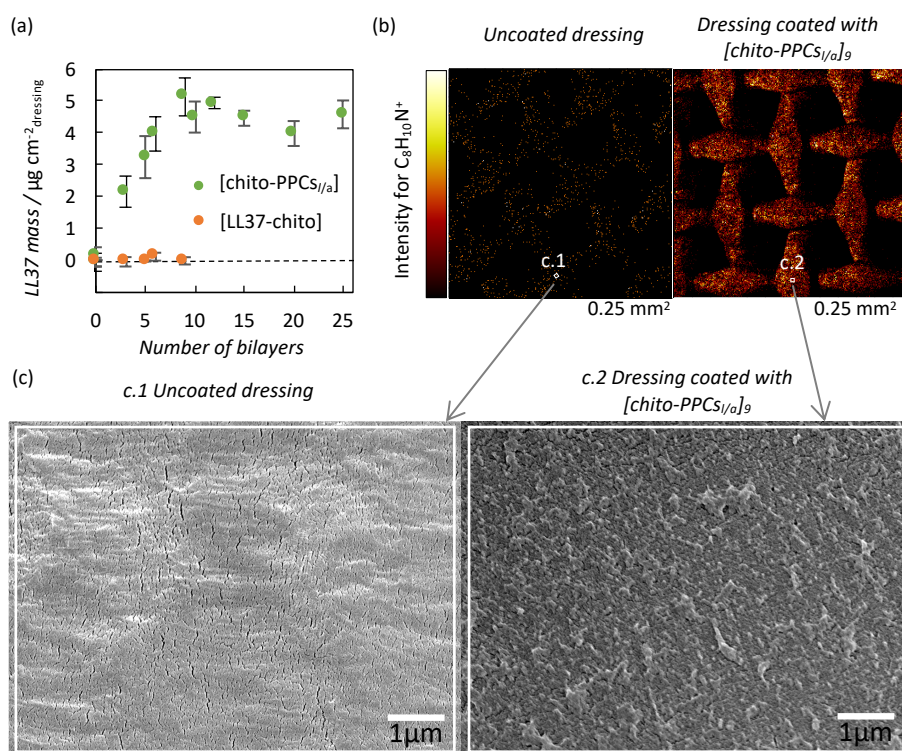


Figure IV.49 – (a) LL37 mass immobilized on a Tegaderm Contact 3M dressing after 3 to 25 [chito-PPCs_{l/a}] bilayers, or 3 to 9 [chito-LL37] bilayers. Standard deviation is presented for n=3 (standard deviation is presented). (b) Intensity map for C₈H₁₀N⁺ ions detected by ToF-SIMS at the surface of an uncoated dressing or a dressing coated with [chito-PPCs_{l/a}]₉. (c) SEM images of an uncoated dressing (c.1) or a dressing coated with [chito-PPCs_{l/a}]₉ (c.2). All multilayers containing LL37 are constructed on a [chito-algi]₂ anchoring layer. The size of SEM images comparatively to the one obtained using ToF-SIMS is presented as the small white squares in intensity maps.

From the Tegaderm Contact 3M manufacturer, the dressing is expected to be held on the wound for a maximum of 7 days. The LL37 release profile from the coated dressing was investigated. Dressings were coated, left to dry at 4°C, and incubated in PBS at 37°C for up to 15 days. The amount of LL37 left

on the bandage was quantified using a BCA assay after 7 h, 1, 2, 3, 5, 10 or 15 days. Results are presented in percent of LL37 released as a function of time in Figure IV.50. It clearly appears from these data that the release profile is divided in two regimes. First, a burst release is observed, during which 60% of the immobilized LL37 is released. Then, from 7 h to 15 days, the 40% left are gradually released. It can be anticipated that the release in PBS is slower than in the wound environment due to the action of proteases and friction with the skin in the latter case. The advantage that the coated dressing may offer through the release of LL37 thus occurs during the first few hours to days. It was thus decided in further *in vivo* experiment to renew the dressing every four days.

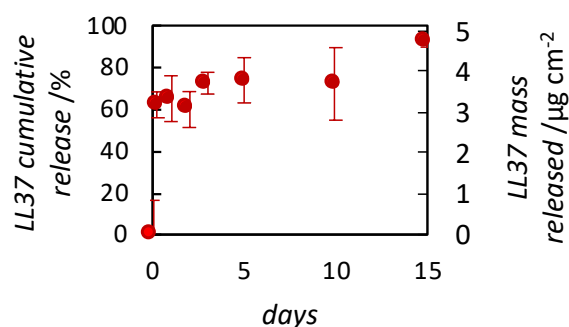


Figure IV.50 – LL37 release profile from [chito-PPCS_{1/2}]₉ coated dressings after 7 h, 1, 2, 3, 5, 10 and 15 days. Error bars show the standard deviation n=3.

5.6. LL37 wound healing tested in vivo

As demonstrated here above, LL37 can be immobilized and released from dressings. The next step was to test whether these coated dressings can improve wound healing in mice. To do so, a protocol adapted from Chereddy *et al.* and Castleberry *et al.* was designed.^{269,289} The procedure is illustrated in Figure IV.51 and is described in details in the materials and methods section. In brief, the coated dressings were kept in place on the wound by sewing them between the skin of mice and a rubber ring. Then, a non-occlusive adhesive fabric was applied above the dressing and held in place with a fixation adhesive tape. This last step is critical. Indeed, during a first trial, an occlusive adhesive was used, which caused moisture accumulation and resulted in an inflammatory reaction. The dressing, the non-occlusive

fabric and the fixation adhesive tape were changed after 4 and 8 days, which involves changing the sewing. The wound healing was assessed by measuring the wound closure after 2, 4, 8 and 11 days.

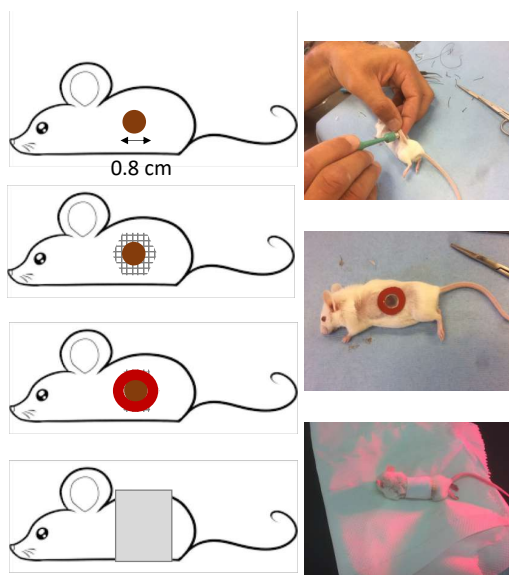


Figure IV.51 – Schematic of the experimental procedure used to test the wound healing on mice. First, a 0.5 cm² piece of skin is removed to obtain a wound. Then, the dressing is applied on the wound and sewed together with a rubber ring. Finally, a non-occlusive bandage is taped above the dressing.

A preliminary test was conducted to verify that the experimental design allows the wound to heal (no inflammation). The sterility of the coating was also challenged. Indeed, the multiple steps that are necessary to construct the LbL coating may be at the origin of wound infection. Finally, the effect of the alginate and the chitosan on the wound was tested. To do so, the same protocol as described hereabove was used for three different systems: (1) no dressing, (2) untreated sterile dressing and (3) dressing coated with [chito-*alg*]₁₁ multilayer (no LL37, in order to separate the effect of the coating from the one of LL37). The coating was achieved in a laminar flow hood. The wound closure was measured in these three conditions and results are presented in Figure IV.52.

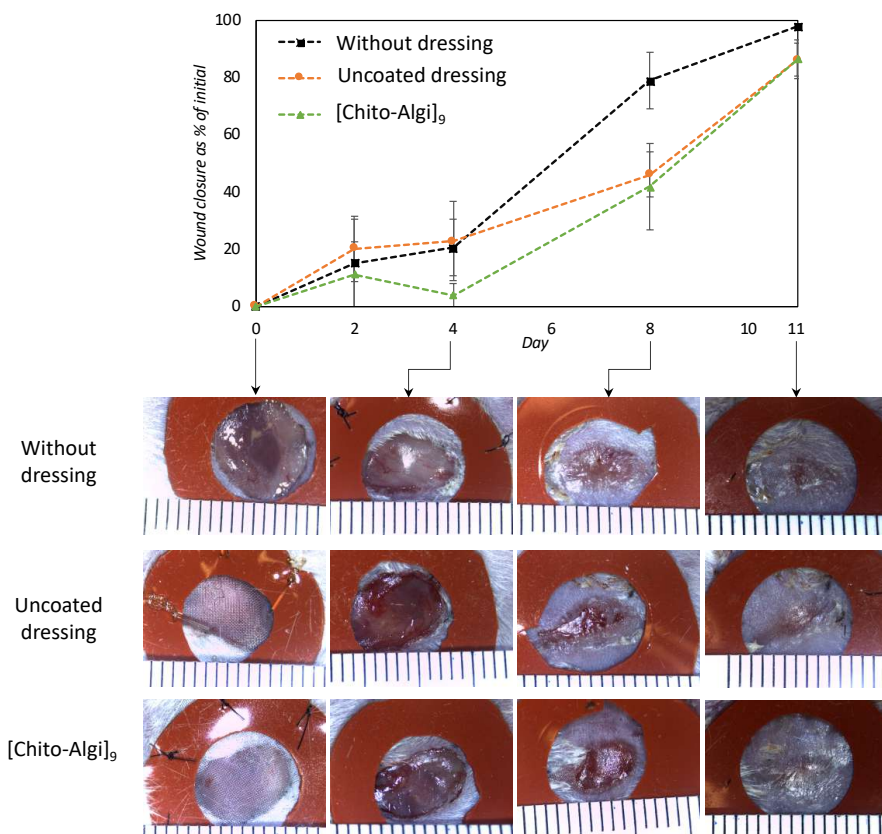


Figure IV.52 – Wound healing expressed as the wound closure compared to the initial wound size as a function of days ($n=4$, *i.e.* four different mice, with standard deviation). Results are presented for a wound that heals without any dressing, an uncoated dressing, and a dressing coated with [chito-algi]₁₁. Images taken after 0, 4, 8 and 11 days are also shown.

Over the 11 days of healing, it appears that the absence of any dressing allows a slightly better healing (the only significant difference in wound closure being after 8 days). Overall, the wound heals well in the three cases and no inflammation nor infection is detected (absence of giant cells, data not shown). As demonstrated by the pictures of the wound, all wound are closed and healed after 11 days. This suggests that the protocol that has been developed allows the wound to heal normally. Moreover, there is no difference between uncoated sterile dressing and the coated (no LL37) dressings. This suggests that the sterility could be maintained despite the repetitive immersion necessary for the construction of the film. Moreover, it

suggests that the presence of alginate and chitosan does not affect the wound healing.

The procedure to test wound healing has been developed. The last step consists in testing whether dressings coated with [chito-PPCs_{I/a}]₉ improve the wound healing. These experiments are now planned and will be carried out as soon as possible.

5.7. Conclusion

The method for protein immobilization that has been developed in this thesis was used to immobilize LL37, an AMP with angiogenic properties, on a dressing. To achieve this, LL37 was first complexed with alginate. In line with literature, this complexation causes the LL37 to take an α -helical conformation. This α -helix formation was shown to be independent of the PPCs_{I/a} concentration. Using intrinsic fluorescence, a high proximity between alginate and LL37 was demonstrated. This confirms the exclusion of water from PPCs_{I/a}, which is congruent with H-bond formation and α -helix formation. LL37 proteolysis by α -C was tested on bare LL37 molecules or on PPCs_{I/a}. It was shown that while PPCs_{I/a} formation provides protection against proteolysis in water, the presence of alginate significantly sensitizes LL37 to α -C activity in TRIS-CaCl₂ buffer. In line with the literature, this result is attributed to a boost of the catalytic effect coming from alginate complexation with the enzyme. This unexpected result, which is well documented in the field of biocatalysis, has never been investigated in the field of drug delivery. It importantly raises the question of a potential detrimental side-effect of PE use for drug delivery systems.

After their formation, PPCs_{I/a} were assembled LbL with chitosan. The LbL growth was first evidenced to be linear as a function of adsorption steps on planar surfaces. Interestingly, the classical bare protein LbL assembly failed to produce multilayers. This confirms the interest of the method developed in this thesis for protein immobilization. Using a BCA assay combined with a o-Ph assay, it was evidenced that the [chito-PPCs_{I/a}] multilayers are made of an equivalent amount of chitosan and LL37, which represent together half of the total hydrated mass of the coating. LL37 was then coated on a Tegaderm

Contact 3M dressing by dip coating. The LL37 amount immobilized on this particular geometry was consistent with the one obtained on planar surfaces. Importantly, the construction of 9 [chito-PPC_{sl/a}] bilayers led to the immobilization of 5 µg of LL37 per cm² of dressing, which should be enough to observe an improved wound healing. Using ToF-SIMS in imaging mode, the presence of LL37 was confirmed and shown to be homogeneously distributed on the dressing surface. Thick multilayers were also observed using SEM, which further confirmed the formation of [chito-PPC_{sl/a}] multilayers. With a view to deliver the LL37 to a wound, its release from coated dressings was assessed in PBS. A burst release was observed for the 7 first hours. This led to a release of 60% of the LL37 content. Then, between 7 hours and 15 days were needed to release the whole content of the multilayer. Finally, a protocol was developed to test *in vivo* whether these coated dressings could promote wound healing. Preliminary experiments were conducted, showing that the successive immersion necessary to the multilayer formation, and the presence of alginate and chitosan have no effect on the wound healing. These preliminary data pave the way for the *in vivo* testing of the effect of LL37-containing multilayers on wound healing.

5.8. Materials and methods

5.8.1 PPC_{sl/a} preparation

PPC_{sl/a} were prepared by mixing an alginate solution (0.5 mM negative charge, *i.e.* 88 µg mL⁻¹, $M_w < 75.000 \text{ g mol}^{-1}$, Pronova UP VLVM, NovaMatrix, Norway) with a LL37 solution (0.1 mM positive charge at pH 5, *i.e.* 75 µg mL⁻¹, 98.3% pure, ProteoGenix, France) (peptide sequence: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES, theoretical molecular mass 4493.37 Da). All solutions were prepared in ultrapure water. The LL37 solution volume was kept constant while the alginate solution was added in such a way that (-)/(+net) varies between 0 and 2. For turbidimetry experiments, only the alginate solution was added to the LL37 solution while for zeta potential, CD and fluorescence, the alginate solution and additional water were added in such a way that LL37 concentration stays constant, *e.g.* 1 mL of LL37 was mixed first with 0.3 mL water and then with 0.1 mL of alginate solution to reach a charge ratio of 0.5.

5.8.2 Turbidimetry

The A_{450} of the suspension was measured using a UV-vis spectrophotometer (DU800, Beckman Coulter, USA). A_{450} was normalized by the dilution factor. Measurements were performed in triplicate.

5.8.3 Zeta potential

A Malvern Zetasizer Nano ZS instrument (Worcestershire, UK) was used to determine the electrophoretic mobility of LL37 and PPCs using a Laser Doppler Velocimetry technique. The zeta potential was calculated using Henry's equation.²⁹⁰ Measurements were performed in triplicate and results are presented together with their standard deviation which was always lower than 0.5 mV.

5.8.4 Circular dichroism

CD spectra were recorded with a Jasco J-810 spectropolarimeter equipped with a thermostated cell holder from 185 to 260 nm in water or ethanol. The LL37 ($53.6 \mu\text{g mL}^{-1}$) and alginate ($2.5 \mu\text{g mL}^{-1}$) concentrations were kept constant for all experiments. When varying the LL37 or PPCs_{I/a} concentration, the higher concentration was sequentially diluted with the solvent to reach other concentrations. All spectra were acquired at 25°C in a quartz cell with 0.1 cm path length at a scan rate of 50 nm min^{-1} , with a 1 nm bandwidth and a 2 s response. The presented spectra correspond to the average of ten accumulations. The spectrum of the solvent used, *i.e.* water or ethanol (95%), was subtracted for all experiments. For PPCs_{I/a}, the spectrum of alginate was also subtracted.

5.8.5 Fluorescence

Fluorescence intensity was measured with a Varian Cary Eclipse fluorescence spectrophotometer. Excitation wavelength was set at 210 nm with a 5 nm bandwidth and emission was recorded and integrated between 250 and 400 nm. Solvents were used as blanks.

5.8.6 LL37 proteolysis

All protein concentrators (Thermo Scientific™ Pierce, cut-off 3000 Da) were first rinsed with 500 μL water by centrifuging them at 15,000 g for 30 min. Indeed, N_3 is present and has to be removed as it interferes with the BCA assay. For the proteolysis in water, a stock solution of $\alpha\text{-C}$ from bovine pancreas ($1.8 \mu\text{g mL}^{-1}$; $\alpha\text{-C}$ purity $\geq 85\%$, Sigma-Aldrich, Steinheim, Germany) was freshly prepared in water. For the proteolysis in TRIS- CaCl_2 buffer, a first solution of $\alpha\text{-C}$ (2 mg mL^{-1}) was reconstituted in 1 mM HCl and 2 mM CaCl_2 , and stored at -20°C for maximum one week. Then, the latter solution was diluted to obtain a stock solution containing $1.8 \mu\text{g mL}^{-1}$ $\alpha\text{-C}$, 200 mM TRIZMA and 20 mM CaCl_2 at pH 7.8. For both $\alpha\text{-C}$ in water and TRIS- CaCl_2 buffer, 175 μL $\alpha\text{-C}$ (stock solution) was mixed with 175 μL of water, LL37 or $\text{PPCs}_{\text{L/A}}$. This yields a solution with a 1:60 (w:w) ratio of $\alpha\text{-C}$:LL37 ($0.9 \mu\text{g mL}^{-1}$: $53 \mu\text{g mL}^{-1}$) in water at pH 5 or 100 mM TRIS with 10 mM CaCl_2 at pH 7.8. In this case LL37 and alginate concentrations were twice higher before two-fold dilution by mixing with $\alpha\text{-C}$. This allows to reach a final concentration of LL37 and alginate that is the same as for all other experiments. Solutions in water were incubated 20h30 at 30°C and the reaction was stopped by adding 5 μL of 0.1 N HCl. Solution in TRIS- CaCl_2 buffer were incubated 23h at 30°C and the reaction was stopped by adding 10 μL of 1 N HCl. Then, solutions were centrifuged at 15,000 g for 40 min on a rinsed protein concentrator. Both the initial amount and the one recovered after centrifugation were quantified using a BCA assay. The BCA assay was conducted by mixing 50 μL of sample with 1 mL of BCA working reactant (50:1, v:v, BCA-A:BCA-B) and incubating at 60°C for 60 min. Then $A_{562 \text{ nm}}$ was recorded using a DU800 spectrophotometer (Beckman Coulter, USA).

5.8.7 Quartz crystal microbalance with dissipation monitoring

Commercially available 4.95 MHz AT-cut gold-coated quartz sensors (purchased from Q-Sense, Stockholm, Sweden) were used. These sensors were washed with a $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$ 1:3 (v:v) solution, successively rinsed with water and ethanol, then exposed to UV-ozone prior to measurement. Layer growth on gold-coated sensors was monitored using a QCM-D monitoring system (Q-Sense E4 system, Q-Sense, Stockholm, Sweden). The solution flow

rate and temperature were set at $20 \mu\text{L min}^{-1}$ and 20°C , respectively. Layers were constructed by successive adsorption steps from the following solutions: alginate at 0.5 g L^{-1} , chitosan at 0.5 g L^{-1} (chito 95/5, Heppe Medical Chitosan GmbH, Germany), LL37 ($53.6 \mu\text{g mL}^{-1}$) and PPCs_{I/a} (algi $2.5 \mu\text{g mL}^{-1}$ and LL37 $53.6 \mu\text{g mL}^{-1}$). The chitosan solution was obtained by first dissolving 100 mg of chitosan in approximately 50 mL water and 3.2 mL HCl 3.7 % at 60°C for 1h. Then, pH was adjusted to pH 5 with NaOH and the volume was brought to exactly 200 mL. Finally, the chitosan solution was filtered with a $0.45 \mu\text{m}$ filter. Each adsorption step of 20 min was followed by rinsing for 10 min with pure buffer. A [chito-algi]₂ anchoring layer was first constructed in all cases. Then LL37 was alternately adsorbed with alginate ([LL37-algi]_n), or chitosan with PPCs_{I/a} ([chito-PPCs_{I/a}]_n). The changes in frequency and D were recorded. Mass adsorbed on QCM-D crystals was assessed by the Sauerbrey equation for the 7th overtone ; $\Delta m = -C \Delta f_7$, where Δm is the shift of adsorbed mass per surface area ($\mu\text{g cm}^{-2}$), Δf_7 the frequency shift measured for the 7th overtone (Hz), and C a constant equal to $17.7 \text{ ng.cm}^{-2}.\text{Hz}^{-1}$ for a 5 MHz crystal.²⁹¹

5.8.8 Chitosan quantification

A stock solution of 0.2 M borate buffer was first prepared by mixing 50 mL of 12.4 mg mL^{-1} boric acid solution with 42.5 mL of 19.0 mg mL^{-1} borax solution, and bringing the volume to 200 mL and pH 8.9. The working reactant was prepared by mixing 0.2 volumes of *o*-Ph (110 mM), 0.2 volumes of N-acetyl-L-cysteine (NAC, 71 mM) and 5 volumes of the 0.2 M borate buffer. The reaction was started by mixing 500 μL of sample with 500 μL of working reactant. The reaction was conducted at room temperature and A_{340} was measured after 1h, 3h and 5h.

The disassembly of the multilayer by H_2SO_4 was tested in a 96-well plate. First, the [chito-algi]₂ – [chito-PPCs_{I/a}]₅ multilayers were constructed in the 96-well plate using the same solution as for QCM-D experiments and the same protocol as for [PAH-PSS]₂ – [PAH-PPCs]₅ multilayers of Chapter 1, 2 and 3. 80 μL of 3.5 % H_2SO_4 were then added to the coated wells and the solution was pipetted up and down along the walls of the well. Then, the wells were rinsed 3 times with the buffer used for the construction (water at

pH 5) and a BCA assay was conducted (same protocol as for Chapter 1, 2 and 3). A_{562} was measured and compared to the one of a well that did not undergo the H_2SO_4 treatment.

Exposure to H_2SO_4 was used to recover the multilayers. The recovered 80 μL were neutralized with 10 μL of 10 N NaOH. Then, 70 μL of water and 340 μL of borate buffer were added. The 500 μL obtained were then mixed with the working reagent containing *o*-Ph and NAC (same as hereabove). A solution with known chitosan, alginate and LL37 amount, which underwent the same successive H_2SO_4 and NaOH additions, was used as a calibration to quantify chitosan and LL37 in the multilayers. It also appears that the presence of SO_4^{2-} and Na^+ in the sample did not change the 3h optimal incubation time neither the sensitivity of the test (data not shown as the same data as in Figure IV.47b are obtained).

5.8.9 LbL film preparation on the dressing

Woven nylon dressings (Tegaderm Contact, 3M) were cut in hexagons of 3 cm^2 to fit in a 12-well polystyrene plate (Greiner Bio-One®) and multilayers were constructed by successive adsorption steps from 1 mL of the same solutions as for QCM-D. All solutions were prepared using autoclaved water. Adsorption steps were carried-out under a laminar flow hood. A 1.4 mL stock solution of alginate, chitosan and $PPCSi/a$ was used for all adsorption steps in each well. The same construction as for QCM-D was realized but it was stopped after $n=0, 3, 5, 6, 9, 10, 15, 20, 25$ bilayers. Each adsorption step lasted 20 min and was followed by rinsing three times with water. The coated bandages were then left to dry at 4°C.

5.8.10 LL37 quantification on coated dressings

Hexagonal dressings coated with $[chito-PPCSi/a]_n$ were cut in 6 triangles (by simply connecting angles), which gave 6 pieces of 0.5 cm^2 . The LL37 amount was quantified using a modified BCA assay (Thermo scientific, Rockford, USA). 50 μL of SDS 1% and 500 μL of BCA reagent A were first added to 2 triangles, *i.e.* 1 cm^2 of coated dressing. The mix was vortexed for 30 s. 500 μL of solution was then collected and added to 520 μL of a 25:1 (v:v) mix of reagent A: reagent B. The obtained solution was incubated at 60°C for 60 min

and A_{562} was measured. Uncoated dressings were used as a blank and calibration was performed with known LL37 amounts. It is worth mentioning that uncoated dressings had to be washed with water before the BCA assay (interferences were observed otherwise).

5.8.11 ToF-SIMS and SEM imaging

Positive ToF-SIMS images were recorded by using an TOF.SIMS⁵ instrument (IONTOF GmbH, Münster, Germany). A Bi_3^{++} liquid metal ion source was used to produce the analytical primary beam (energy 60 keV). An AC target current of 0.003 pA was used with the analytical burst mode (1 pulse selected with the sine blanker). Only positive secondary ion species were analyzed. A raster of 2048 x 2048 pixels over an area of 500 μm x 500 μm was used. The total primary ion beam dose for each analyzed area was always kept below 3×10^{10} ions. cm^2 , ensuring static conditions. Lateral resolution of 0.3 mm and mass resolution $m/\Delta m > 2500$ at 120 m/z were maintained for acquisition of both images and corresponding spectra for positive ions. Charge compensation was done by electron flood gun ($E_k=20$ eV). All data processings were carried out using the software supplied by the instrument manufacturer, SurfaceLab (version 6.5).

Dressings were coated with a 8 nm thick gold layer. Images were acquired with a JSM-7600F (JEOL, Zaventem, Belgium) scanning electron microscope at 5 keV.

5.8.12 LL37 release

3 cm^2 hexagons of dressing coated with [chito-PPCs_{1/a}]₉ were immersed in 0.5 mL of PBS buffer (Gibco™). The space between wells was filled with water to saturate the air in water and avoid excessive evaporation from the wells. The plates were sealed with parafilm and left under agitation at 150 rpm and 37°C. The LL37 left on the dressings was evaluated after 7 h, 1, 2, 3, 5, 10 and 15 days. Before the BCA assay, dressings were briefly washed in water to remove any PBS solution and left to dry at 4°C overnight.

5.8.13 Materials and methods: *in vivo* wound healing assay

Six to seven-week-old SWISS female mice (Janvier, BE) were randomly selected and grouped following the standardized animal experimental protocols. Animals were from the same breed, age and near body weight (as delivered by supplier). Temperature and RH were maintained constant in the animal facility. Mice were anesthetized with isoflurane (Isoflo, Zoetig) and the dorsal area was shaved just before the surgery.

Full-thickness excisional wounds were created on either side of the dorsal midline, approximately 3.5 cm distal from the ears using an 8 mm round skin biopsy punch (Kai Europe GMBH, DE). Dressings were held on the wound by sewing to the skin a 0.5-mm-thick silicone (Grace biolabs, UK) donut-shaped splints (OD=20mm, ID= 10 mm) with 6-0 nylon sutures (Monosof, USA; as described in Figure IV.51). In order to investigate re-epithelialization as a major factor in wound healing, splints were used to avoid skin contraction. A non-occlusive adhesive fabric (Mefix, Mölnlycke) was then taped to the wound and covered with a fixation adhesive tape (Leukoplast pro LF, BSN medical). The wound healing was evaluated by measuring the size of the wound after 2, 4, 8, 12 days and comparing it to the initial size. The dressing, the silicone ring, the non-occlusive fabric and the fixation adhesive tape were changed after 4 and 8 days. The animal studies were approved by the animal care and ethical committee of medical sector of UCLouvain.

5.9. Supporting information

5.9.1 *o*-phthalaldehyde assay optimization

The *o*-Ph assay was developed by Larionova *et al.*, based on the reaction of *o*-Ph and N-acetyl-L-cysteine (NAC) with amines.²⁸⁷ This reaction, which is presented in Figure IV.53b, produces 1-alkylthio-2-substituted isoindole (AAsI) which absorbs light at 340 nm (A_{340}). The amount of AAsI produced by the reaction increases proportionally to the amine concentration. Importantly, the spectrophotometrically active product (AAsI) is subject to degradation and specific incubation times have to be respected. Moreover, Larionova *et al.* have shown that the presence of a polyanion such as alginate

lowers the reaction rate.²⁸⁷ This was attributed to the hindered access of *o*-Ph to the amine, caused by the complexation between positively charged amines (the reaction is carried out at pH 8.9) and negatively charged polyanions. This effect can be mitigated by carefully selecting the incubation time, as it is only a matter of kinetics. Preliminary tests were thus carried out in which the reaction was stopped after 1h, 3h and 5h, and A_{340} was measured for increasing amounts of either chitosan alone or chitosan mixed with an equal mass of alginate, as presented in in Figure IV.53c, d and e. After 1 h, a lower absorbance is obtained when alginate is present together with chitosan as it decreases the reaction rate. If a sufficient time is allowed for the reaction to proceed, *i.e.* 3h, all amines have reacted and A_{340} reaches the same value both in presence and absence of alginate.²⁸⁷ After 5h, the AAsI that is produced by the reaction converts *via* a sulfur-oxygen rearrangement into 2,3-dihydro-1*H*-isoindole-1-one, which leads to a decrease in A_{340} .²⁹² An incubation time of 3h was thus chosen to mitigate the effect of alginate, while maintaining a stable AAsI. Since amines are also present in LL37, the *o*-Ph assay was also performed on increasing amounts of LL37. It appears (Figure IV.47b) that LL37 indeed gives a signal, which is however significantly lower than the one of chitosan.

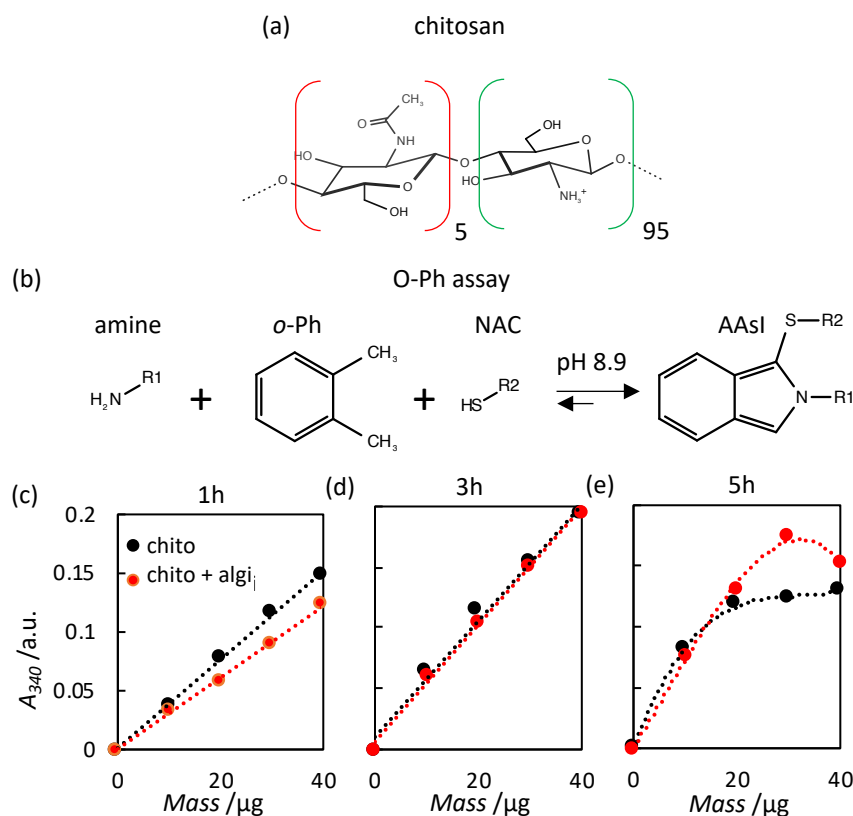


Figure IV.53 – (a) chitosan structure. (b) Amine reaction with *o*-Ph and NAC, and subsequent formation of AAI. – A_{340} of AAI as a function of chitosan mass for pure chitosan or chitosan mixed with an equal mass of alginate after 1h (c), 3h (d) or 5h (e).

5.9.2 Multilayer recovery for *o*-Ph assay

Now that the assays have been developed in solution, multilayers have to be recovered from the surface to determine their composition. The issue is that, while the BCA reactant disassembles the multilayer (see Chapter 2), the *o*-Ph assay fails to remove it from the surface (data not shown). The BCA reactant was tentatively used to trigger the disassembly of multilayers constructed in 96-well plates and the *o*-Ph assay was subsequently performed in the same wells. There was however a strong interference between the assays (data not shown). Different methods were then tested to trigger the disassembly and perform the *o*-Ph assay. To do so, [chito-PPC_{SI/a}]₅ multilayers were constructed in a 96-well plate and exposed to SDS 1%, NaOH 1 N, HCl 1 N, BCA-A reactant (which does not interfere with *o*-Ph assay, data not shown)

or H_2SO_4 3.5%. A BCA assay performed on multilayers not exposed v.s. exposed to each treatment allowed the disassembly to be evidenced. Results are presented Figure IV.54a for the single or double exposure to 3.5% H_2SO_4 (the solution was pipetted up and down), which was the only successful method. The H_2SO_4 solutions were then recovered and neutralized with NaOH , and an *o*-Ph assay was conducted (see schematic in Figure IV.54b).

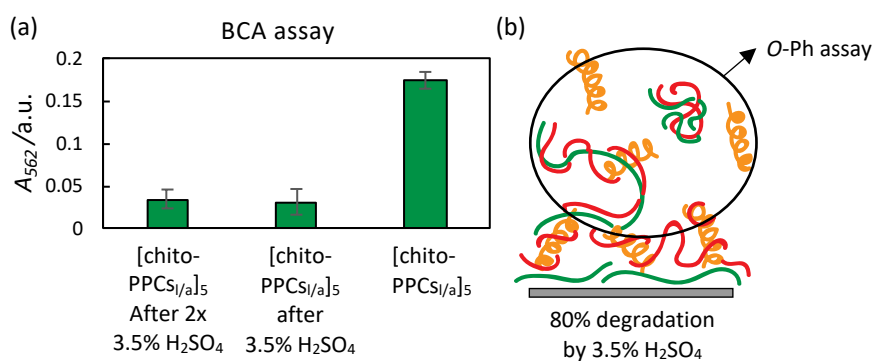
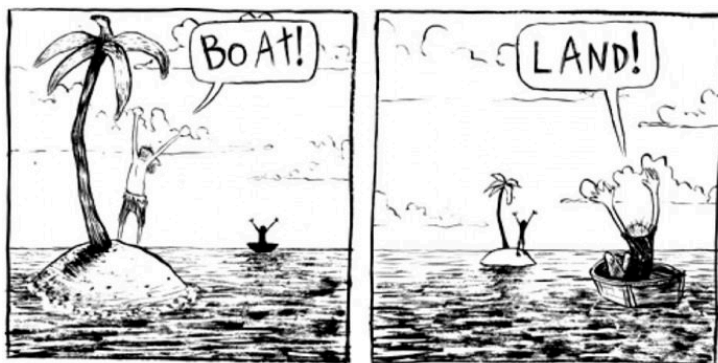


Figure IV.54 – (a) BCA dosage of a [chito-PPCsI/a]₅ multilayer constructed in a 96-well plate and subsequently exposed to either 2 times or 1 time H_2SO_4 (3.5%), or to nothing. (b) schematic of the multilayer disassembly upon H_2SO_4 exposure.

Part V: Conclusion and future work



1. Conclusion

1.1. Summary of achievements

This thesis aimed at providing a way to facilitate the use of the LbL method for the surface immobilization of proteins. Following an extensive review of the literature, it was found that the heterogeneity of a given protein and amongst proteins is the major setback of the use of the LbL method. Therefore, it was proposed to standardize protein properties *via* their complexation with PEs. The resulting PPCs could then be used as a new building block of the LbL method. The developed method was thus expected to be robust and universal, *i.e.* applicable to any kind of protein.

The first step was to create PPCs and control their charge. It has been demonstrated for Lyz complexation with PSS_i (PPCs²) and LL37 complexation with alginate (PPCs_{l/a} – also for Lyz complexation with alginate and LL37 complexation with PSS_i, see appendices 3, p237, and for GOx complexation with chitosan, see Appendices 3, p239, and the master thesis of Ivan Dutrieux, 2017) that the PPCs charge can be controlled by adjusting the stoichiometry of the complex. This was expressed as a negative-to-positive charge ratio (-)/(+net) (or the opposite for GOx which is negatively charged at the investigated pH). In all cases, the addition of the PE to the protein resulted in a charge inversion, *i.e.* the PPCs takes the charge of the added PE, between (-)/(+net) $\approx$ 1 and 1.5. For all tested PPCs, the resulting complex can

be assembled LbL with an oppositely charged PE, as demonstrated with chitosan and PAH.

Importantly, it was demonstrated for PPCs² (Lyz-PSS_I complexes) that their LbL assembly with PAH is independent of the Lyz charge, and that it depends solely on the PE-PE interaction. Indeed, PPC² core is reported neutral, with the PE chains exposed outside the complex. Upon LbL assembly, the PE chains of the PPC interact with the other oppositely charged PE. The result is that only the PE-PE interaction governs the assembly and the protein remains trapped in the neutral core of the PPC. For these conditions to be met, it was evidenced that the PE ionization degree should not be maximal, so that it is not able to disassemble the PPCs upon its LbL assembly. The independence of the protein charge could allow to theoretically immobilize any protein using the PPCs-based method. This result is highly promising since the failure of the classical assembly LbL for the surface immobilization of any protein originates in its dependence on the protein charge (a dependence which was demonstrated in this thesis). A further proof of this universality is that LL37 was successively immobilized with other PEs while the classical LbL assembly failed for this peptide. The potential of PPCs for the LbL assembly of any protein with any PE is also further confirmed with the data presented in Appendix (Part VI:2, p237), where it is shown that Lyz-PSS_I, Lyz-alginate, LL37-PSS_I and LL37-alginate complexes can be assembled LbL with PAH and that in every case, the resulting multilayer growth is much better compared to the classical bare protein assembly. Moreover, it was demonstrated for PPCs² and PPCs_{I/a} that much more protein can be immobilized on the surface using PPCs compared to the classical assembly (in any pH or I condition).

Keeping a high protein bioactivity is critical for all applications requiring their immobilization. As a proof of concept for the design of a heterogenous biocatalyst, a 3D-printed reactor was coated with a [PAH-PPCs²]₅ multilayer and a suspension of lyophilized bacteria, *i.e.* the natural substrate of Lyz, was flowed through the reactor. In about one hour, the bacteria suspension became transparent, indicating that Lyz had hydrolyzed all cells walls. In order to achieve a better sensitivity, the same multilayers were constructed in a multiwell plate. As a matter of comparison, multilayers made *via* the

classical route, *i.e.* bare Lyz LbL assembly with an oppositely charged PE, were also constructed in a multiwell plate. For both type of multilayers, the enzymatic activity of Lyz was assessed using (GlcNAc)₃-MeU. The results show that Lyz immobilized in PPCs-based multilayers is more active than after its immobilization via the classical route. This was attributed to a higher hydration level of the latter.

Interestingly, it was shown in Chapter 4 that two oppositely charged PEs and a protein do not coexist in the same self-assembled object in suspension. In contrast, the alternate adsorption of PPCs with an oppositely charged PE lead to a self-assembly where all three charged species can coexist. The path along which the multilayer is constructed, and the use of a solid surface are thus critical for the stability of the system. Just as for the classical LbL, the PPCs-based multilayers exist thanks to the alternate adsorption of oppositely charged species on a surface. If the very same components were adsorbed together, no multilayer would be constructed. The multilayer is thus in an out-of-equilibrium state (this is hardly new). With the classical LbL, the exciting news was that a multilayered architecture could be constructed. The two PEs (or the protein and the PE) were, however, expected to favorably interact at interfaces since they were known to form PECs (or PPCs) in solution. Through the LbL assembly of PPCs with a counter PE, there is a new feature that emerges. Indeed, the path that was used here to construct the self-assembled films not only allows obtaining a particular architecture, but it enables the three molecules to coexist while they would not in suspension. This key feature resulted in a system with new properties, as further evidenced in this thesis.

As demonstrated in Chapter 2, if a PE with a high ionization degree is assembled using the LbL approach with a PPC, the protein may be removed from the assembly. Even if this results in the failure of the use of PPCs to immobilize any protein in these conditions, the multilayer that is obtained presents very particular characteristics. Indeed, the use of the protein as a sacrificial element led to a multilayer that contains solely two oppositely charged PE (PAH and PSS_i in our case), yet, its architecture completely differs from a multilayer that is constructed *via* the alternate adsorption of the very

same two oppositely charged PEs (no protein used). Especially, it was demonstrated that these particular multilayers are exceptionally hydrated, and that their dehydration is irreversible. After dehydration, they become undistinguishable from multilayers constructed via the classical alternate adsorption of two oppositely charged PEs. In order to record water sorption/desorption isotherms, a method that allows to continuously vary the RH of the air in contact with a PEM-coated QCM-D sensor was used. This method, which was not used before for PEMs, allowed to evidence that continuous rather than discrete RH variations are needed to fully grasp the swelling/deswelling behavior of interfacial self-assemblies.

The last property that was evidenced is that the coexistence of these three charged species allows to release a higher fraction of intact proteins. Usually, in order to switch from a stable to an unstable mode, it is needed to switch from an attractive to a repulsive mode, or to add a chemical moiety that is able to compete with the film components. Both approaches require particular PEs and protein properties (charge inversion, etc.). Here, by solely changing the pH and/or I, the species with the higher attractive force binds together and a large fraction of the third one is released free. In particular, it was demonstrated that the use of PPCs allows a higher Lyz amount to be immobilized and a higher enzymatic activity to be recovered after release. This leads to a total enzymatic activity which is up to three-fold higher when compared to the classical LbL assembly. This interesting self-reorganization might prove useful for the design of systems that release intact molecules.

The potential of the method that has been developed was finally tested for a biomedical application. The aim was to create an antimicrobial and angiogenic coating for dressings *via* the surface immobilization of LL37. LL37 was first complexed with alginate to form PPCs_{I/a} and the properties of the formed complexes were investigated in solution. Besides the ability to control the PPCs_{I/a} charge, which was already demonstrated for PPCs², it appears that LL37 takes an α -helical conformation upon complexation with alginate. This is an interesting feature as LL37 bioactivity is related to its α -helical form. Peptides are highly sensitive to the hydrolytic activity of proteases that are present in the wound environment. LL37 sensitivity to

chymotrypsin was thus tested when it is a free molecule or complexed in PPCs_{I/a}. It appears that PPCs_{I/a} protect LL37 against chymotrypsin activity in pure water. However, when PPCs_{I/a} are in TRIS-CaCl₂ buffer, LL37 sensitivity to chymotrypsin significantly increases compared to the one of non complexed LL37 molecules. This LL37 sensitization was attributed to the formation of a chymotrypsin-alginate complex, which subsequently improves the hydrolytic activity of the enzyme. In a view to immobilize LL37, PPCs_{I/a} or bare LL37 molecules were alternately adsorbed with respectively chitosan or alginate on a Tegaderm Contact dressing. It was demonstrated that the classical bare protein LbL assembly fails to immobilize any LL37, while up to 5 µg of LL37 per cm² were immobilized using PPCs_{I/a}. This confirms the potential of PPCs-based LbL assembly. The LL37 release profile was investigated and it appears that 60% of the LL37 immobilized is released within the first 7 hours. The remaining 40% is released over the 15 following days. Finally, a set-up was developed in order to test *in vivo* the effect of LL37-containing multilayers on wound healing.

The method that was developed throughout this thesis thus has a true potential for protein immobilization, which will be beneficial to fields requiring bioactivity on the surface of inert materials. Importantly, new features emerged from the use of PPCs in LbL assemblies: (1) the assembly is independent of the protein charge, (2) the protein can be used as a sacrificial element to create new PEMs architectures, and (3) a higher fraction of intact molecule can be recovered upon its release from the multilayer. These features were obtained only *via* investigating new assembly construction pathways, which stresses the importance of assembling common building blocks through new routes to obtain novel functionalities.

1.2. Lessons learned

Constructing LbL thin films is the simplest and most intuitive way to order molecules at interfaces – it is not surprising that it has been intensively used. However, in my opinion, this method suffers from this naive picture.

The first consequence is that multilayers are always represented with simple cartoons corresponding to the method rather than to the real structure of

the self-assembly. This is fine, except when one then assumes that the multilayer is made of the constituents that were used to grow it, and that it possesses the structure depicted by the simplified cartoons. When a multilayer is constructed, its composition and structure should always be fully investigated and cannot be predicted based on the method nor the constituents. Understanding the interactions and the competition between soluble and surface-immobilized compounds is of utmost importance, as well as taking care of effects related to subtle details of the assembly process.

The second consequence of this naive picture is that an incremental strategy is usually chosen when a LbL with specific functions is desired. With this strategy, a model LbL assembly is gradually improved to finally obtain the desired functions. This allows the use of materials that are not expensive or easy to detect to optimize the conditions that lead to a good LbL growth, a stable multilayer, or a multilayer that releases its content in a controlled manner. Then, the PE or the protein is replaced by the more expensive/less easy to detect material, or is constructed on a different surface geometry that is required for the application. This strategy emerges from the laconic assumption that alternating the adsorption of oppositely charged species is the main driving force of the LbL self-assembly, and that any similar macromolecule would self-assemble the same way. However, the LbL assembly of proteins with PE depends on a wide range of parameters such as protein nature, PE nature, concentration, ions and counterions nature, adsorption sequence, temperature, relative humidity, solvent, surface geometry and chemistry, and each of which can drastically change the resulting multilayers. The translation from the model to the application thus cannot be predicted. Therefore, I suggest choosing materials (PE, protein, surface, etc.) that fully match the specifications of the application and to use them from the beginning, even if it sometimes makes the analysis of the composition, the stability and the structure more challenging.

2. Future work

2.1. Fundamental investigations

Throughout this thesis, the LbL assembly of PPCs with PE has shifted from the understanding of their mechanism of formation to the development of new materials. Nevertheless, the fundamental understanding of the PEMs that were created is critical.

When reviewing literature, there is a strikingly higher number of papers and methods that allow to characterize the state of multilayers compared to their dynamics. Except in Chapter 3, only the state of the films in given conditions was investigated in this thesis. Transitions between different states were also evidenced in Chapter 4, but the multilayer was not observed upon reorganization.

QCM-D is one of the best methods to monitor a dynamic process. For example, thermal transitions were observed in dry and hydrated LbL assemblies that exhibit linear and exponential growths. In particular, PEMs that contain PEs with a higher segmental mobility, *i.e.* those that undergoes exponential growth, displayed a thermal transition akin to a glass transition between 49°C and 56°C.²⁹³ In Chapter 3, it has been shown that the use of Lyz as a sacrificial element in the LbL assembly leads to thick multilayers with a very high hydration level. A high swelling degree implies that many charges are uncompensated in the hydrated state. We can thus expect a high PE segmental mobility. As a consequence, glass transitions might be observed upon temperature increase in the QCM-D. If the dehydration irreversibly changes the structure, and the resulting PEM is the same as the linearly growing [PAH-PSS]_i LbL assembly, such glass transitions should not be observed anymore after dehydration.

Another way to study the dynamic of the systems could be to monitor the LL37 intrinsic fluorescence as the multilayer undergoes reorganization. Indeed, it has been clearly evidenced in Chapter 5 that LL37 in PPCs_{i/a} has a completely different fluorescence when compared to LL37 free in water. As a consequence, the fluorescence of a multilayer as a function of time could

allow the monitor the release of intact LL37 from multilayers. Similarly, LL37 conformation is different when it is free in water or in PPCs_{II/a}. CD measurement could thus allow to monitor the release of intact LL37 molecules.

2.2. Application-oriented outlook

2.2.1 *Reaction-free enzyme colocalization in diblock polyampholyte for improved tandem reaction*

It was demonstrated in Chapter 4 that the presence of the PE in the proximity of the enzyme could significantly enhance its biological activity. In this thesis, a pH shift in the PE microenvironment was invoked. Other ways to improve enzymatic reactions were already reported in literature. In particular, the amelioration of tandem reactions of enzymes *via* their colocalization was reported.^{282,294}

For future work, we propose to choose two enzymes that work in cascade, *i.e.* the product of the first is the substrate of the second, and to immobilize them in a PPC using a diblock polyampholyte (DPA). The principle is that each block of the polyampholyte complexes one type of enzyme. This would result in a proteins-DPA complex (PsDPAC). The two enzymes should be oppositely charged at their working pH (pH at which the reaction is carried out), so that one protein complexes the negative DPA moiety while the other complexes the positive DPA moiety. The most studied cascade reaction is the HRP-GOx tandem reaction. In this reaction, GOx transforms glucose into CO₂ and H₂O₂, and then the HRP uses H₂O₂ to oxidize various organic compounds.^{282,294} The charge as a function of pH was computed for these two enzymes (using 1CF3 and 1HCH pdb files for GOx and HRP) and results are presented in Figure V.1a. It appears that between pH 5 and pH 7, HRP is positively charged while GOx is negatively charged. As a consequence, it could be possible to complex GOx with a positively charged DPA moiety and HRP with a negatively charged moiety. However, the latter complexation is not straightforward. Indeed, when both DPA moieties are oppositely charged, an intra-PE complex forms and the DPA precipitates.²⁹⁵ As it was demonstrated in this thesis, the solution to this problem stands in the path to follow. Instead of mixing all

constituent at once, it might be possible to first complex one moiety with a protein, then the other, by carefully selecting the complexation conditions. Indeed, if one block is uncharged, the latter forms a neutral micelle and the charged one stays extended/soluble in water (see Figure V.1b①). In this case, complexation with HRP is possible. Then, by decreasing the pH, the second moiety gets charged and can be complexed with GOx (Figure V.1b②). In other words, by sequentially complexing the DPA with different proteins at different pH values, it might be possible to prevent the formation of an intra-PE complex in the DPA and colocalize the proteins in the PsDPAC. It is worth mentioning that the conditions of self-assembly and the nature of the DPA might result in PsDPAC with completely different architectures, which could in turn have an effect on the obtained tandem reaction.

A well-studied DPA is poly(PSS-*b*-2-vinylpyridine) (PSS-*b*-P2VP). The PSS moiety is a strong polyacid and is thus always negatively charged. P2VP is a weak polybase and its ionization degree depends on pH. PE charge prediction as a function of pH is not straightforward due to the close proximity of charged species that influence their respective pKa. When it comes to DPA, it is even more difficult to predict the ionization degree at different pH values. Indeed, the complexation of the oppositely charged moiety drastically shifts the pKa of the charged groups. For PSS-*b*-P2VP, Varoqui *et al.* measured the ionization degree as a function of pH and compared it to P2VP alone (without the PSS moiety).²⁹⁵ Their data are presented in Figure V.1c and show a large shift of the PSS-*b*-P2VP pKa to more alkaline values. Interestingly, while the pKa of P2VP (a classical PE) is around 2, the pKa of PSS-*b*-P2VP is at pH 5.2, which is the same as the one of pyridine single molecules. A possible explanation is that when P2VP gets charged, it complexes PSS. As a consequence, the effect of the charge proximity is cancelled since charges are paired. The result is that the ionization degree as a function of pH of the P2VP moiety resembles the one of monomers in solution (no effect of charge proximity on the pKa shift). The result is that around pH 7, P2VP has a low ionization degree and adopts the structure depicted in Figure V.1b①. Therefore, it can be complexed with positively-charged HRP. Then, when pH is decreased close to the pKa of PSS-*b*-P2VP, *i.e.* around pH 5, the P2VP moiety gets charged and can be complexed with GOx

as schematized in Figure V.1b①. Of course, there is a risk that when P2VP moieties get charged, they displace HRP and complex with the PSS moieties. This risk is mitigated by the low ionization degree of P2VP (around 50%) at pH 5. Indeed, we have demonstrated in Chapter 2 and Chapter 5 that a lower ionization degree results in lower ability to displace a complex. After that the PsDPAC is formed, the cascade reaction should be tested and compared to the one of a mixed solution containing the same amount of free HRP and GOx. These PsDPAC could be useful in suspension, the products of the reactions being simply separated from the complex by centrifugation of the later. PsDPAC could also be integrated in LbL assemblies by alternate adsorption with an oppositely charged PE (as it was demonstrated for PPCs). Indeed, As for PPCs, the charge of the complex can be tuned. For PsDPAC, the stoichiometry between the negatively and the positively charged moieties dictates the charge of the final complex.²⁹⁵

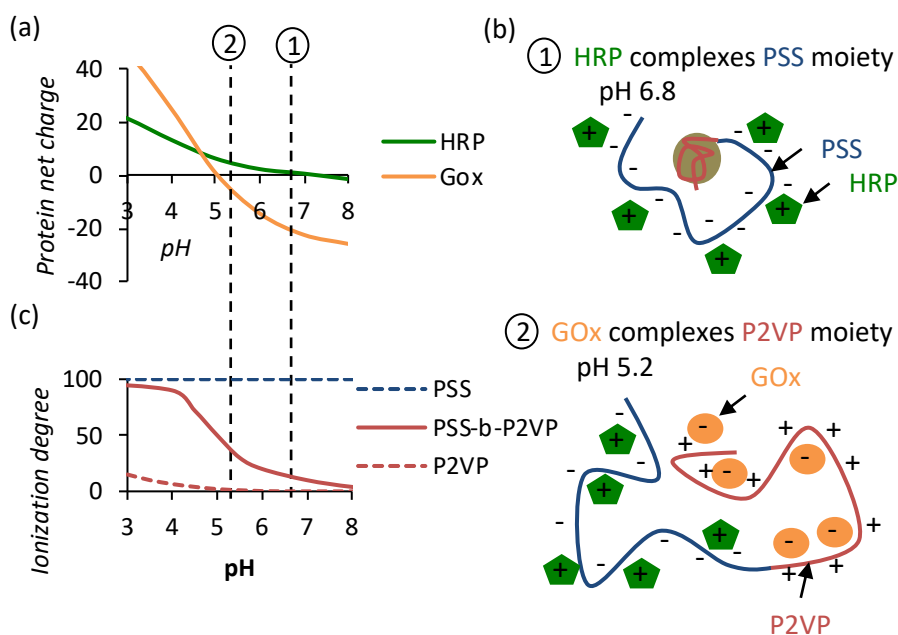


Figure V.1 – (a) HRP and GOx net charge as a function of pH (computed from PDB2PQR). – (b) Schematic of the complexation steps. (1) HRP complexes the PSS moiety at the higher pH possible where HRP is positive. (2) GOx complexes P2VP moiety at the lower pH possible where GOx is negatively charged. – (c) PSS, PSS-b-P2VP and P2VP charge as a function of pH.²⁹⁵

2.2.2 PPCs-based multilayer stability improvement for heterogenous biocatalysis and biosensing

In Chapter 5, the LbL assembly of PPCs with PE proved advantageous for drug delivery. Indeed, it was shown that a much higher activity could be maintained for the released proteins. This was attributed to the self-reorganization of the multilayer, resulting in the competitive binding of the two PEs and the subsequent release of a higher fraction of intact protein molecules. While this feature is valuable for the delivery of proteins, it is a major drawback for applications that aim at preserving the active protein immobilized, *e.g.* for heterogeneous biocatalysis and biosensing. In the same Chapter, it was shown that the LbL assembly of single protein molecules with PE allows, upon building in certain conditions, to create multilayers that are stable. Unfortunately, it was also shown that less than 10% of activity was maintained in these films.

In this frame, it is thus substantial to find ways to stabilize the multilayer created with PPCs to avoid the release of proteins. Luckily, PPCs-based multilayers are composed of two PEs and one protein, in contrast with the classical LbL assembly of protein with one PE. This allows using strategies to reticulate the film with a much lower probability to cross-link the protein active site. Indeed, cross-linking of amines in a [PAH-PPCs] multilayer will likely result in a lower protein deactivation compared to [Lyz-PSS]. Interestingly, if the PPCs-based method is used for heterogeneous biocatalysis and biosensing, the system can be designed so that both PE possess cross-linkable functions that are not present on the protein surface. For example, click chemistry could be used to cross-link only the PEs, therefore sparing the protein from any chemical reaction.²⁹⁶

We also suggest creating PPCs-based multilayers in conditions that lead to a high amount of immobilized protein, *i.e.* in which the counter polyion has an ionization degree lower than 100% and PPCs are assembled at low I (see Chapter 2). Then, these multilayers should be cross-linked *via* a reaction that involves one or both PE used. After cross-linking, the conditions should be changed and both protein leaching and enzymatic activity should be investigated. Interestingly, the classical theory of LbL assemblies suggests

that by screening the electrostatic interactions or by decreasing the PE ionization degree, it might be possible to swell the multilayer, even if the multilayer is cross-linked.²⁹⁷ This could in turn result in higher substrate diffusion in the PE network and consequently a higher protein activity could be obtained. However, as demonstrated in Chapter 3, PPCs-based multilayers do not behave the same way as classical LbL assemblies and a higher swelling degree might be obtained when ionization is maximal and I is minimal. These features should be tested.

2.2.3 Protein immobilization for drug delivery

The method that has been developed during this thesis will be beneficial to any application that requires the immobilization and subsequent release of active proteins. In the following, we will non-exhaustively present proteins that could be immobilized and how the LbL assembly of PPCs with PE could be worthwhile. As reported by Lu *et al.* in a recent review, there is an urgent need to use materials or formulations (including their form(s) after response) with excellent biocompatibility as the first step when conceiving the strategy.⁵⁸ Therefore, only highly biocompatible materials will be used in the following.

a) LL37 to promote wound healing

Results obtained with LL37 are highly promising and should be further investigated. Importantly, the delivery system relies on the self-reorganization of the multilayer to release intact LL37. While evidences were presented for [PAH-PPCs²] films (immobilizing Lyz), the use of other PEs might influence the mechanism of construction or delivery. Indeed, chitosan and alginate have different hydrophobicities and pKa compared to PSS and PAH. The method that is based on the LL37 intrinsic fluorescence, which was proposed hereabove could be used to assess for the release of uncomplexed LL37. In order to verify the release of LL37 from PPCs_{I/a}, the method based on light sheet microscopy recently developed by Chen *et al.* could be used.²⁹⁸

b) rhBMP-2 for bone regeneration and repair

Recombinant human bone morphogenetic protein 2 (rhBMP-2) has a unique capability to promote bone regeneration.²⁹⁹ It is involved in the hedgehog

pathway, TGF beta signaling pathway, cytokine-cytokine receptor interaction, cardiac cell differentiation and epithelial to mesenchymal transition.³⁰⁰ Importantly, it is its potential to induce osteoblast differentiation that has made of rhBMP-2 the spearhead of therapy in nonunion bone defect.²⁹⁹ In this way, a large number of different delivery systems have been investigated to control the sustained release of rhBMP-2. In a recent review, it is reported that the current available carriers present problems such as ectopic bone growth (bone present in abnormal sites), low protein delivery and protein inactivation. With respect to the former, it appears that the released rhBMP-2 has to be confined in the bone defect region.²⁹⁹ Moreover, an optimal carrier should possess a release kinetics that is neither a bolus injection or low dose prolonged release.³⁰¹ Indeed, the first would lead to excessive BMP-2 concentration far from the bone defect, which could cause ectopic bone growth. The second would result in an ineffective BMP-2 concentration. In their comprehensive review, Agrawal *et al.* reported that, depending on the carrier, improved osteointegration was achieved by releasing only a few μg to 100 μg of rhBMP-2 per implant (delivery duration ranges between 4 and 16 weeks).²⁹⁹

Using PPCs, rhBMP-2 masses that are of this order of magnitude could be easily immobilized on an implant surface. The protein could then be released free and highly active by self-reorganization of the multilayer. However, an issue that has not been tackled in this thesis is the control of protein release kinetics. This has to be investigated, especially for implants that, contrarily to bandages, are not intended to be replaced after few days. An elegant way that has been proposed earlier is the insertion of components between the layers of a LbL assembly to slightly improve their stability so that it can release its content over a longer time period.³⁰² In this frame, we suggest to add a bioinspired catechol-modified PEM every few bilayers of PPCs-PE.³⁰³ This could result in the successive release of each PPCs-PE bilayer interspersed with the one of the catechol-modified PEM.

Actually, rhBMP-2 is very similar to Lyz as it is a small positively charged protein. Therefore, we suggest to complex rhBMP-2 with alginate or heparin. Heparin has osteointegrative properties, however, in a view to release intact

proteins, it might be easier to use alginate, which is much less charged. The resulting PPCs should then be integrated in a multilayer system with chitosan, one of the only positively-charged biocompatible PE. Alongside rhBMP-2 immobilization, we suggest to immobilize Lyz using the very same protocol (same PE) and to investigate its enzymatic activity once released. Indeed, rhBMP-2 is a structural analog of Lyz. If Lyz enzymatic activity is recovered after its release, we could expect the same from the system immobilizing rhBMP-2. (The purpose of this is to quantitatively evaluate the activity loss due to the immobilization of the protein. This is not practical for rhBMP-2 as it is a growth factor and only cellular response can be investigated. In contrast, an accurate loss of enzymatic activity can be measured for Lyz.) As already presented, we suggest alternating a catechol-modified PEM every few bilayers of PPCs. The size of either the catechol-modified PEM or the PPCs bilayer sequence should be varied and its influence on the release kinetics should be investigated.

c) Antigens for transdermal protein delivery

The transdermal delivery of proteins *via* microneedles (MNs) is becoming topical. This method allows to painlessly translocate proteins to the systemic circulation.⁸⁴ Different ways to deliver proteins using MNs exist and are represented in Figure V.2; (a) solid MNs are coated with the protein to deliver, (b) the MNs remain in the skin and dissolve to deliver the protein, (c) similarly to the latter, the MNs stay in the skin and degrade over time, and (d) the degradation/dissociation of polymer-based MNs depends on the particular conditions that are present in the skin, *i.e.* bioresponsive MNs. PPCs-based multilayers are highly sensitive and readily release intact proteins. They could thus be either used as coating of solid MNs or combined with all other MNs to create dual functions (release from the coated and from the core MNs).

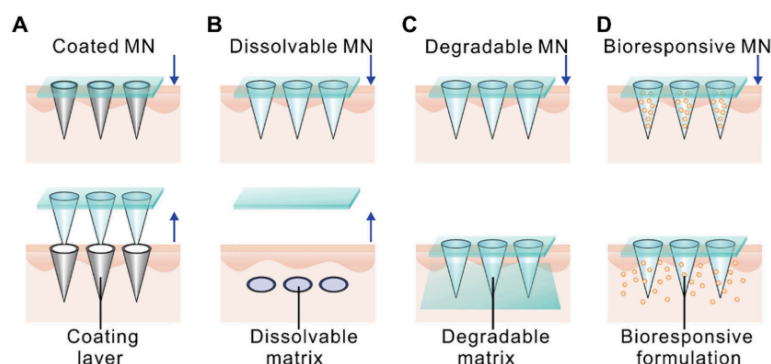


Figure V.2 – Different strategies that can be used to deliver drugs using MNs. Reproduced from Ye *et al.* with permission of Elsevier.⁸⁴

2.3. Future work related to unexpected observations

In Chapter 5, it was found that the presence of alginate could significantly improve the hydrolytic activity of α -C against LL37. Given the number of drug delivery systems using PE that are currently under development, this issue should be investigated. In particular, the peptide sensitivity to proteolytic activity is already a major issue for their delivery. Finding that the delivery system increases this sensitivity may prove to be a severe drawback in the use of PEs as drug carriers.

In order to investigate this issue, we propose to adapt the protocol developed by Chereddy *et al.*²⁶⁹ The new procedure would be to add LL37 alone or LL37 mixed with alginate (or another naturally occurring PE such as heparin) to a wound. After 0, 4, 8 and 12 days of wound healing, tissues would be collected and analyzed by MS. Contrarily to the protocol developed by Chereddy, emphasis would be put on evidencing masses that corresponds to the hydrolyzed peptide, and to compare it to the whole peptide.

2.4. Broad application-oriented outlook

Overall, the method that has been developed will be beneficial to any application requiring immobilized protein at interfaces. In all cases, the step of immobilization is facilitated by its independence on the protein charge state. For the design of new drug delivery systems and biomaterials, the improved activity after release is highly promising. The only pitfall is the

control on the release profile of the protein. The latter can be mitigated using the methods that were proposed in this last section. The method will also be beneficial to applications requiring active proteins to be stable at the interface, *e.g.* heterogenous biocatalysis and biosensing, via the integration of new cross-linkable functions in the multilayer.

Part VI: Appendices

1. Preliminary results on PPCs formation

The abbreviation PSS_i will not be used in this section as different PSS M_w were used.

In this thesis, a certain PE nature (PSS) and molar mass (7,540 g mol⁻¹) was chosen. This choice was motivated by primary results, which will be presented in the following. First, the effect of PE molar mass and nature (PSS *versus* PAA) on PPCs formation was investigated using turbidimetry and UV-dosage. Results show that the main parameters that influence PPCs formation are the PE length and ionization degree (in a given pH and I condition). It was thus decided to use PSS rather than the PAA since the ionization degree of the latter changes with pH. The effect of pH on PPCs made with different PSS was also assessed using turbidimetry and DLS. In line the results obtained when PSS was compared to PAA, it appears that PSS length is critical. In particular, PPCs with an extended length (R_e) smaller than the protein diameter behaves differently compared to those having a R_e higher than Lyz diameter.

With the aim to alternate the adsorption of PPCs with a positively charged PE, they have to take negative value and possess an external corona of PE.¹⁶⁴ We hypothesized that PSS with a R_e higher than the Lyz diameter would be more effective in (i) overcompensating Lyz charges and (ii) creating a PE corona around PPCs. Two PSS that were tested had a R_e higher than Lyz diameter, *i.e.* PSS of 7,540 and 70,000 g mol⁻¹. It appears that both led to PPCs of similar size but the one made with PSS of 7,540 g mol⁻¹ scattered much more light. This latter feature was attributed to denser PPCs. Somewhat arbitrarily, we decided to use PSS with a $M_w=7,540$ g mol⁻¹ in this thesis. Our hunch was that denser PPCs would lead to a higher density of Lyz molecules immobilized on the surface. Nevertheless, it clearly appears in Chapter 2 that soluble PPCs are much better assembled with an oppositely charged PE. It would be interesting to achieve the same experiments as the one that were done in Chapter 1 and 2 with PE of different length. Indeed, PPCs with different morphologies, *i.e.* made with PE of different M_w , might results in improved LbL assembly.

1.1. Effect of PE nature and molar mass

Three different molar masses (M_w) were investigated for PSS and PAA. As a matter of comparison, similar M_w for chosen for both PEs. For PSS, M_w of 1,690 (PSS1690), 7,540 (PSS7540) and 70,000 (PSS70000) g mol^{-1} were chosen. For PAA, M_w of 2,100 (PSS2100), 5,100 (PSS5100) and 15,000 (PSS15000) g mol^{-1} were chosen.

First, Lyz complexation with these different PE was investigated at pH 7.5 and $I=10$ mM. The turbidity of the solution was measured as a function of PE addition to Lyz. As in Chapter 1, the addition of PE to Lyz was expressed as a ratio of charge $(-)/(+net)$, which was computed as the ratio of PE charges to the net structural charge of Lyz at pH 7.5, i.e. +8.0. For PSS, the charge was taken as the number of monomers since it is a strong polyacid and its ionization degree is always of 100%. Contrarily to PSS, PAA is a weak polyacid and its charge depends on pH. At pH 7.5, an ionization degree of 42% was considered.³⁰⁴ For both PPCs, the PE was added until a turbidity plateau was obtained. In order to confirm PPCs formation, PPCs with different $(-)/(+net)$ were constructed and subsequently centrifugated at 9500 g. As in Chapter 1, the amount found in the pellet was then quantified. Results of this quantification are presented with turbidity for PSS in Figure VI.1 and for PAA in Figure VI.3.

PPCs were then constructed at $I \approx 0, 10, 200$ or 800 mM (a pH of 7.5 was maintained). The turbidity, as a function of the $(-)/(+net)$ and $I^{1/2}$ was measured. Results are presented under the form of an isoresponse map (Figure VI.2 for PSS and Figure VI.4 for PAA). κ^{-1} , which indicates the distance over which electrostatic interactions are significant, is proportional to the inverse of $I^{1/2}$. Maps and charts built with $I^{1/2}$ as a scale can thus be interpreted in terms of κ^{-1} . When pH and I conditions are mentioned, they are valid for the building of PPCs as well as for the measurement condition.

1.1.1 PSS complexation with Lyz

It appears for both PSS 1690, PSS 7540 and PSS 70000 that the amount of Lyz found in the pellet after centrifugation increases following the trend

observed by turbidimetry (see Figure VI.1). This suggests that turbidity can be linked to PPCs formation.

Now looking at the isoresponse maps presented in Figure VI.2, it appears that the turbidity of PPCs made with PSS of 7,540 g mol⁻¹ (PPCs 7540) and 70,000 g mol⁻¹ (PPCs 70000) only differs in magnitude, while PPCs made with PSS of 1,690 g mol⁻¹ (PPCs 1690) present a different dependence on (-)/(+net) and I. For PPCs 7540 and PPCs 70000, a non-monotonic dependence of turbidity on I is observed. This can be explained by a balance between the increasingly compliant conformation of PSS and less effective electrostatic interactions as I rises. When PSS flexibility increases with increasing I, it leads to a possible better accommodation of PSS to Lyz surface charges, with therefore a higher number of PSS monomers per chain interacting with each protein molecule. This might result in a higher density and bigger complexes. In consequence, more light is scattered and turbidity increases. On the other hand, electrostatic interactions decrease with increasing I due to charge screening and no PPCs are detected at high I. This model of balance between PSS conformation and electrostatic interaction was discussed in detail in Chapter 2.

With respect to PPCs 1690, it appears that insoluble complexes are observed only at low I, I ≈ 0 mM and 10 mM (3.16 mM^{1/2}; see Figure VI.2). Interestingly, this can be explained using the same model of interaction that was introduced in Chapter 2. Indeed, PSS 7540 and PSS 70000 have an extended chain length (R_e) of respectively 9 nm and 83.5 nm, which is longer than Lyz diameter (3,7 nm).¹⁹⁹ This makes the accommodation of PSS 7540 and 70000 to the Lyz surface charge a critical parameter. Contrarily, PSS 1690 is shorter than the Lyz diameter, *i.e.* PSS 1690 R_e is 2 nm. In the latter case, the polymer flexibility can only improve the accommodation to surface charge in a limited fashion. The dependence to I that is observed is thus mainly due to the decreased electrostatic interaction.

The difference in magnitude of the turbidity of PPCs 7540 and PPCs 70000 can be explained by the fact that the PPCs 70000 is less dense and therefore scatters less light. Indeed, since PSS 70000 is longer, it leads to a higher

degree of cross linking between chains and more charges that are not compensated by Lyz. This hypothesis is supported by the turbidity plateau that is observed for slightly higher $(-)/(+net)$ values for PPCs 70000 compared to PPCs 7540, which suggests that more PSS 70000 has to be added to overcompensate Lyz charge. For both PPCs 7540 and 70000, the PPCs unfold when $(-)/(+net)$ is increased above a certain threshold-value that depends on I . In both cases, this dependence scales as following: $(-)/(+net) = 0.2 * I^{1/2}$. The PPCs unfolding at high $(-)/(+net)$ is thus probably due to electrostatic. Indeed, PPCs stability increases by lowering the magnitude of the electrostatic interactions.^{160,159,182–184}

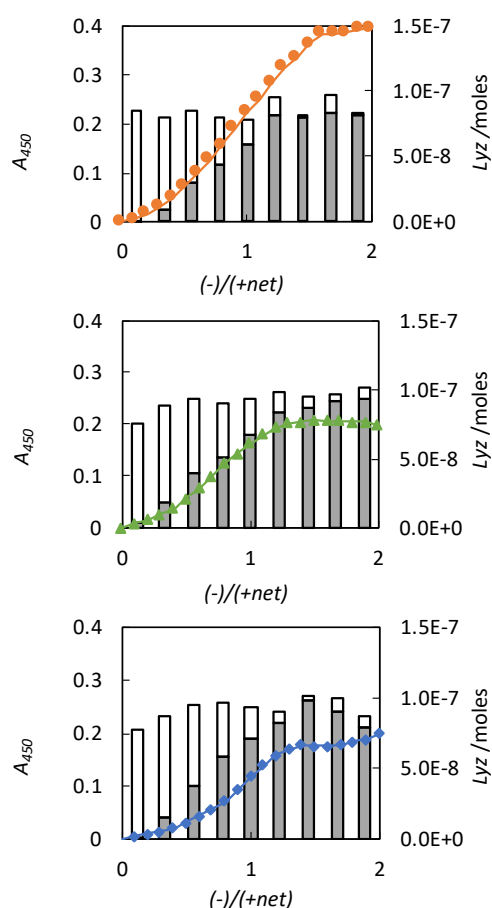


Figure VI.1 – UV dosage and turbidity at $I=10$ mM for PSS 1690 (N=8) (circle), PSS 7540 (N=36) (triangle), PSS 70,000 (N=340) (losange) (0.5 mM of negative charge) added to Lyz (0.1 mM) at pH 7.5 (buffered with HEPES).

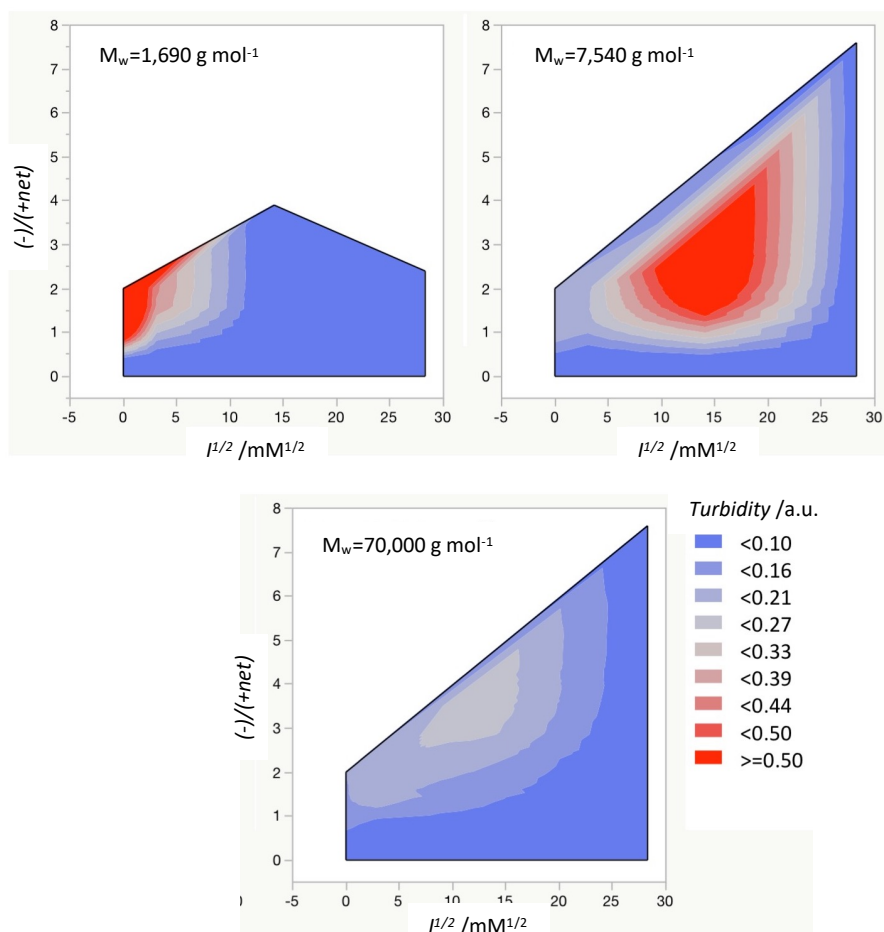


Figure VI.2 – Isoresponse map of the turbidity as a function of $(-)/(+net)$ and $I^{1/2}$ for Lyz complexed with PSS of different M_w , i.e. respectively 1,690 g mol⁻¹, 7,540 g mol⁻¹ and 70,000 g mol⁻¹. The turbidity is defined as the A₄₅₀ of PPCs suspensions. The x-axis is presented as $I^{1/2}$, meaning that this axis is inversely proportional to κ^{-1} . Complexation was carried-out at pH 7.5 in HEPES buffer. I was adjusted by adding NaCl.

1.1.2 PAA complexation with Lyz

With respect to the complexation of Lyz with PAA, the very same trend is observed compared to PSS. First, the turbidity can be effectively linked to complex formation as witnessed in Figure VI.3. Indeed, for all PAA lengths, the amount of Lyz found in the pellet after centrifugation at 9500 g, *i.e.* the Lyz that is complexed, correlate with the trend observed by turbidimetry.

The PAA of 2,100 g mol⁻¹ (PAA 2100) has a R_e shorter than Lyz diameter, *i.e.* 2.6 nm. Therefore, as witnessed for PSS 1690, this results in a complexation that is mainly driven by electrostatic interactions and turbidity decreases with increasing I . For PAA of 5,100 g mol⁻¹ (PAA 5100) and 15,000 g mol⁻¹ (PAA 15000), both have R_e longer than Lyz diameter, *i.e.* respectively 6.4 nm and 18.7 nm. This results, as witnessed for PSS 7540 and PSS 70000, in a non-monotonic dependence of turbidity on I .

It is however important to point out that, while the trend is the same compared to PSS, the position of the maximum turbidity appears at lower I . Indeed, PAA is a weak polyacid and its ionization degree is of 42% while PSS is 100% ionized. The electrostatic interaction is thus weaker for PAA compared to PSS and its contribution to PPCs formation is smaller at lower I .

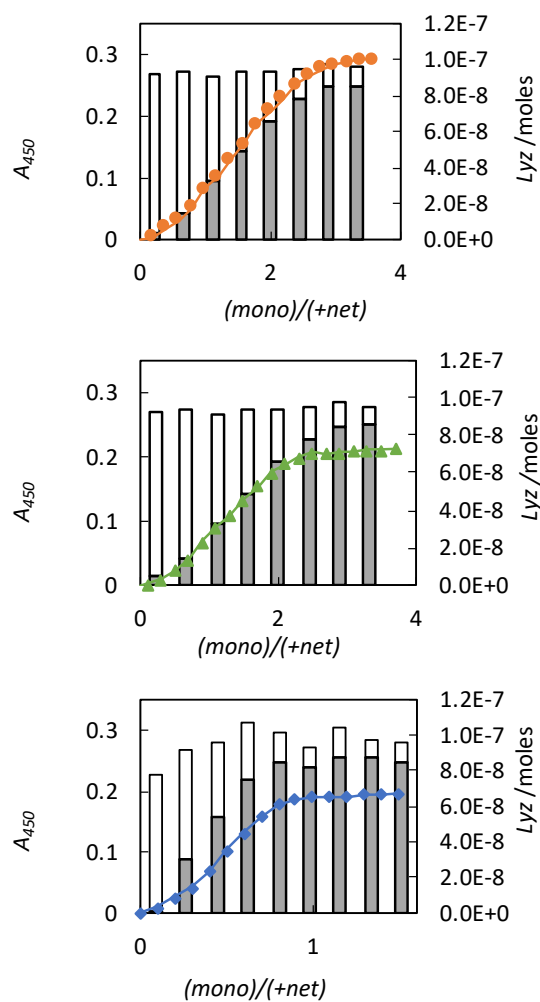


Figure VI.3 – UV dosage and turbidity at $I=10$ mM for PAA 2100 (N=21), PAA 5100 (N=54), PAA 15,000 (N=160) (0.5 mM of negative charge) added to Lyz (0.1 mM) at pH 7.5 (buffered with HEPES).

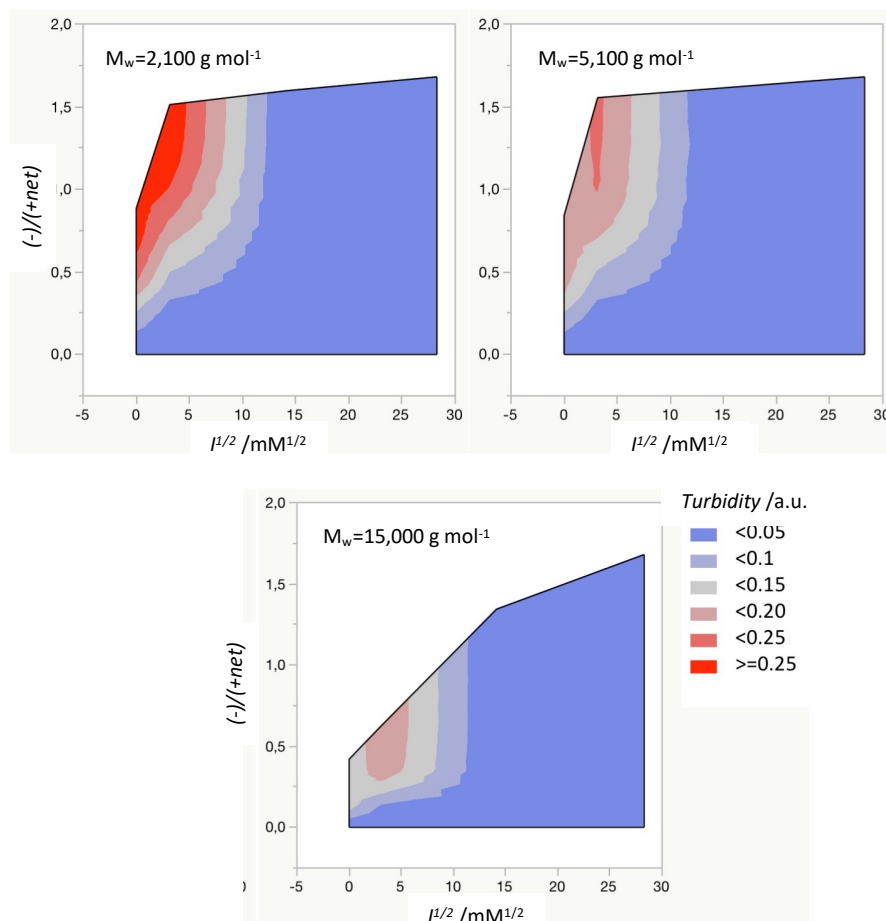


Figure VI.4 – Isoresponse map of the turbidity as a function of $(-)/(+net)$ and $I^{1/2}$ for Lyz complexed with PAA of different M_w , i.e. respectively $2,100 \text{ g mol}^{-1}$, $5,100 \text{ g mol}^{-1}$ and $15,000 \text{ g mol}^{-1}$. The turbidity is defined as the A_{450} of PPCs suspensions. The x-axis is presented as $I^{1/2}$, meaning that this axis is inversely proportional to κ^{-1} . Complexation was carried-out at pH 7.5 in HEPES buffer. I was adjusted by adding NaCl.

1.2. Effect of pH on PSS complexation with Lyz

The effect of the pH on the complexation of Lyz with PSS 1690, PSS 7540 and PSS 70000 was then evaluated. The same simple turbidimetric assay was used and the pH of a PPCs suspension was increased from 7.5 to 11.75 and decreased from 7.5 to 3. Turbidity of the PPCs suspension as a function of pH is presented in Figure VI.5a. It appears that PPCs are stable on a wide range of pH for all PSS length. When the pH reaches values close to the IEP, PPCs disassemble. This is not surprising since PSS is negatively charged and Lyz

takes a negative charge above pH 11.35. Interestingly, PSS length scales with PPCs stability at high pH. This confirms that the PE accommodation to surface charge is favorable to PPCs formation and stabilizes it.

Then, the size of the PPCs as a function of $(-)/(+net)$ was measured using DLS at pH 7.5 and $I=10mM$. Once more, the PSS 7540 and PSS 70000, *i.e.* the PSS that have a R_e greater than L_{yz} diameter, behave in the similar ways. In both cases, a plateau is observed, followed by a decrease in R_h when $(-)/(+net)$ is increased above 1.5. This result is consistent with the decreased turbidity and decreased amount of L_{yz} found in the pellet after centrifugation that are presented in Figure VI.1. For PSS 7540, the maximal size reached is $1\ \mu m$ while for PSS 70 000 it reaches $2\ \mu m$. Since the same mass of polymer was added in both cases (the number of charges is equal to the number of monomer), this result confirms the hypothesis that PPCs 70000 are less dense and thus scatters less light.

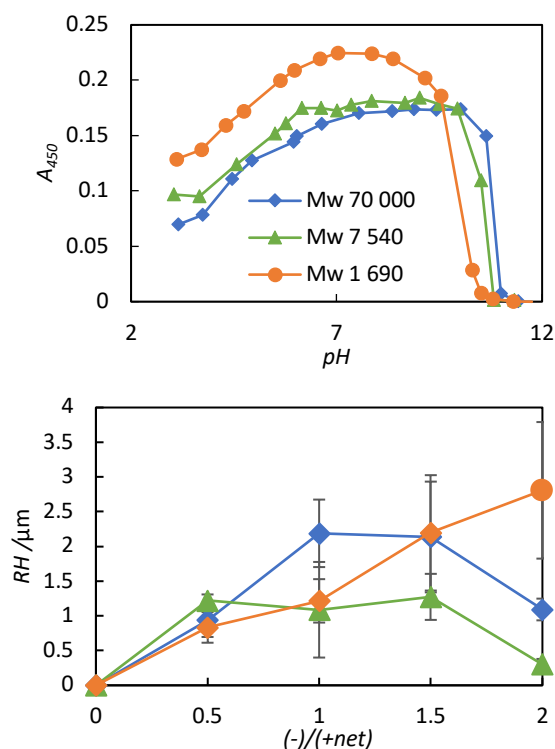


Figure VI.5 – (a) turbidity as a function of pH at $I=10$ mM for PSS 1690 ($N=8$) (circle), PSS 7540 ($N=36$) (triangle), PSS 70000 ($N=340$) (losange) (0.5 mM of negative charge) added to Lyz (0.1 mM) at $(-)/(+net)=1.5$. (b) DLS at $I=10$ mM for PSS1690 ($N=8$) (circle), PSS 7540 ($N=36$) (triangle), PSS 70000 ($N=340$) (losange) (0.5 mM of negative charge) added to Lyz (0.1 mM) at pH 7.5 (buffered with HEPES). Population of Lyz (around 3nm) in yellow circle.

In conclusion, turbidity can be used to assess rapidly for the PPCs formation. Indeed, it was shown that the turbidity increase that is observed upon PSS addition to Lyz is associated to gradual PPCs formation. Turbidity was thus used to assess the effect of the PE nature, PE length, I , pH and charge ratio on PPCs formation. With respect to polymer length, two distinct situations were evidenced. In the first, the PE is shorter than the Lyz diameter and only electrostatic seems to regulate the PPCs formation. In the second, the PE is longer than the Lyz diameter and the balance between PE accommodation to surface charge and electrostatic interactions drives the assembly. The effect of the polymer nature was investigated by complexing Lyz with PAA, a weak polyacid, or PSS, a strong polyacid. It appears that the trend is the same for both, suggesting that changes in PE will not result in antagonistic effects

on PPCs formation. I has a non-monotonic effect on PPCs formation. When it increases, the increasingly favorable compliant PE formation is counterbalanced by decreased electrostatic interactions. With respect to the effect of pH, it appears that PPCs can be constructed on a wide range of pH, provided that Lyz is oppositely charged to the PE. When approaching Lyz iep, the PPCs stability scales with PE length, suggesting that the accommodation of PE to Lyz surface charge highly contributes to PPCs formation. Finally, the size of PPCs is in the micro-meter range and scales with with turbidity, once more supporting that turbidity can be an appropriate method for fast detection of PPCs formation. With respect to the effect of (-)/(+net), results suggest that bigger PPCs are obtained around charge equimolarity. A decrease in size and turbidity is concomitant to Lyz charge overcompensation by PSS that are longer than the protein diameter.

2. Layer-by-Layer assembly of Lysozyme-PSS_I, Lysozyme-alginate, LL37-PSS_I or LL37-alginate complexes with PAH

In the following, Lyz and LL37 were complexed with either PSS_I or alginate. Turbidimetry was used to monitor complexation. The procedure and the concentration of species are the same as the one presented in Chapter 1 and 5. Results of the complexation as a function of charge ratio $(-)/(+net)$ are presented in Figure VI.6. It appears that the PE systematically forms a complex with the protein, which is observed as an increase of A_{450} as $(-)/(+net)$ increases. In all cases, a turbidity plateau is reached when $(-)/(+net)$ is around 1. As presented in Chapter 1 and Chapter 2, this is attributed to the protein charges overcompensation by the PE charges. The PPCs charge inversion, *i.e.* the PPCs takes the charge of the PE that is added, is thus expected to occurs above $(-)/(+net)=1$. In all cases, complexes formed at $(-)/(+net)=2$ were assembled LbL with PAH. As a matter of comparison, the classical LbL assembly was also carried-out. In the latter case, the component used to create the PPCs are simply alternately adsorbed to form a multilayer (bare protein molecules). The construction was monitored by QCM-D and results are expressed in frequency shift or mass as a function of adsorption steps (both a proportional). It appears that all PPCs allow a much better LbL growth, *i.e.* a much higher multilayer mass, to be obtained compared to classical LbL assembly of bare protein molecules. Interestingly, when alginate is assembled LbL with Lyz (Figure VI.6b) or LL37 (Figure VI.6d), it systematically fails to grow a multilayer, while very good LbL growths are obtained with PPCs.

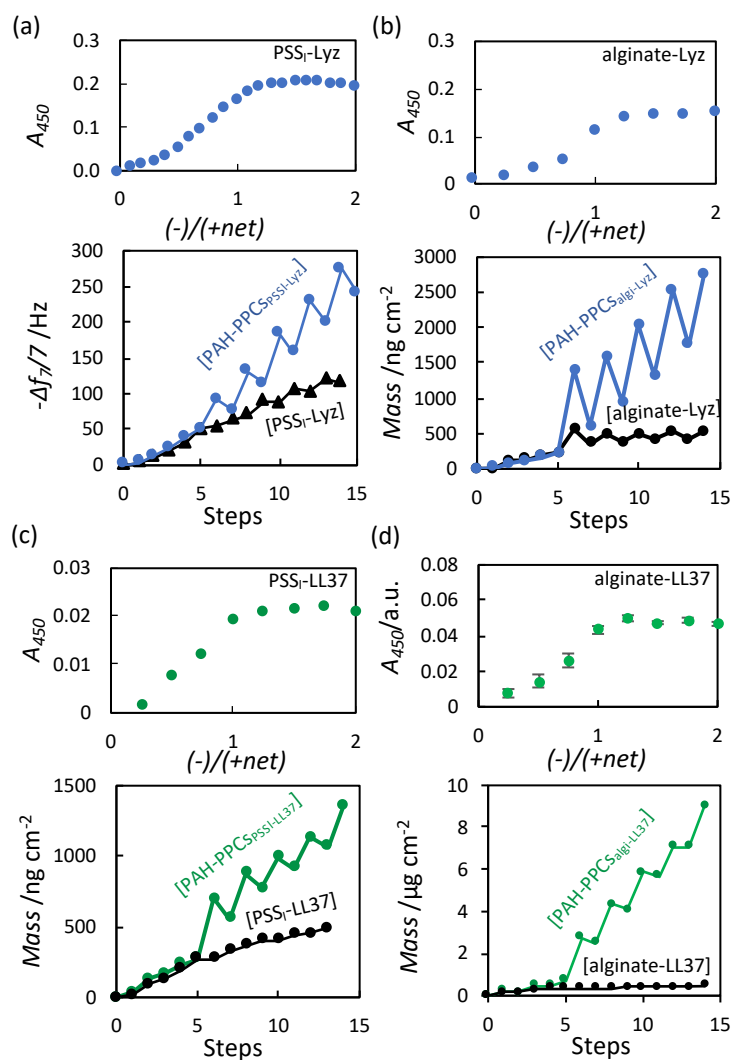


Figure VI.6 – Lyz or LL37 complexation with PSSi or alginate as a function of $(-)/(+net)$ monitored by turbidimetry. (a) Lyz complexation with PSSi at pH 7.5 and $I=10$ mM, (b) Lyz complexation with alginate at pH 5 and $I=0$ mM, (c) LL37 complexation with PSSi at pH 5 and $I=0$ mM, and (d) LL37 complexation with alginate at pH 5 and $I=0$ mM. PPCs with a $(-)/(+net)=2$ are then assembled LbL with PAH. The classical LbL assembly of bare protein molecule is also tested (black curve). The LbL growth is measured by QCM-D.

3. GOx-PAH complex

GOx was complexed with PAH using the very same protocol as in Chapter 1. The only difference being that GOx is negatively charged and, therefore complexed with PAH. As for results presented in Figure IV.1a of Chapter 1, the turbidity as a function of PE addition to the protein was measured (expressed as +)/(-net). The PPCs formed were also centrifuged to check for their formation. Results are presented in Figure VI.7 and show the very same trend as the one obtained when Lyz is complexed with PSS_I (excepted that now the protein is negative and the PE is positive).

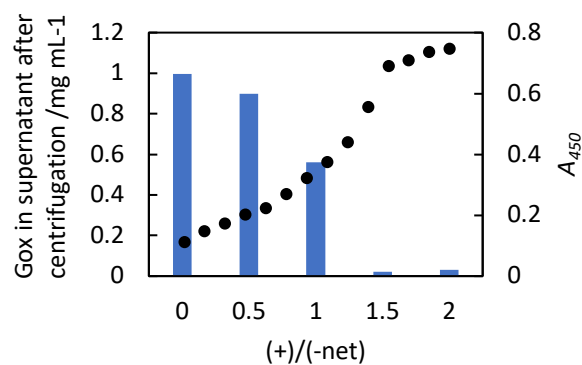


Figure VI.7 - Normalized A₄₅₀(●) of a GOx solution undergoing PAH addition in such a way that positive-to-negative charge ratio varies between 0 and 2 at pH 7.5 in HEPES buffer. Lyz amount assessed by UV spectrophotometry in the supernatant (bars) after PPCs centrifugation at 9500 g for 15 min.

Part VII: References

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