

Faculté des bioingénieurs

Tailoring surfaces using protein- polyelectrolyte complexes and cluster ion beams

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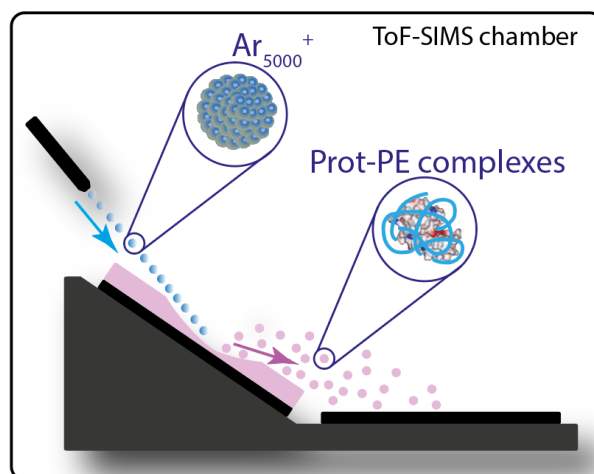
To every person that will read this master thesis, I wish you will enjoy reading it as much as I enjoyed writing it.

Abstract

The biofunctionalization of surfaces, *i.e.* immobilization of proteins at interfaces, is a cornerstone technology in diverse fields of application. Deposition from aqueous protein solutions has proven efficient for some utilizations. However, such methods suffer from several drawbacks, reducing the range of achievable functions. Among those disadvantages, the inherent drying step often causes problems such as the loss of 3D conformation of immobilized proteins, a lack of control on the spatial distribution of the engineered layer, *etc.* Therefore, recent research aims at the development of new methods outpacing the drawbacks of current ones.

This master thesis investigates the elaboration of a new protein immobilization technique that uses large argon clusters produced by a Time-of-Flight Secondary-Ion Mass Spectrometer (ToF-SIMS) in order to transfer proteins from one surface rich in proteins to another clean one. The idea is to take advantage of the low energy per atom of such clusters to induce the sputtering of intact proteins under ultra-high vacuum. For the purpose of granting proteins with an extra protection, they were complexed with oppositely-charged polyelectrolytes (PE) resulting in protein-PE complexes (PPCs). Three different PPCs containing protein of different sizes were considered during this thesis.

First, the synthesis of the different PPCs was investigated with the aim of optimizing the different parameters of the complexation. Moreover, the efficiency of the PPCs production method and the activity of complexed proteins were assessed. Using optimized parameters, more than 80% of the proteins were found to be complexed with their corresponding PE. The complexed proteins tend to lose part of their activity but it was shown that this one is retrievable. Second, PPCs were transferred with a 10keV Ar_{5000}^+ cluster ion beam inside the ToF-SIMS chamber. Finally, the collecting surface was characterized by ToF-SIMS and multivariate analysis. It appeared that protein material was successfully transferred with a semi-circular deposition pattern. Other characterization methods that aim to determine whether proteins were transferred intact and to quantify their amount and biological activity are presented.



List of Abbreviations

AFM	Atomic Force Microscope
Ar-GCIB	Argon Gas Cluster Ion Beam
BCA	BiCinchoninic Acid
Chi	Deacetylated chitosan
ESI	ElectroSpray Ionization
Ins	Insulin
Ins-Chi	Insulin-Deacetylated chitosan complexes
LbL	Layer by Layer
LMIG	Liquid Metal Ion Gun
Lys	Lysozyme
Lys-PSS	Lysozyme-PolyStyrene Sulfonate complexes
MALDI	Matrix Assisted Laser Desorption Ionization
MVA	MultiVariate Analysis
M & M	Materials and Methods
PAA	PolyAcrylic Acid
PAH	PolyAllylamine Hydrochloride
PCA	Principal Component analysis
PE	PolyElectrolyte
pMS	preparative Mass Spectrometry
PPCs	Protein Polyelectrolyte Complexes
PSS	PolyStyrene Sulfonate
PCx	Principal Component number x
QCM	Quartz Crystal Microbalance
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
ToF-SIMS	Time of Flight Secondary Ion Mass Spectrometry
ToF	Time of Flight
Tryp	Trypsin
Tryp-PAA	Trypsin-PolyAcrylic Acid complexes

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1 Introduction

The biofunctionalization of surfaces with thin layers of proteins underlies innumerable applications in various fields. For those, the efficiency, *i.e.* the biological activity of the adsorbed layers depends on different parameters: the amount and nature of the proteins adsorbed, their interaction with the surface, their integrity and the molecular and supramolecular organization of the layers [1]. Those parameters will influence the conformation and the accessibility of the proteins [2] [3].

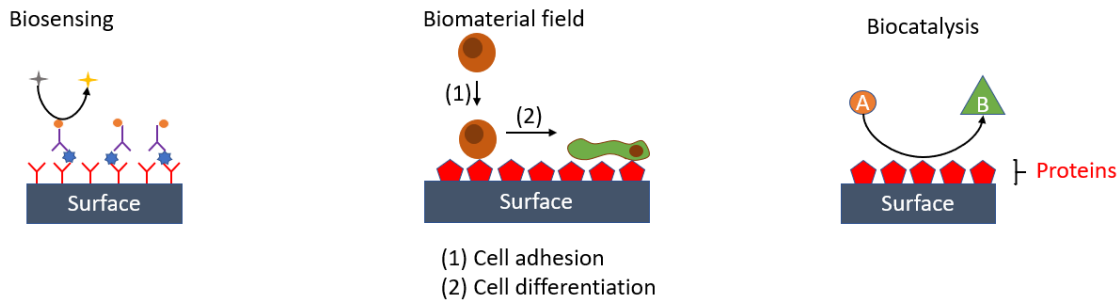


Figure 1.1: Application examples of biofunctionalized surfaces

Some examples of applications of biofunctionalized surfaces are shown in figure 1.1. Biofunctionalized surfaces can be used in analytical chemistry. Prieto-Simón *et al.* investigated the use of immobilized specific bioreceptors, *i.e.* antibodies, onto electrode materials in order to quantify a bacteriophage MS2. This quantification method uses a typical sandwich assay, also known as Elisa assay. A detection limit enhancement by a factor of 10^8 has been observed compared to classical direct assays. Moreover, the direct assay is subject to a strong matrix effect due to non specific adsorption when analyzing complex samples such as river water where this method showed no significative matrix effect [4].

Proteins at interfaces are also a cornerstone in the biomaterial field since those are involved in the adhesion and differentiation of cells on the material by mimicking the extracellular matrix [5].

Biofunctionalized surfaces are interesting too in the biocatalysis field. Indeed, enzymes present great catalytic properties in water at low temperature but cannot be used in continuous processes since those are soluble. Therefore the ability to immobilize enzymes at an heterogeneous interface enables continuous processes to operate using enzymes [6].

Numerous other applications are found in water treatment, pharmacological field and many other fields [7][8][9].

1.1 Methods used for the production of biofunctionalized surfaces

Now that the importance of biofunctionalized surfaces has been underlined, this section will describe different techniques that are currently used to obtain those surfaces. Their advantages and disadvantages will be briefly discussed.

1.1.1 Direct adsorption from solution

Adsorption from an aqueous solution is by far the easiest way to anchor protein at interfaces. Indeed, most proteins will spontaneously adsorb on most surfaces with more or less affinity depending on the protein primary structure and on the surface properties [2]. Dee *et al* outlined the different parameters for proteins and surfaces that govern protein adsorption from solution. Table 1.1 shows the important parameters of the protein and table 1.2 shows the ones of the surface [3]. Moreover, it is possible to influence the adsorption by changing the external conditions of the solutions (temperature, pH, pressure, concentration of the protein, *etc.*). In general, the adsorption is endothermic, thus the adsorption is mainly governed by entropic gain [2].

Table 1.1: Parameters of the protein influencing the adsorption [3]

Protein property	Effect on adsorption
Size	Larger proteins can bind to more sites on the surface
Charge	Protein near their isoelectric point adsorb more readily
Structure stability	Less stable proteins can unfold to a greater extent and bind to more surface site
Unfolding rate	Proteins that unfolds quickly will anchor to the surface more rapidly

Table 1.2: Parameters of the surface influencing the adsorption [3]

Surface Feature	Effect on adsorption
Topography	Greater roughness increases the surface area available for interactions with proteins
Composition	Chemical composition of the surface determines the intermolecular forces governing the adsorption
Hydrophobicity	Hydrophobic surfaces tend to bind more proteins
Heterogeneity	Nonuniformity of surface characteristics results in different domains that interact differently with proteins
Potential	Surface potential influences the interactions with proteins

On apolar surfaces, the adsorption is driven by the dehydration of hydrophobic group in the protein. Indeed, those groups will tend to anchor at the hydrophobic surface regardless of its charge. When it comes to polar surfaces, proteins can be divided in two categories based on their internal cohesion: a "soft" protein has a weak internal cohesion, making it capable of relaxing on a surface by undergoing structural changes. This leads to an increase of its conformational entropy and thus favors the adsorption. On the other hand, a "hard" protein has a strong internal cohesion, preventing structural changes. Those proteins will preserve their conformation and thus adsorb less on surfaces [2].

This method has the advantage to be quite simple and has already been proven efficient for numerous uses. Nevertheless, surfaces tailored using this method present some drawbacks that prevent those to be used for most applications: (I) this technique restricts the thickness of the deposited film to monolayers, limiting thus the number of proteins that can be anchored [1]. (II) As previously mentioned, proteins tend more or less to change their conformation upon adsorption on a surface leading to loss their native structure and thus, their biological activity. (III) Moreover, proteins can anchor with an orientation that will hide their active site, preventing substrates from reaching it, even if the native conformation is kept [2]. Figure 1.2 present a schematic view of an interface obtained by direct adsorption.



Figure 1.2: Schematic representation of an interface obtained by direct adsorption [2]

1.1.2 Spin coating

Spin coating is a widespread method used to create uniform thin film (few nm to μm) of very different natures. Indeed, it can be used to coat surfaces with photoresist, organic semiconductors, metal oxide precursors, ... And also proteins [10]. Figure 1.3 shows the principle of spin coating. First, the substrate (surface) is covered by a drop of solution containing the molecule of interest dissolved in a solvent. Then, the substrate is rotated at high speed causing the ejection of most of the liquid and, at the same time, the substrate is dried by an airflow. When the solvent is fully evaporated, the surface exhibits a homogeneous coating [10].

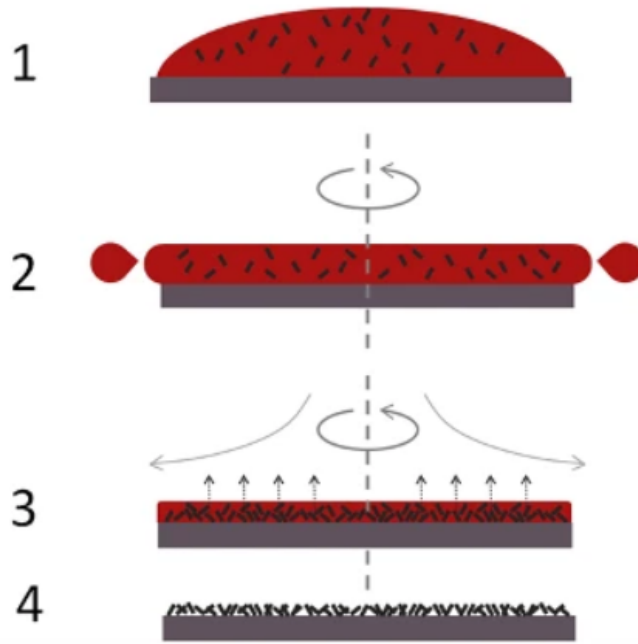


Figure 1.3: Schematic representation of the spin coating technique [10]

The thickness of the coating can be engineered. Indeed, the thickness of the film generally is inversely proportional to the square root of the angular velocity (see equation 1.1). Thus, when the spinning speed (ω) is increased by a factor of four, the thickness of the layer (t) is divided by two. Nevertheless, the speed cannot be inferior to 500-600 rpm otherwise the coating will not be homogeneous. Note that, in general, the thickness of the layer grows linearly with the concentration of the spin coated solution [10][11].

$$t \propto \frac{1}{\sqrt{\omega}} \quad (1.1)$$

The main advantages of this method are that it is simple, easily tunable and applicable to a wide range of molecules/substrates. However, spin coating is applicable only as a batch process and has a low material usage, around 10% which is a constrain since proteins are valuable material. Therefore, spin coating is a great method for the study of different thin films but it is not appropriate for the production of biofunctionalized surfaces at larger scale [10].

1.1.3 Dry drop

Dry drop is a method where a droplet of protein solution is deposited on a surface and then dried. This method is straightforward and the amount of proteins immobilized is adjustable by varying the concentration and the volume of the droplet (see figure 1.4). The resulting morphology of the deposited proteins depends on mass transport inside the droplet during the drying step. This transport is regulated by the competition between capillary forces and surface tension gradients. Furthermore, other parameters also play a role: the concentration of salt, the temperature, the nature of the substrate, the relative humidity, *etc.* [12]

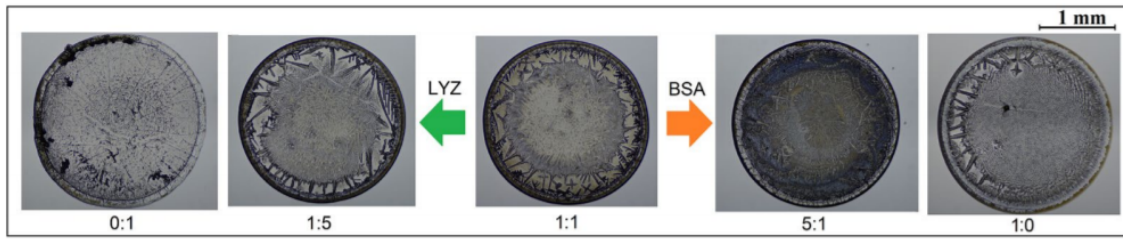


Figure 1.4: Dry drop of lysozyme (LYZ) and bovine serum albumin (BSA) mixture in different relative concentration [12]

The major drawback of this technique is the heterogeneity of the resulting protein film, as an effect of drying. This can be observed on figure 1.4. Actually, the mass transport phenomenon is complex and thus not manageable. This is problematic for some applications because interfaces created by dry drop are not perfectly reproducible.

For most applications, the immobilized proteins should keep their conformation. The techniques presented above are relatively easy to perform but lack of protections for protein conformation. Moreover, it is barely impossible to properly control the thickness and the homogeneity of layers manufactured with those techniques. Recently, new techniques have been elaborated in order to override those limitations. The following sections will present some relevant ones.

1.1.4 Layer by layer assemblies (LBL)

The layer by layer methodology relies on the sequential adsorption in mild conditions of oppositely charged polyelectrolytes (PE). Each adsorption will add a layer to the system (see figure 1.5). Those adsorptions are driven mainly by electrostatic interactions. Hydrophobic interactions and hydrogen bonding also play a role [9].

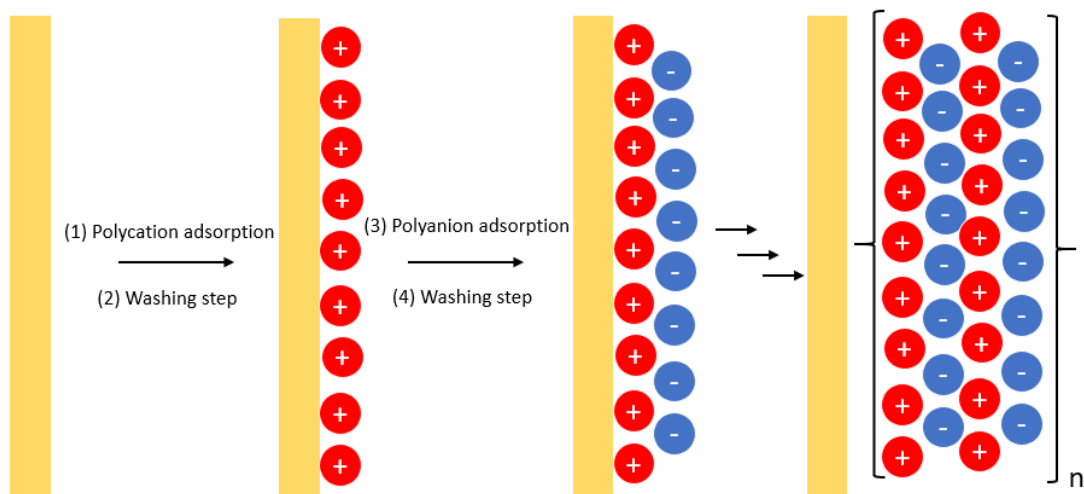


Figure 1.5: Illustration of the layer by layer methodology, inspired from [13]

It is possible to replace one of the PE by a protein that has the same charge sign (see figure 1.6). This will provide proteins with a highly hydrated environment that favors the retention of its native conformation and thus its activity. External parameters such as pH, ionic strength or PE nature must be optimized to ensure sustainable system growth. Bio-functionalized surfaces made by an optimized LBL approach present an enhanced activity compared to simple adsorption from a solution because more proteins can be anchored on the surface [1][13].

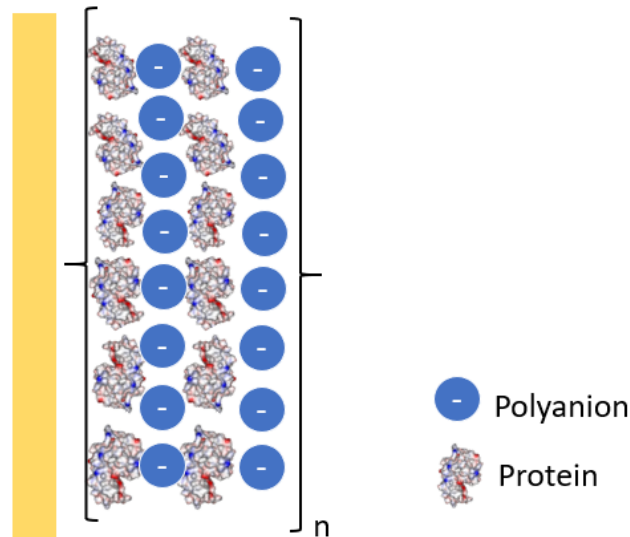


Figure 1.6: Illustration of biofunctionalized LBL

Vander Straeten *et al.* showed that it is also possible to include proteins, as complexes, in a PE layer instead of replacing the PE. Indeed, when a protein and a PE of opposite charge are mixed together, they may form a so-called "protein-polyelectrolyte complex"(PPCs). More information about those complexes will be presented in section 1.4 of this thesis. The study of such systems showed an enhancement of lysozyme, *i.e.* a globular protein, activity upon adsorption of a PPCs-PE LBL compared to a protein-PE LBL, see figure 1.7, PAH (Polyallylamine hydrochloride) and PSS (Polystyrene sulfonate) are two PE and Lys stands for lysozyme. In (a), the activity of the LBL is measured at each step of its building and in (b) the specific activity of the two LBL is shown [1].

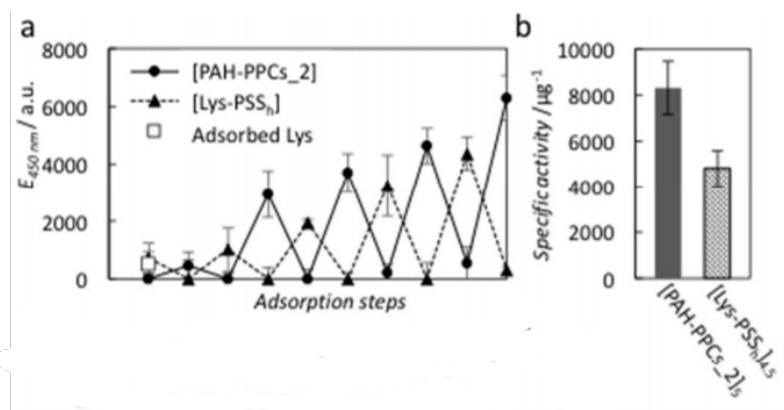


Figure 1.7: Activity comparison between PPCs-PE LBL, protein-PE LBL and adsorbed lysozyme. (a) in function of the adsorption step. (b) LBL specific activities [1]

This method, even though less straightforward, has many advantages: (I) it is applicable on most surfaces, regardless of its chemistry and geometry; (II) the architecture of the film can be tuned by playing on the external conditions; (III) a large quantity of proteins can be adsorbed in comparison with simple adsorption; (IV) proteins are more likely to retain their native conformation due to the solvated environment [13].

However, when the biofunctionalized surface is dried, a reorganization of the anchored layers is possible. This may lead to an uncontrolled spatial distribution, *i.e.* a clumping of the molecules while the solvent is removed, and/or proteins denaturation resulting in a decrease in the efficiency of the interface. Note that this phenomenon is strongly influenced by the drying conditions [14].

1.1.5 Preparative mass spectrometry

In order to overcome the drying issues mentioned above, solvent free approaches were developed. Those techniques are referred to as preparative mass spectrometry (pMS) as they derive mass spectrometer from their primary analytical function. Figure 1.8 illustrates the principle of pMS: First, macromolecules, *e.g.* proteins, are ionized by soft ionization sources such as electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI). This results in a wide mass range of ionized fragments and molecular ions of the macromolecule. Second, those ions pass through a mass filter which sorts them in order to create an ion beam only composed by the ions of interest, *e.g.* the protein molecular ion. The mass filter is a typical mass spectrometer analyzer that has been locked on the desired m/z ratio. Finally, this m/z filtered ion beam is focused on a surface in order to nanostructure it. Note that the whole process is performed under ultra high vacuum for the purpose of avoiding non specific collisions with gaseous molecules that could induce furthermore fragmentation [15].

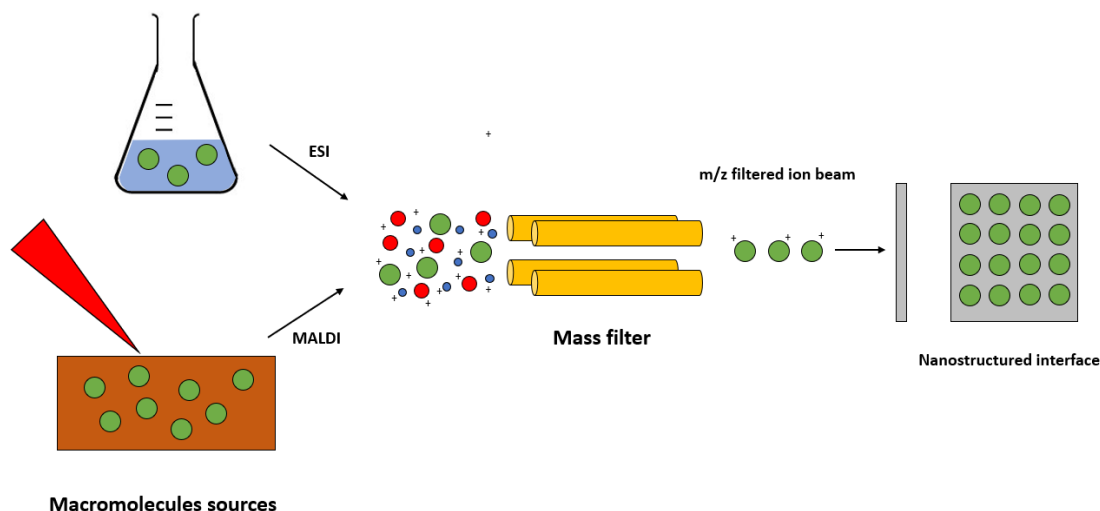


Figure 1.8: Illustration of pMS principle, inspired from [16]

Soft landing, *i.e.* a subcategory of pMS, has shown promising results for the deposition of proteins. This technique uses a mass filtered ion beam with low translational kinetic energy which prevents proteins from being damaged throughout the deposition process [16]. Ouyang *et al.* were able to separate and soft land a mixture of lysozyme and trypsin, two enzymes of different masses. Furthermore, the biological activity of those enzymes was maintained upon soft landing [17]. Soft landing presents specific advantages compared to previously mentioned wet techniques: (I) by avoiding a drying step, soft landing is not subject to the clumping effect described in the previous section or to solvent contamination; (II) soft landing enables a precise control of the surface composition and surface coverage at small scale unreachable using conventional techniques; (III) soft landed proteins may retain their 3D conformation and activity [18].

However, the pMS technique is subject to one major issue: the low current intensity of the mass filtered ion beam. This is due to both instrumental limitations and the very low ionization yield for large macromolecules [18]. This low current intensity prevents the use of pMS for the production of large scale (cm^2) biofunctionalized interfaces.

This thesis will investigate a solvent free approach for the production of biofunctionalized surfaces using protein polyelectrolyte complexes and a time-of-flight secondary-ion mass spectrometer. The following sections will be dedicated to a short bibliographic review of those, after what, the thesis framework will be presented.

1.2 Time-of-flight Secondary-ion mass spectrometry

1.2.1 Overview

Time-of-Flight Secondary-Ion Mass Spectrometry or ToF-SIMS is an ultra high vacuum surface analysis technique (see figure 1.9) developed following the work of Alfred Benninghoven and his team in the 60s [19].

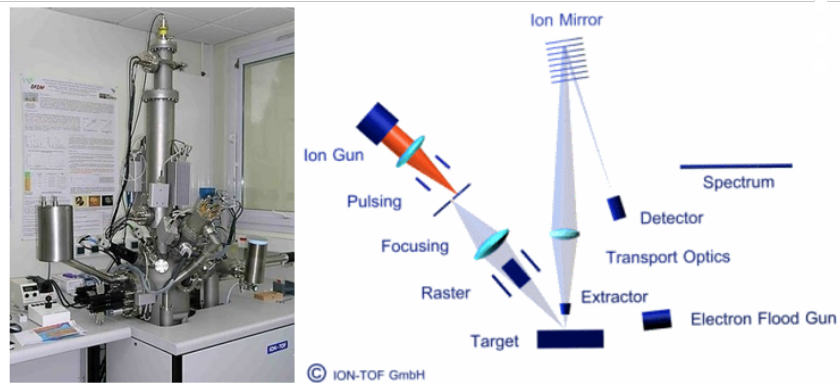


Figure 1.9: Time-of-flight secondary ion mass spectrometer [20]

Originally, the device analyzes ions emitted by a surface when bombarded by a high energetic (between 1 and 25 keV) pulsed atomic ion beam, of which the atoms are referred to as primary particles. When those particles hit the surface, their energy is transferred to the topmost surface atoms leading to a cascade of billiard ball type collision. Some collisions return to the surface inducing the sputtering of partially ionized plume of atoms and clusters, referred to as secondary particles (see figure 1.10). The ionization can occur by different mechanisms such as electron exchange, proton transfer or adduct formation. Nevertheless, most of the ejected material remains neutral, about 99%. This technique is restricted to surface analysis and has a high depth resolution, about 1nm. Actually, about 95% of the ejected particles comes from the top two layers of the sample. For organic samples, this percentage is slightly lower. ToF SIMS measurements provide a spatial resolution of a few hundreds of nm [19].

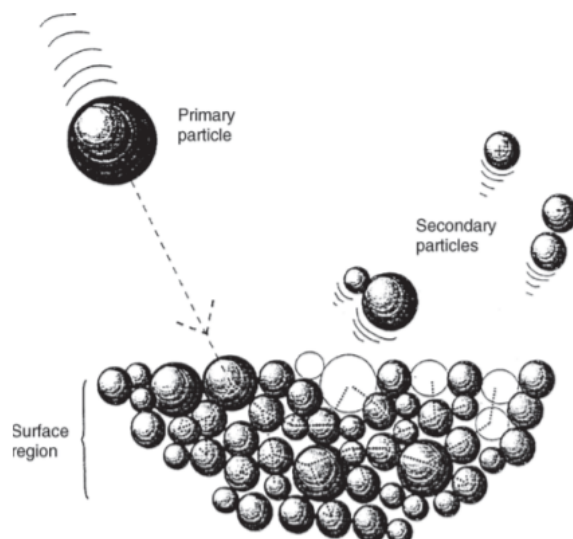


Figure 1.10: Schematic representation of material sputtering in SIMS [19]

This method can seem destructive at first sight. However, in typical routine analysis, also known as "*static SIMS*", the dose of primary ion impinging on the surface is very low (less than 10^{13} ions/cm²). Therefore, less than 1% of the atoms/molecules from the top layer gets hit. Statistically, the chance of a given location on the surface being impacted twice is close to zero. The spectral information can thus be considered as coming from an undamaged surface [19]. Other types of analysis are possible using the ToF-SIMS, those will be discussed further in section 1.2.3.

The charged part of the sputtered material is then extracted into a time-of-flight (ToF) analyzer. Ions entering this drift tube are accelerated by an electric field of known strength, providing all ions with the same kinetic energy, that then travel along a known flight path. The velocity of each ions will thus only be governed by its m/z ratio, m being the mass of the fragment and z its formal charge. In SIMS, most of the charged fragments carry only one charge ($z=1$). Thus, ions are separated and counted according to their mass (see figure 1.11). The analyzer can work either in positive (cations extracted) or in negative (anions extracted) mode [21] [22]. The main advantage of the ToF analyzer is that it is capable of analyzing simultaneously the complete range of secondary ions which is essential for both organic and inorganic samples [19].

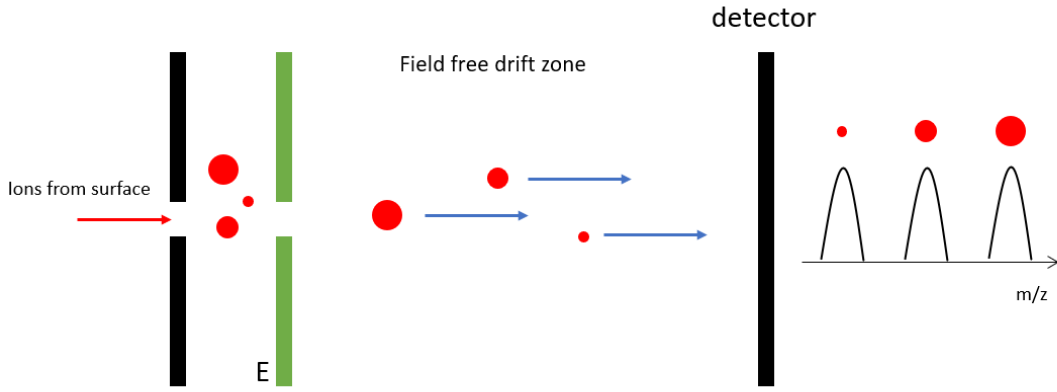


Figure 1.11: Illustration of a time of flight analyzer, inspired from [22]

Each peak of the spectrum (example spectrum is given on figure 1.12) can be related, more or less easily, to a fragment of the analyzed sample. Given the high mass resolution of the ToF instrument, peaks provide chemical information that is complementary to other surface analysis techniques such as X-ray photoelectron spectrometry (XPS) or Fourier transform infrared spectroscopy (FTIR) [24].

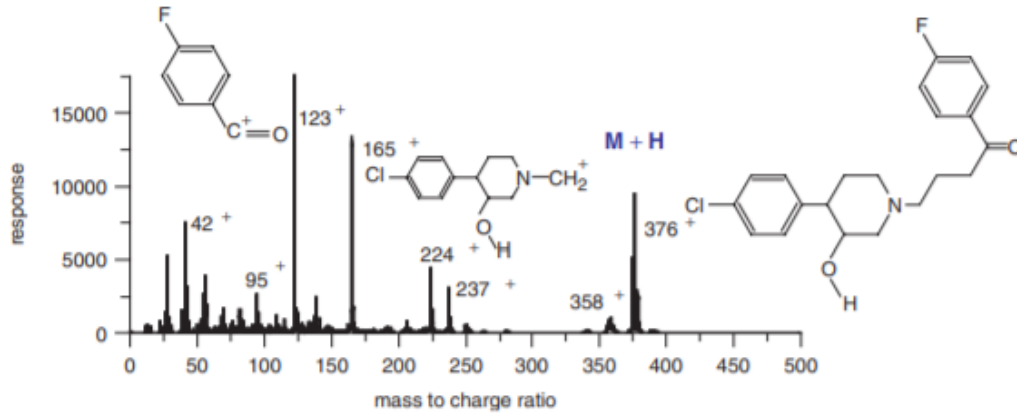


Figure 1.12: Positive spectrum of halperidol [19]

The intensity of each peaks can be described by different parameters presented in equation 1.2 [19]:

$$I_m = I_p y_m \alpha^+ \theta_m \eta \quad (1.2)$$

Where:

- I_m is the secondary ion current of species m

- I_p is the primary ion flux
- y_m is the sputter yield, *i.e.* the number of emitted species m , neutral or ionic, per primary particle impact
- α^+ is the ionization probability to positive ions
- θ_m is the fractional concentration of m in the sample
- η is the transmission factor to the analyzer

The sputter yield and the ionization probability are two fundamental parameters. y_m increases with primary flux, with primary particle charge, mass and energy (maximum between 5-50keV) and with increasing incident angle (maximum between 60 and 80° with regards to the surface normal). On the other hand, the ionization probability is strongly influenced by different exchange phenomena between the surface and the leaving species. The chemical state of the surface is thus critical. For example, the ion yield of an element in a metal or its oxide will often differ by orders of magnitude because of an electronegativity effect on the ionization process. Indeed, in the oxide form, the electrons of the metal atoms are attracted by oxygen atoms. Therefore, the metal atoms will more easily be sputtered as ions Me^+ in the oxide than in the metal. This matrix effect influences the relative proportion of each of the sputtered ions [19].

1.2.2 Cluster ion beam

Clusters, *i.e.* small polyatomic aggregates, ion beams have been developed in the sixties for the deposition of thin film [25]. Researchers discovered only later that increasing the mass of the primary ion particles, *i.e.* using clusters for analytical purposes, resulted in a non linear enhancement of the sputtering yield [23]. Many methods exist for the production of clusters, one of those, called supersonic expansion, is shown on figure 1.13. The big picture is that a highly pressured gas, *e.g.* argon, undergoes an adiabatic expansion through a supersonic nozzle, causing the gas to condense into clusters. Those clusters are then ionized by electron bombardment. Finally, those are accelerated to their final kinetic energy, between 1 and 25 keV [21] [26].

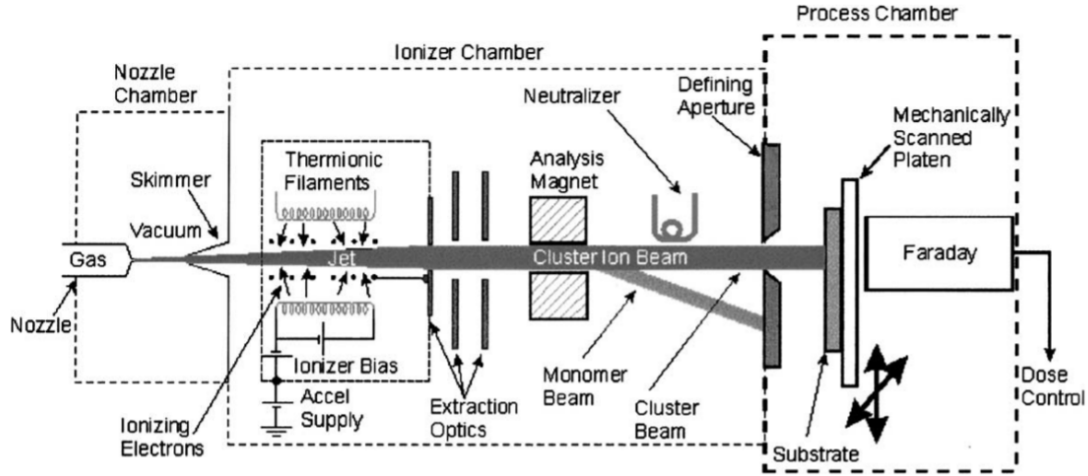


Figure 1.13: Production of clusters by supersonic expansion [26]

When a cluster impacts the sample, it breaks apart and its energy is spread between all surface atoms. Equation 1.3 describes the energy carried by the split atoms of the cluster, E_c . E_0 refers to the initial energy of the cluster, M_c to the mass of the constituent atoms and M_t to the total mass of the cluster. The energy per atoms is thus directly linked to the size of the clusters [21].

$$E_c = E_0 \frac{M_c}{M_t} \quad (1.3)$$

The impact energy of the incident particles is thus significantly reduced using cluster ion beams. As a consequence, the fragmentation is lowered and the chemical structure of the subsurface region is preserved, enabling molecular depth profiling (see section 1.2.3). On the other hand, atomic beams with the same total energy penetrate deeper in the material. This difference has been modelled (see figure 1.14) [21]. Note that for small cluster of heavy elements, like Bi_5^+ or Au_3^+ , the penetration depth and damage cross section are comparable with their equivalent atomic beams [23].

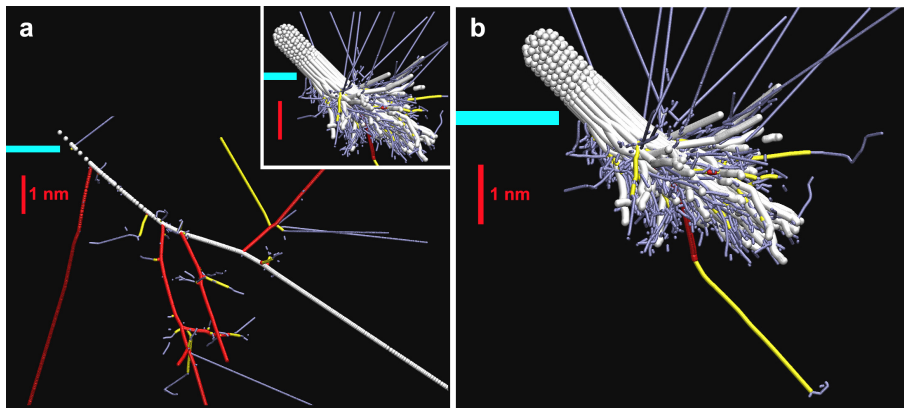


Figure 1.14: Impact depth modelling: (a) atomic ion beam (b) cluster ion beam [21]

It is possible to make clusters of different size from different elements in different states

(gas, liquid or even solid). For the record, here are some examples: Bi_5^+ , SF_5^+ , C_{60}^+ , Ar_n^{+1} . As mentioned earlier, the use of clusters instead of atomic ion beams results in an enhanced sputtering yield and lower fragmentation, *e.g.* for Bi_5^+ some ion yields are increased by a factor of 100 to 1000 compared to its atomic equivalent Bi^+ . Moreover, the use of clusters enables bigger mass fragment to be sputtered, up to 10-12kDa [23] [27].

1.2.3 Modes of analysis

Different modes of analysis are available using a ToF-SIMS device. Some of those will be presented in this section.

Spectrum of small patches

In this mode, the ion beam browses randomly a small patch, *e.g.* $500\mu\text{m}^2$, for a short period of time, *e.g.* 60s. Secondary ions are counted by the ToF analyzer and a spectrum is generated.

Surface imaging

In this mode, the ion beam travels all along a surface. The interface is divided in multiple small patches that are shortly analyzed. The data obtained can be gathered in order to make a chemical map of the surface (see figure 1.15). The secondary ion intensity for a given mass is tracked as a function of its position on the sample [21].

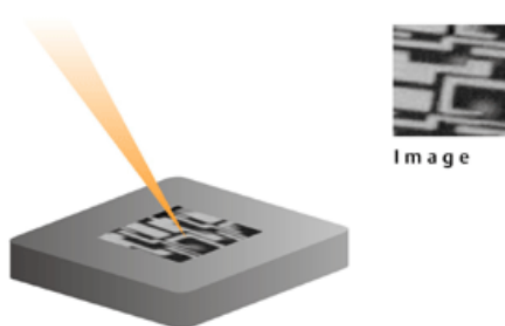


Figure 1.15: Surface imaging [20]

Molecular depth profiling

This mode uses alternatively two ion beams: one, *e.g.* Bi_5^+ , is used to analyze the surface while the other, *e.g.* Ar_{3000}^+ , is digging the surface. Therefore, the sample is analyzed "layer by layer". With this technique, the surface is damaged by the second ion beam. The "static" assumption mentioned earlier is exceeded. Depth profiling method are thus referred as "dynamic SIMS" in opposition to static SIMS.

As mentioned earlier, the introduction of clusters in SIMS significantly decreased the sub-surface damages induced by the impinging ion beam. This enables the molecular identity

¹n is included between 500 and 10 000

of molecules located in the sample to be preserved. That is why this method is referred as molecular depth profiling. [19] [20].

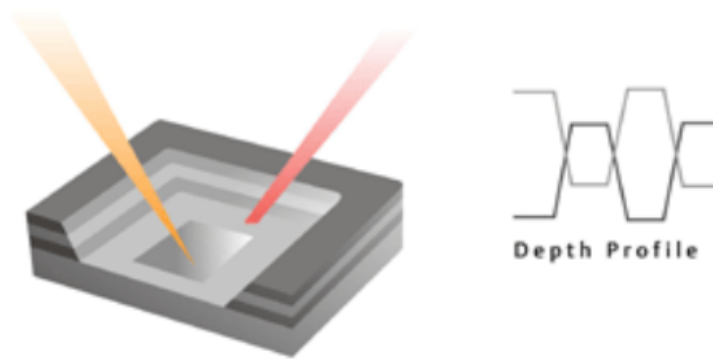


Figure 1.16: Depth profiling [20]

Complete 3D analysis

3D analysis combines the previously cited mode in order to investigate complex structure. Alternatively, one ion beam digs the surface then another realizes a 2D mapping, resulting in a complete 3D map of the analyzed sample. It is particularly interesting because it enables the visualization of the position, composition and shape of features and defects of the sample [20].

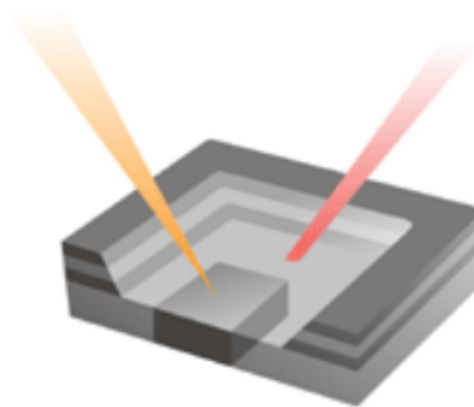


Figure 1.17: 3D analysis [20]

1.3 Protein-polyelectrolyte complexes

1.3.1 Overview

Protein-polyelectrolyte complexes (PPCs) are coacervates, *i.e.* colloidal droplet formed by the interactions between a protein and a polyelectrolyte [28]. The charged amino acids of the protein surface interact electrostatically with the charged PE. Proteins and PE interact simultaneously with multiple other partners (see figure 1.18). Those systems have the characteristics to be very stable and provide a highly hydrated environment for proteins. [28].

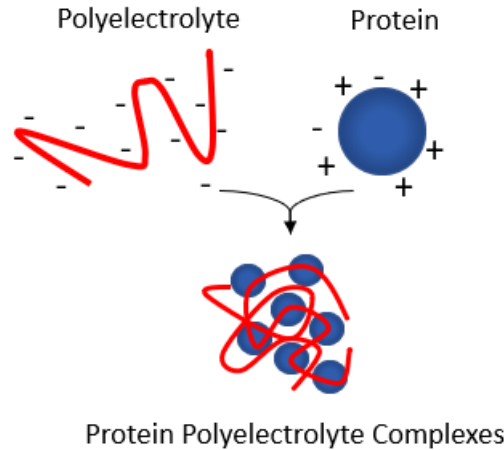


Figure 1.18: Protein-polyelectrolyte complexes formation

PPCs formation can occur with many PE of different nature *via* various interactions. This section focuses on hydrophilic flexible PE. The driving force of the complexation is controverted in the literature: Some affirm that it is mainly due to a counter ion release force, the PE chain in this case becomes a multivalent counter ion of the protein. The release of counter ions from both protein and PE would lead to a major gain of entropy for the system [29]. Others consider that only coulombic interactions (both attractive & repulsive) between the two polymers contribute to the binding energy [28].

Kayitmazer *et al.* outlined different parameters that influence the complexation: Since PE-proteins interactions are mainly electrostatic, the formation of complex will be favored in low ionic strength medium [30]. Indeed, high concentrations of ions in the medium would reduce the strength of the interactions between PE and proteins due to a screening effect. Temperature influences the behavior of the PE chains. Nevertheless, working with proteins only allows a short range of temperatures to prevent them from denaturation. Its effect is thus neglected in most studies. The pH of the solution is also critical. Actually, the global charge of the protein is dictated by the ionization of its amino acids which strongly depends on the pH. The isoelectric point (pI), *i.e.* the pH value for which the positive charge of acidic amino acids is compensated by the negative charge of basic amino acids leading to a neutral global charge, is a crucial information for engineering PPCs. Indeed, when $\text{pH} < \text{pI}$, the protein carries a positive global charge. While when $\text{pH} > \text{pI}$, the protein

carries a negative global charge. Furthermore, pH also dictates the charge of weak PE, *i.e.* PE for which the charge comes from weak acid/basic functions². The strength of electrostatic interactions between PE and proteins can thus be controlled by adjusting the pH. The PE and protein natures also play a role. The strength of interaction will determine the structure of the complexed protein and thus its functionality. Those parameters have to be optimized regarding the desired application [30]. Plenty of PE-proteins couples and their complexation conditions are referenced in the literature [31] [32] [33].

1.3.2 Applications

During the past few decades, PPCs have been widely investigated for applications in numerous fields. Some of those are presented in this section:

PPCs ionic gels for drug delivery and entrapment

With the emergence of protein/peptide based drugs, effective delivery systems had to be developed. Therefore, charged hydrogel, *i.e.* 3D polymeric structures that can absorb a large volume of water, manufactured using PPCs have been investigated. Indeed, those structures can entrap large amounts of proteins. The release of proteins can then be triggered by external stimulation that initiates the swelling of the hydrogel (see figure 1.19). Several studies already showed promising results notably in wound management or treatment of osteoporosis [28] [34] [35].

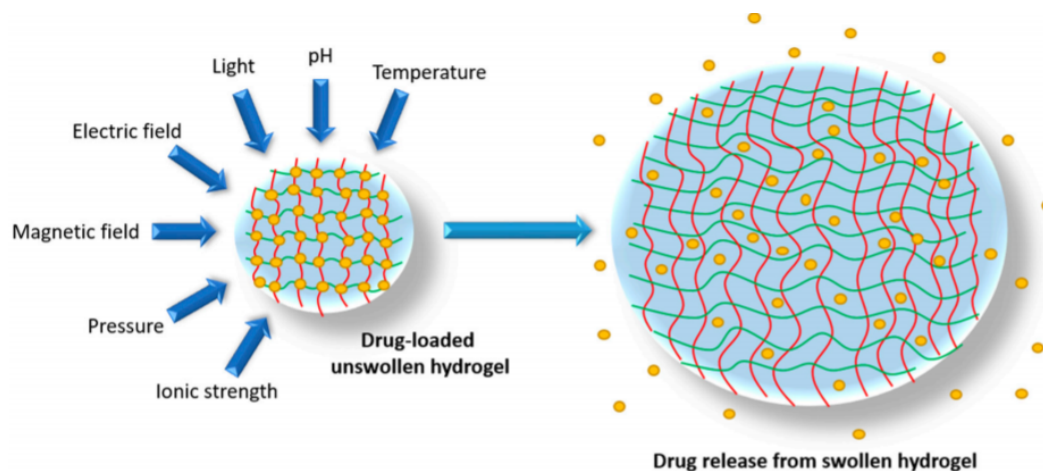


Figure 1.19: Response to external stimuli of PPCs based hydrogels [35]

PPCs for protein immobilization

This application has been discussed within the framework of lysozyme-polystyrene sulfonate complexes in section 1.1.4 of this thesis. This technique has also been used for the immobilization of other proteins such as β -glucosidase or biosensors [28].

²In opposition with strong PE that always carry the same charge regardless of the pH

PPCs for protein purification

A phase separation can be performed by adding PE to a protein mixture. Indeed, PPCs can easily be separated because of their colloidal dimensions. Some polymers show remarkable selectivity for specific protein, *e.g.* commercial polycation PDADMAC is very selective to bovine serum albumin and other proteins having the same pI [28].

1.4 The iBeam project

This thesis is carried out in the framework of the iBeam project, a multi disciplinary research project coordinated by Prof. A. Delcorte and co-promoted by Prof. C. Dupont and Prof. C. Lauzin gathering about ten people with different background: bio and civil engineers, chemists and physicists.

A part of the project is dedicated to develop a novel solvent-free approach for the production of biofunctionalized surfaces and ultimately building up a specifically designed instrument for this method. The idea, inspired from the study led by M. Lorenz *et al.* [36], of this new technique is to derive the cluster ion beam of a ToF-SIMS from its analytical function in order to transfer proteins from a surface full of proteins (target) to a clean surface (collector) under ultra high vacuum (see figure 1.20).

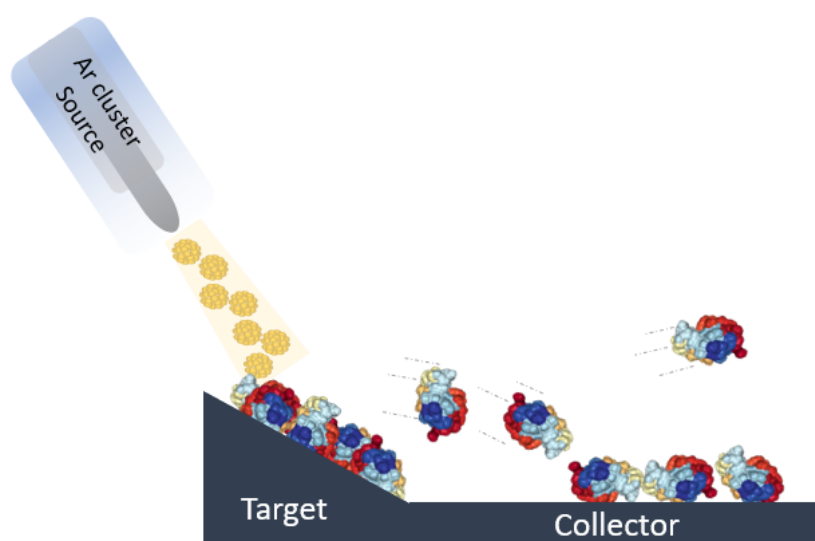


Figure 1.20: Transfer of proteins using ToF-SIMS, with the permission of V. Delmez

The main advantage of this technique compared to previously mentioned solvent-free method is that a mass selection of the sputtered material is not mandatory, enabling the transfer of neutral intact molecules. In addition, the SIMS ion beam does not require the molecules of interest to be in a solution or in a matrix. Furthermore, the use of large argon cluster beam reduces the fragmentation of the material compared to atomic/smaller cluster beams due to the low energy per atom of such clusters [37].

1.5 Objectives and methodology of the thesis

Since the start of the iBeam project, transfer of small proteins ($<15\text{kDa}$) has already been performed and proved successful. However, the fragmentation of those proteins is still important. This thesis investigates the transfer of protein-polyelectrolyte complexes. The complexation of proteins is expected to provide them with a protection from fragmentation that would lead to an increase of the intact transferred material.

During this thesis, three PPCs built with proteins of increasing size were studied: insulin (5,8 kDa) - chitosan, lysozyme (15kDa) - polystyrene sulfonate and trypsin (26 kDa) - polyacrylic acid. These are model proteins investigated by the iBeam project. A comparison between the transfer of pure proteins and PPCs will thus be possible. For all three complexes the methodological steps were the following:

1. Find optimal conditions to synthesize PPCs and characterize the efficiency of the complexation and the activity (if relevant) of the proteins inside the complexes.
2. Transfer the protein-polyelectrolyte complexes from target to collector using an ion beam.
3. Characterize the collector: assess the presence and the quantity of intact functional proteins, assess the topography, the chemical distribution and the thickness of the transferred layer.
4. Compare the obtained results with pure protein transfers.

2 Results and discussion

This chapter presents the experimental results obtained during this thesis. Unfortunately, due to the current health crisis, this chapter is shorter than expected because some of the planned experimentation could not be performed. Chapter 4 will focus on describing these missing experiments and give some perspectives.

First, a focus will be made on the methodology used for the production of PPCs. The optimization of the complexation method was done *via* turbidimetry measurements. Then, the efficiency of the complexation was assessed using BCA assays. Finally, enzymatic activity assays were performed to assess the biological activity of the complexed proteins.

The second part of this chapter will be dedicated to transfer experiments. All surface experiments were performed on small crystalline silicon wafers (see figure 2.1). The transfer procedure will then be presented.

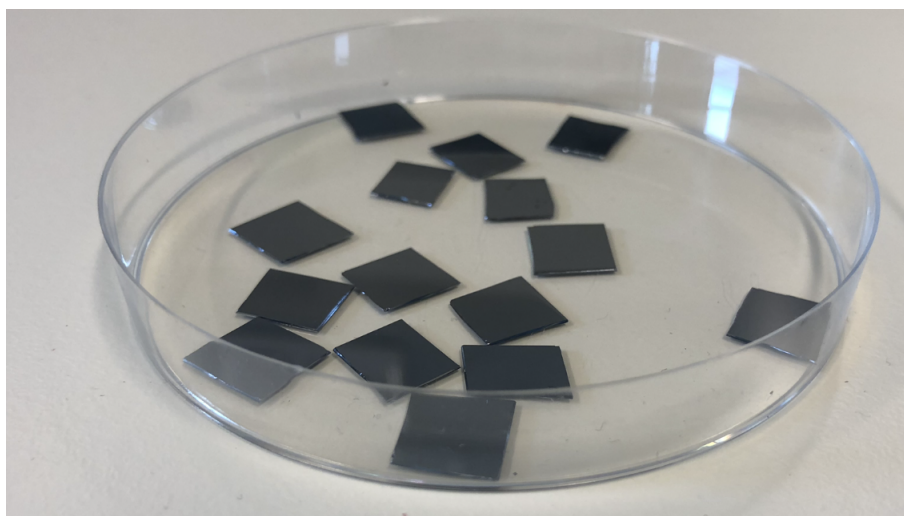


Figure 2.1: Silicon wafers

Then the transferred surfaces were characterized. Therefore, ToF-SIMS measurements were performed on the collector surface. Those measurements were compared to reference surfaces obtained by spin coating PPCs on Si wafers. In order to do so, several principal component analyses were performed.

Experimental protocols and ToF-SIMS analysis parameters are presented in the chapter 5 of this thesis.

2.1 PPCs synthesis

First experiments were focused on developing a method for the production of PPCs. For each chosen protein, the method consists in preparing solutions by mixing the proteins with a PE. Three parameters are critical: (I) the choice of PE; (II) the medium conditions, *i.e.* ionic strength and pH; (III) the protein/polyelectrolyte ratio. The two first parameters were chosen according to the literature (see figure 2.2): lysozyme was complexed with polystyrene sulfonate (PSS) at pH 5,5 (complex referred as Lys-PSS) [31]; trypsin was complexed with polyacrylic acid (PAA) at pH 4 (Tryp-PAA) [32]; insulin was complexed with deacetylated chitosan (Chi) at pH 6,5 (Ins-Chi) [33]. Note that this pH is strangely high since Chi is only soluble up to pH five. The pH of complexation will be investigated later.

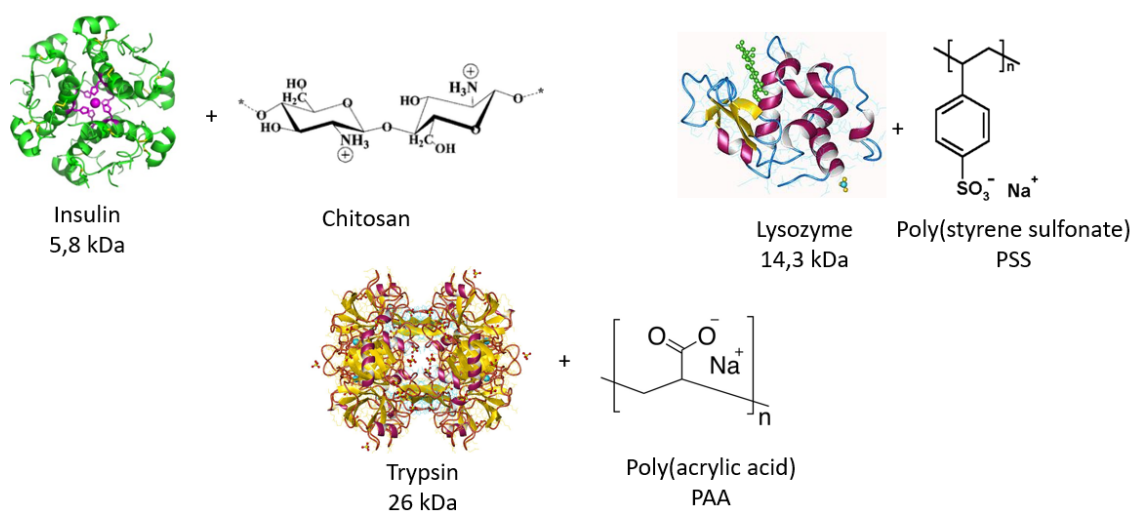


Figure 2.2: Protein-polyelectrolyte complexes investigated in the present work

The third parameter, protein-polyelectrolyte ratio was optimized *via* turbidimetry measurements. Furthermore, the efficiency of the complexation and the biological activity of complexed proteins were characterized using BCA and enzymatic assays. For each techniques, the objectives and principles of the measurements will be described, then obtained results will be presented for each protein-PE couple. Finally, the results will be discussed.

2.1.1 Protein/polyelectrolyte ratio optimization

Optimized protein/polyelectrolyte ratios for the production of the different PPCs investigated in the framework of this thesis can be found in the literature. The complexations in those studies were performed in buffer solutions. The addition of buffer increase the ionic strength of the solution, weakening the interaction between proteins and PE. This is interesting for applications in drug delivery for example [33]. Nevertheless, for transfer experiments, those interactions have to be maximized in order to grant proteins with the strongest protections upon transfer. This assumption will be discussed later. Moreover,

the buffer components will act as contaminants in SIMS measurements, as those will induce proteins non related peaks, rendering data interpretation more challenging. For these reasons, the protein/polyelectrolyte ratios were optimized for suspension without buffer, *i.e.* suspension containing only protein and PE in ultra pure water. The pH of the suspension was fixed by the addition of HCl or NaOH. Suspension containing increasing quantity of PE were analyzed *via* turbidimetry measurements.

Turbidimetry is a widespread analytical technique used for colloidal suspensions. It is based on the fact that the intensity of light going through those cloudy solutions decreases according to the size and amount of small particles in reason of light scattering (see figure 2.3) [38].

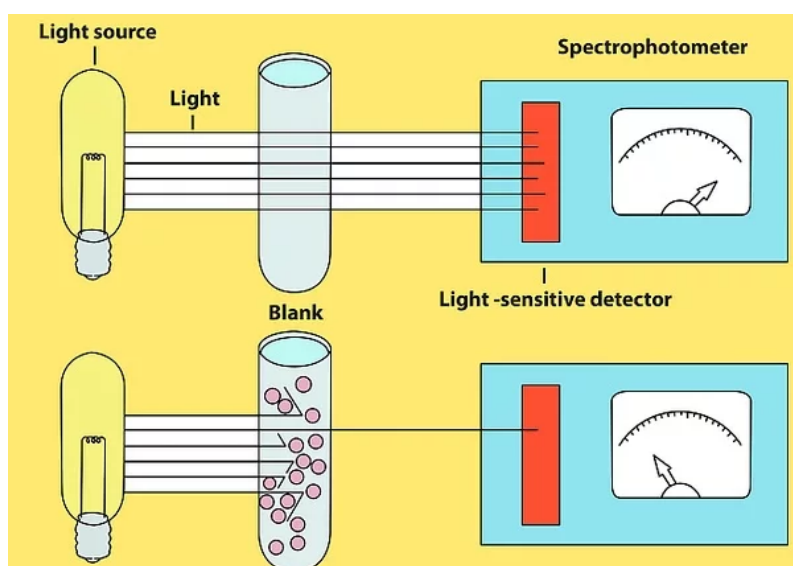


Figure 2.3: Illustration of the turbidimetry principle [39]

The optimal polyelectrolytes/proteins ratio was chosen as the one that leads to high turbidimetric signal¹, since it is related to higher amount/larger PPCs, while using minimum amount of PE in order to build PPCs with the highest protein content possible.

Lys-PSS

Lysozyme carries a net positive charge of 10 at pH=5,5. This charge was calculated by the PROPKA software [40] based on the primary structure of lysozyme referenced in a protein data bank, PDB in this case [41]. The charge of PSS was calculated by multiplying the charge per monomer by the number of monomers in the chain. Since PSS is a strong PE, each repeating unit carries one negative charge (see figure 2.2) and the number of monomer residues is obtained using the equation below. Thereby, PSS carries a negative charge of -36,6/molecule.

¹*i.e.* high absorbance

$$\text{Number of monomer residues} = \frac{\text{Molar mass of PSS chain}}{\text{Molar mass of PSS monomer}} = \frac{7540}{206} = 36,6$$

Figure 2.4 shows the absorbance measured at 450nm for Lys-PSS solutions as a function of the $(-)/(+net)$ charge ratio. The absorbance increases with increasing $(-)/(+net)$, *i.e.* PSS addition, until it reaches a plateau at $(-)/(+net) = 1,25$. The charge ratio chosen for further experiments is 1 because it seems to ensure an appreciable complexation with the minimum amount of PE.

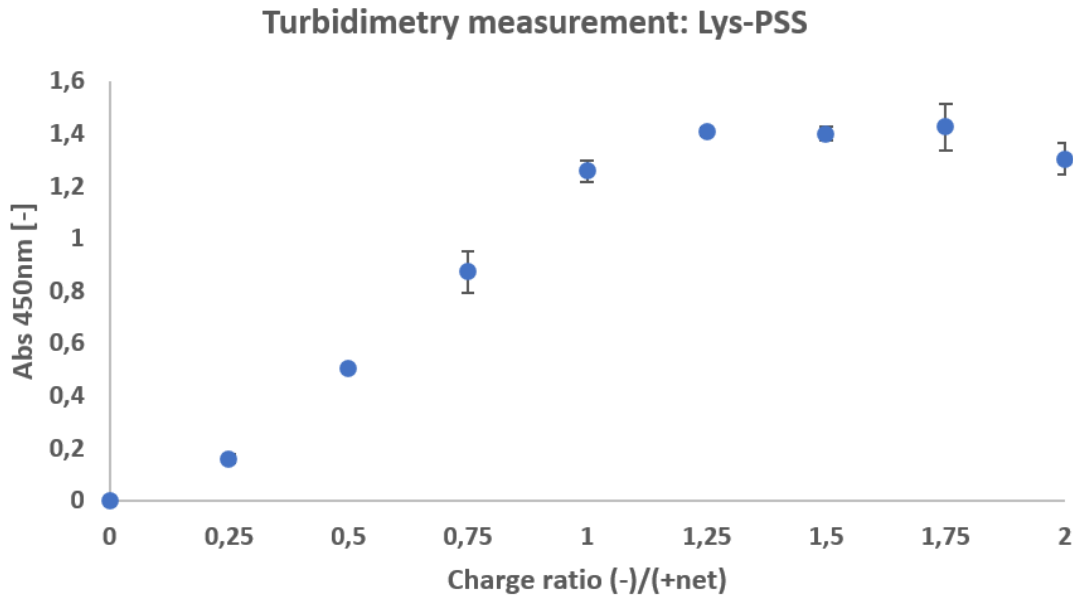


Figure 2.4: Turbidimetry measurement: absorbance of Lys-PSS complexes as a function of the charge ratio

Tryp-PAA

Trypsin carries a positive net charge of 19 at pH=4 [40]. Since PAA is a weak polyelectrolyte with carboxylic acid functions (see figure 2.2), its charge depends on the pH. Therefore, it is not possible to evaluate this charge the same way as for PSS because all repeating units are not charged. Indeed, the apparent pKa of the carboxylic functions changes as a function of the number of protonated monomers. In simpler words, the proton of the acid function will leave the PE chain less easily if the surrounding functions are already negatively charged because of colombic interactions [42].

Therefore, the PAA/trypsin ratio was optimized as a mass ratio (see figure 2.5). Once again, the absorbance increases with increasing mass ratio, *i.e.* PAA addition, until it reaches a plateau at PAA/Trypsin = 0,5. This mass ratio was used for further experiments.

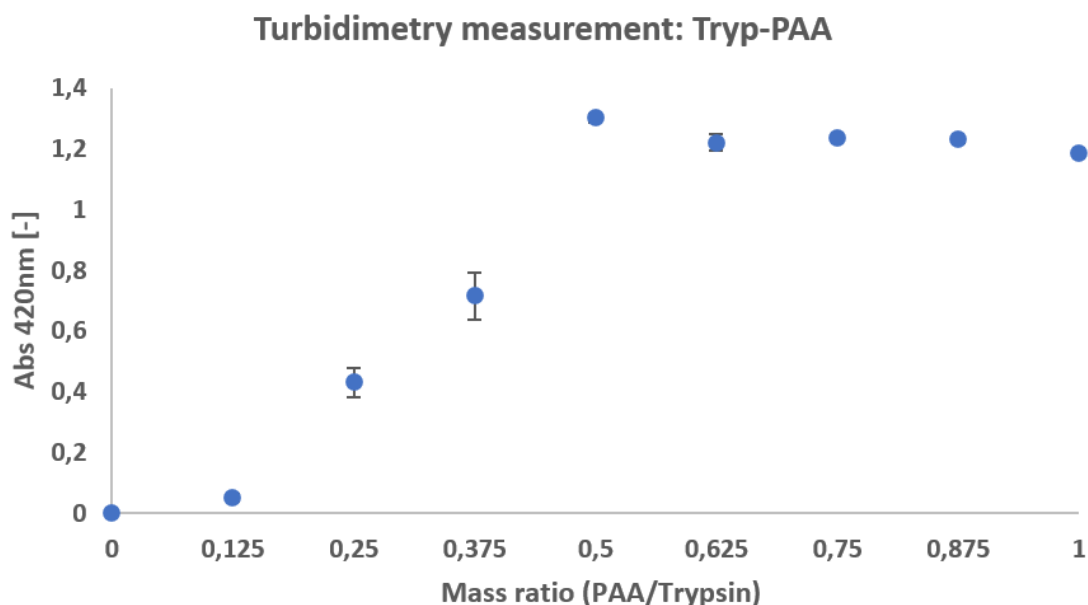


Figure 2.5: Turbidimetry measurement: absorbance of Tryp-PAA complexes as a function of the mass ratio

Ins-Chi

Insulin carries a negative net charge of -1 at pH=6,5 [40]. Chi is a positively charged weak PE. Turbidimetry measurements of Ins-Chi in different ratios were performed at pH=6,5. Unfortunately, no complex formation could be confirmed. Figure 2.6 shows the turbidimetry measured for Ins-Chi solutions in different mass ratios. The absorbance increases with increasing mass ratio, *i.e.* chitosan addition. Since chitosan is not soluble in water at pH=6,5, it forms a colloidal suspension that absorb light and thus gives a signal in turbidimetry.

The increase in absorbance seems to be related to the increase in chitosan content rather than complex formation. Indeed, the analytical blank (blue point at mass ratio=0) contains the same amount of chitosan as the solution with a mass ratio of 1 and their absorbance is statistically not different (bilateral Student test, $\alpha = 0,05$). Even though, it is also possible that insulin complexes with Chi and forms PPCs having the same size as Chi particles. Therefore, no conclusion was drawn from this experiment.

Actually, working at pH=6,5, as referenced in the literature [33], can be criticized: as the driving force of the complexation is the interaction between charged molecules, it does not really make sense to work with Chi at this pH because it is not soluble. Indeed, a lot of Chi molecules are lost in the bulk of the solid particles and thus cannot interact with insulin molecules. The following section presents another approach that aims to assess the success of the complexation at different pH.

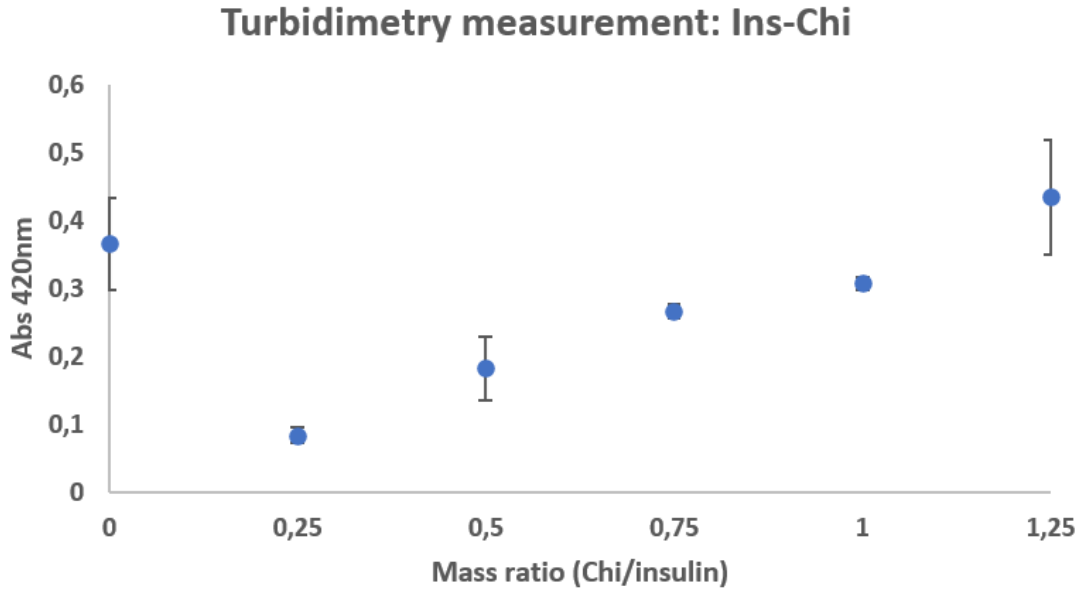


Figure 2.6: Turbidimetry measurement of Ins-Chi complexes as a function of the mass ratio

Results summary and discussion

Table 2.1 summaries the optimal protein/PE ratio assessed by turbidimetry measurements.

Table 2.1: Summary of turbidimetry results

PPCs	Optimal ratio
Lys-PSS	1 (in charge)
Tryp-PAA	0,5 (in mass)
Ins-Chito	N.A.

Note that the net charge of proteins presented in this section was calculated from their primary structures, *i.e.* the charge of each amino acids constituting the protein are summed resulting in the net charges given in this section. Although it is a rapid and easy way to get a rough idea of the protein charges, it does not take into account the spatial distribution of those charges. Indeed, proteins may exhibit oppositely charged patches on their surface. This is illustrated for lysozyme and insulin on figure 2.7. Therefore, a protein and PE carrying the same net charge could potentially complex together [28]. This idea will be investigated for Ins-Chi complexes in the following section. Moreover, thinking in terms of protein/PE charge ratio, as it was done for Lys-PSS in this section or in the literature [1], could seem representative of the reality at first sight. Nevertheless, it is not since it does not take into account the impact of the charge spatial distribution.

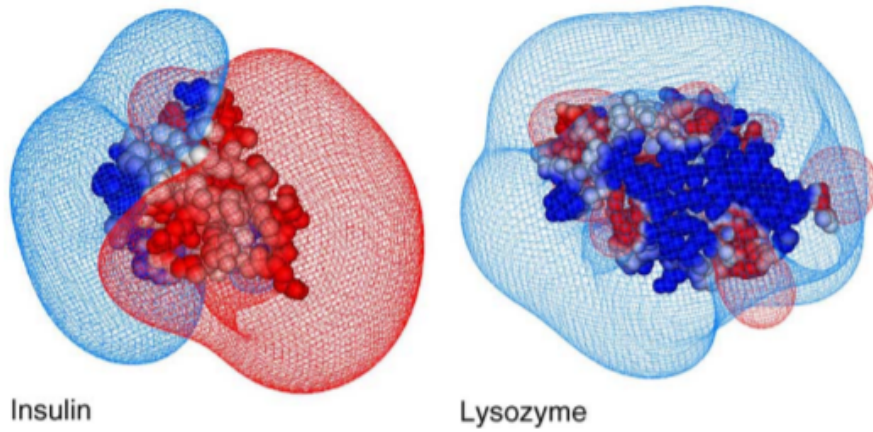


Figure 2.7: Surface electrostatic potential of lysozyme and insulin at pH=7, blue: positive potentials and red: negative potential [28]

The shape of the graphs obtained for Lys-PSS and Tryp-PAA complexes is similar to results obtained from other PPCs produced in the laboratory: the absorbance increases with increasing PE content (for constant amount of proteins) until it reaches a plateau. The beginning of this plateau can be interpreted as a stoichiometric equivalence between proteins and PE. After this point, the maximum amount of proteins is complexed and further addition of PE will not lead to the formation of more complexes.

2.1.2 Efficiency of the complexation: the BCA supernatant assay

In order to assess the efficiency of the complexes formation, *i.e.* the relative amount of proteins that actually complex, BCA assays were performed on the non complexed part of PPCs solutions.

The BCA assay is a protein quantification assay which relies on the ability of proteins to efficiently reduce Cu^{2+} into Cu^+ (Biuret reaction). The reaction of copper is mainly caused by the side chain of three amino acids: Cysteine (also cystines), tyrosine and tryptophan. Moreover, it also reacts with the general backbone of proteins, minimizing the compositional difference between proteins. The reduced copper then reacts with the bicinchoninic acid leading to the formation of a purple complex that absorbs light at 562nm (see figure 2.8). The measured absorbance can be related to the concentration of proteins in the medium by means of a calibration curve made from known proteins concentration solutions [43].

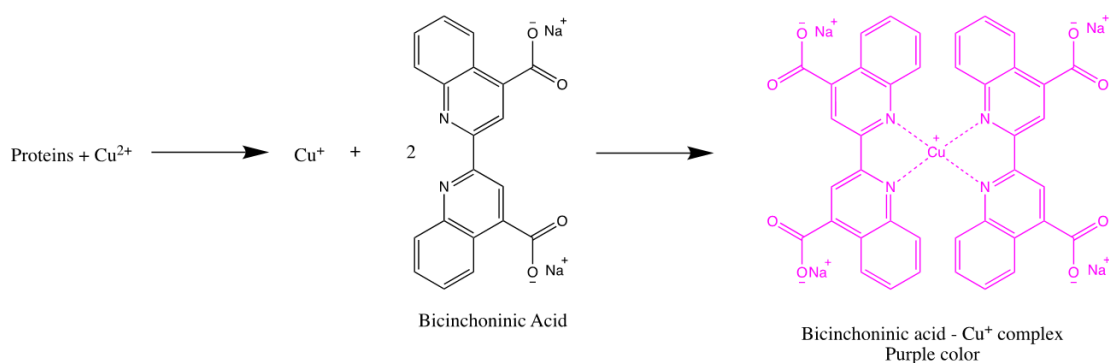


Figure 2.8: Reaction of the BCA assay, with the permission of V. Delmez

The efficiency of the complexation was assessed following the methodology presented on figure 2.9. Complexes were synthesized in Eppendorf tubes using the parameters mentioned previously. Then, the solutions were centrifuged 10 min at 8000 rpm for the purpose of precipitating the PPCs at the bottom of the Eppendorf. Finally, the quantity of proteins left in the supernatant, *i.e.* non complexed proteins, was assessed by performing a BCA assay.

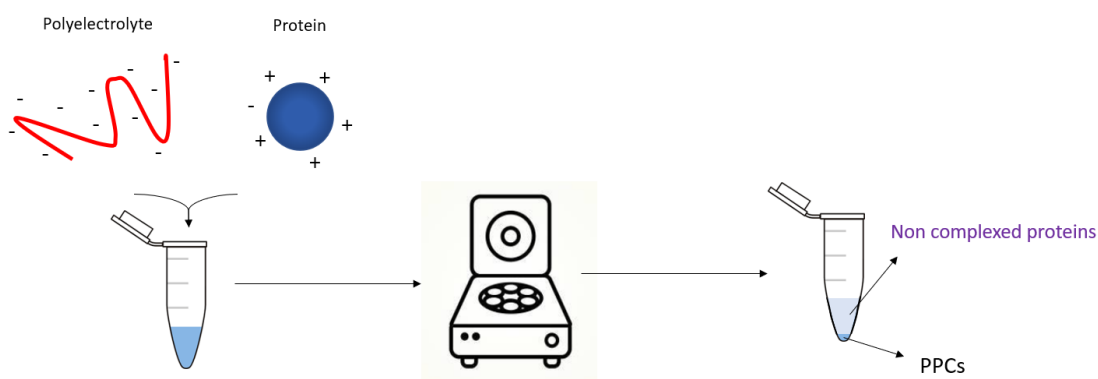


Figure 2.9: Illustration of the BCA supernatant assay principle

Lys-PSS

Figure 2.10 shows the obtained results. Lys-PSS complexes were made so that, if no complexation was observed, 10 μg of lysozyme would be quantified in the supernatant solution (red dotted line). A calibration curve was obtained using free, *i.e.* non complexed, lysozyme solutions (blue dots). A polynomial regression of degree 2 was performed with the calibration data. The choice of regression was made according to the one that fits the best with experimental data. Only $1,14 \pm 0,22 \mu\text{g}$ of lysozyme was left in the supernatant (orange point on the graph), meaning that $88,6 \pm 2,2\%$ of the protein was complexed.

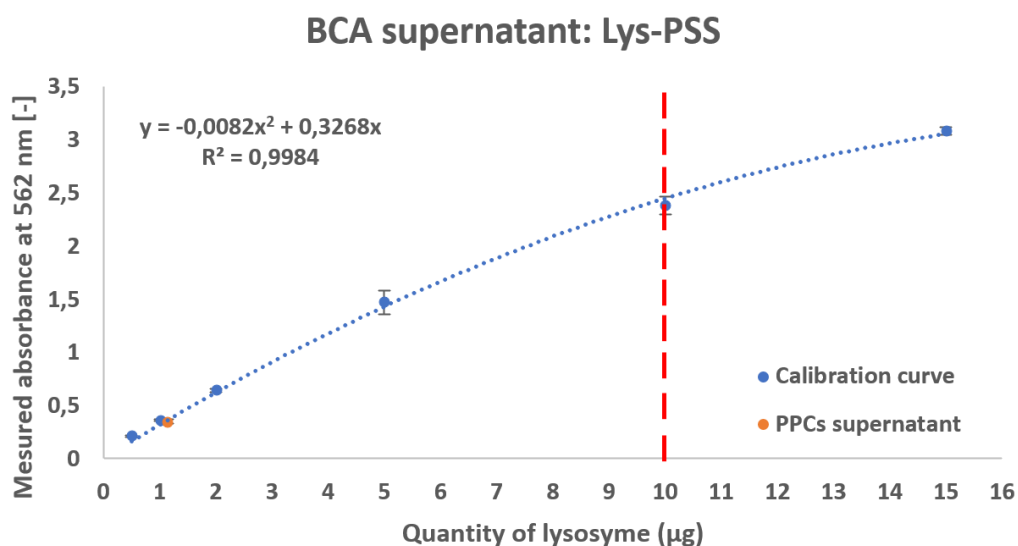


Figure 2.10: BCA supernatant: Lys-PSS

Tryp-PAA

Figure 2.11 shows the obtained results. Tryp-PAA complexes were made so that, if no complexation was observed, 5 µg of trypsin would be quantified in the supernatant solution (red dotted line). A calibration curve was obtained using free trypsin solutions (blue dots). A linear regression was performed with the calibration data. Only $0,89 \pm 0,061 \mu\text{g}$ of trypsin was left in the supernatant (orange point), meaning that $82,2 \pm 1,22\%$ of the proteins were complexed.

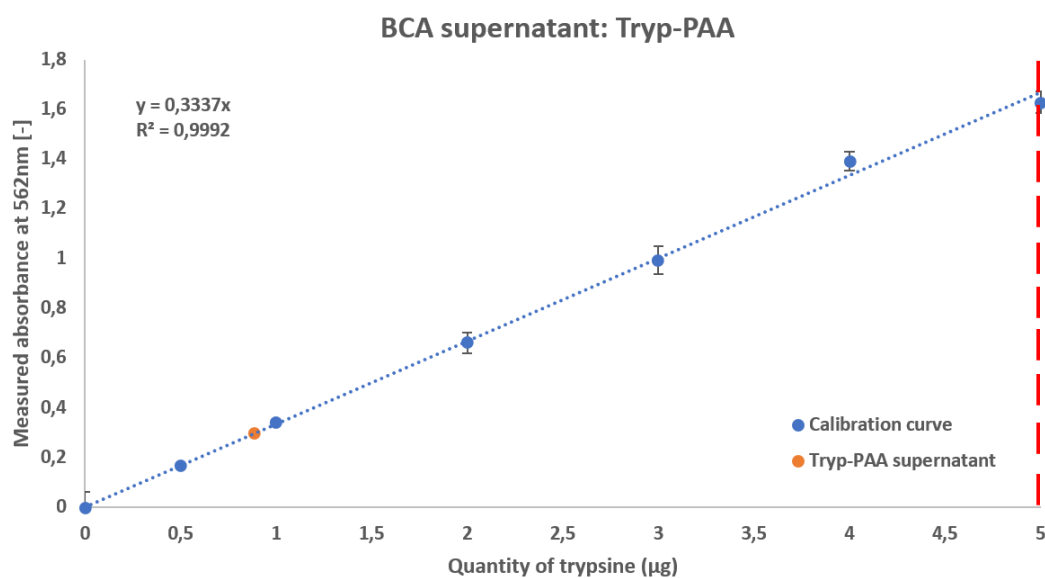


Figure 2.11: BCA supernatant: Tryp-PAA

Ins-Chi

The complexation of insulin with Chi could not be confirmed *via* turbidimetry measurements. The BCA supernatant assay was performed with Ins-Chi solution at different pH values ranging from 3 to 7. Therefore, multiple conditions for the formation of Ins-Chi complexes were tested. At different pH, the spatial distribution of charge on insulin and the charge density of Chi are modified. Moreover, Chi is soluble at pH=3 to 5. PPCs solution were made with an arbitrary chosen mass ratio of one, *i.e.* as much insulin as Chi in mass.

Figure 2.12 shows the obtained results. Ins-Chi complexes were made so that, if no complexation was observed, 5 μg of insulin would be quantified from the supernatant solution (red dotted line). A calibration curve was obtained using free insulin solutions at pH=5,5 (blue dots). A linear regression was performed with the calibration data.

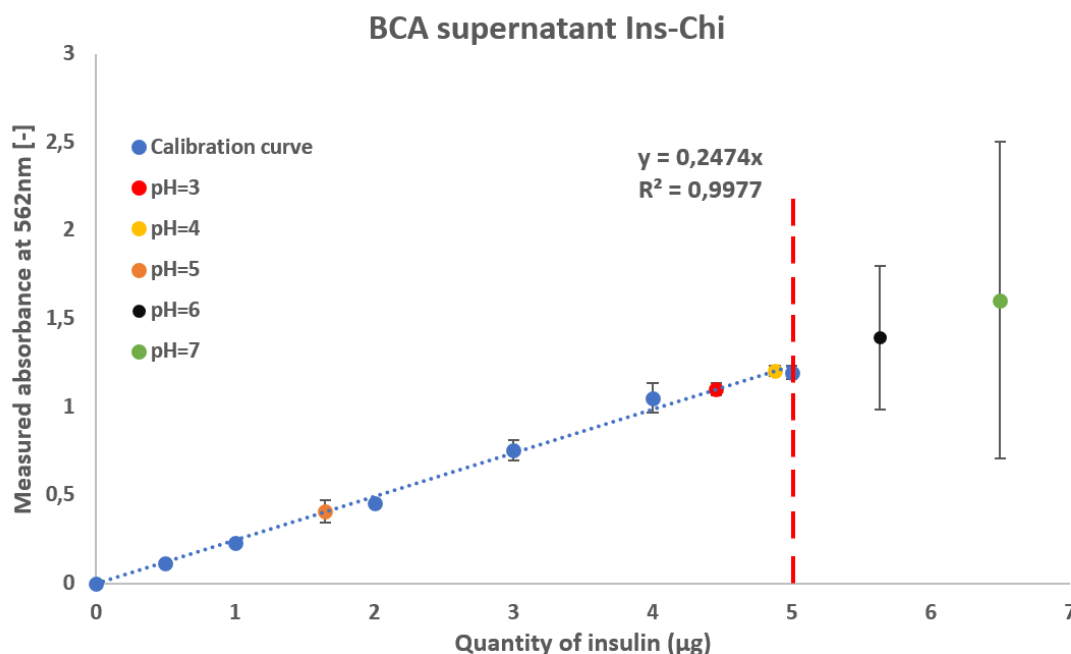


Figure 2.12: BCA supernatant: Ins-Chi

Table 2.2 present the quantity of insulin left in the supernatant at different experimented pH values (also represented in color on the graph). Note that the deacetylated chitosan used is deacetylated at 95%. Therefore, 5% of the chitosan presents a peptide bond which reacts with the BCA reagent. Therefore, those results will only be treated qualitatively, keeping in mind that the efficiencies (% complexed) presented in the table are underestimated.

Table 2.2: Efficiency of the Ins-Chi complexation as a function of the pH

pH	Insulin in supernatant (μg)	% complexed
3	$4,46 \pm 0,34$	$10,8 \pm 6,8$
4	$4,88 \pm 0,28$	$2,4 \pm 5,6$
5	$1,65 \pm 0,63$	$67 \pm 12,71$
6	$5,6 \pm 4,1$	-12 ± 82
7	$6,5 \pm 9,027$	-30 ± 180

It appears that insulin and chitosan complexation is only efficient at pH=5. The net charge of insulin at this pH is +1 and Chi is also positively charged. Therefore, it seems that the surface of insulin molecules presents negative charges despite its net charge. Also, as Chi is soluble in water at this pH, more molecules are available for complexation. Note that the insulin/Chi ratio used for this test was arbitrary chosen and thus should be optimized at pH=5 *via* turbidimetric measurements.

At lower pH value, complexation appears to be less effective. this could be explained by the fact that proteins carry more positive charge at lower pH value. Insulin would thus carry too much positive charge to interact with Chi.

At pH=6-7, the results cannot be interpreted due to the important variation between measurements. As chitosan is not soluble in this pH range, the interactions between insulin and chitosan particles become a complex kinetic phenomenon inducing strong variation in the results observed. Moreover, there is a competition between insulin and Chi molecules to bind to Chi molecules. As complexation was observed at pH=5, this pH value was selected and no further experiments were performed to understand what exactly happens at pH where Chi is not soluble.

Results summary and discussion

This experiment showed that most of the proteins complexed with their corresponding PE. The efficiency of the complexation method of each PPCs is summarized in table 2.3. Note that the supernatant was considered to be composed only by non complexed proteins. This assumption was actually made for practical purpose. Indeed, the supernatant also contains small soluble PPCs that did not precipitate because of their low mass. Nevertheless, the impact of those small PPCs could not be measured using the BCA supernatant assay. Therefore, one should understand that the complexation efficiencies assessed in this section are underestimated and that the real proportion of complexed proteins is slightly greater.

Table 2.3: Summary of BCA supernatant results

PPCs	Complexation efficiency (%)
Lys-PSS	$88,6 \pm 2,2$
Tryp-PAA	$82,2 \pm 1,21$
Ins-Chi	$67 \pm 12,7$

2.1.3 Biological activity of complexed proteins

The biological activity of PPCs is another important feature to assess. Indeed, the complexation should not alter this activity too much otherwise the use of PPCs would be pointless. At least, the activity should be retrievable.

This biological functionality was assessed using enzymatic activity assay. This kind of test is only relevant for lysozyme and trypsin which are enzymes. Since insulin is a peptide hormone, it shows no enzymatic activity. Only complexed lysozyme activity could be assessed.

Lys-PSS

Lysozyme is a globular protein implied in the bacterial defense of different animals. Indeed, lysozyme catalytically cleaves a glycosidic bond in the peptidoglycan present in the cell wall of bacteria. This results in the loss of cellular membrane integrity and further cell death. The activity assay reagent, the 4-methylumbelliferyl β -D-N,N,N-triacetylchitotrioside, is a non fluorescent substrate that exhibit the same binding site for lysozyme as peptidoglycan. Therefore, the protein is able to break this reagent, leading to the formation of a fluorescent product (see figure 2.13). As a result, the activity of lysozyme can be assessed, providing that a calibration curve is first established [45].

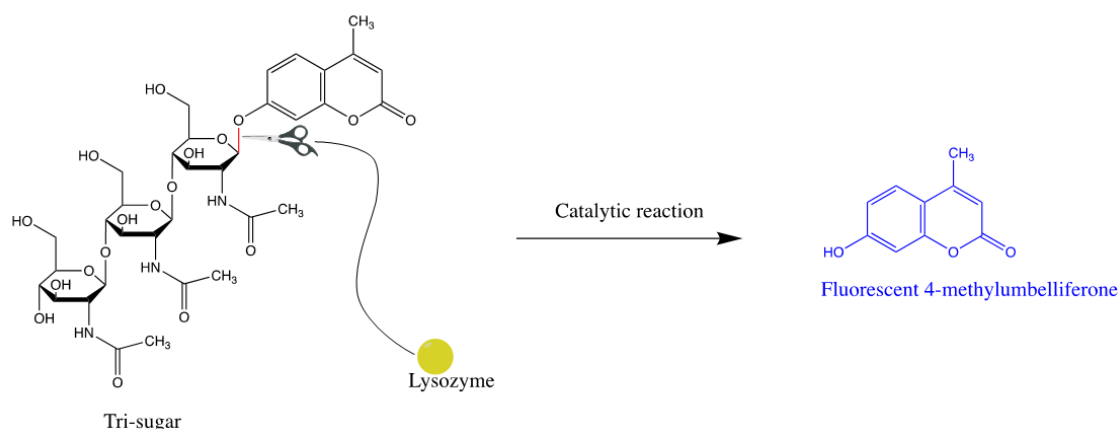


Figure 2.13: Reaction of the enzymatic activity assay of lysozyme, with the permission of V. Delmez

Figure 2.14 shows the obtained results for the enzymatic activity assay of Lys-PSS complexes. A calibration curve was obtained using free lysozyme solutions (blue dots). A linear regression was performed with the calibration data. Lys-PSS complexes were made with $5\text{ }\mu\text{g}$ of lysozyme. Those complexes exhibit the same activity as $2,4 \pm 0,76\mu\text{g}$ of free lysozyme (orange dot). Meaning that upon complexation with PSS, lysozyme loses about 50 % of its activity.

Enzymatic activity assay: Lys-PSS

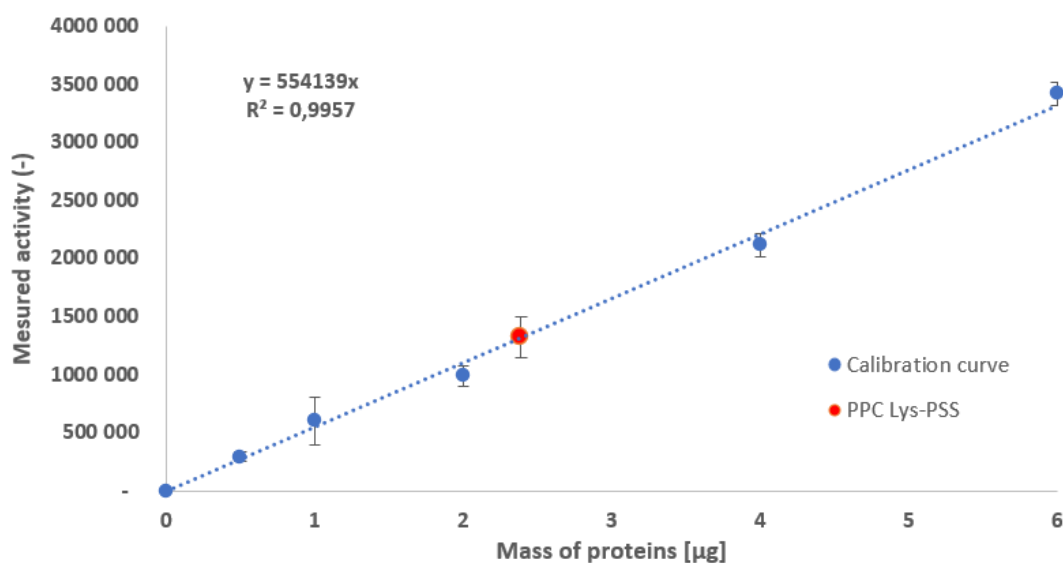


Figure 2.14: Measured fluorescence as a function of the quantity of free lysozyme

It was found in the literature that it is possible to release lysozyme from Lys-PSS complexes by adding another polyelectrolyte, the poly (allylamine hydrochloride) (PAH). PAH is a weak polycation (see figure 2.15 (a)) that is fully charged at pH=5. Since PSS has a stronger affinity for PAH than for lysozyme, lysozyme is released in solution upon the addition of PAH (see figure 2.15 (b)) [46]. Another enzymatic activity assay was performed to investigate this phenomenon. Results are shown on figure 2.16. Lys-PSS complexes with PAH addition shows the same activity as $4,86 \pm 0,33 \mu\text{g}$ of free lysozyme (green dot on the graph). This means that, as expected, the activity of lysozyme was fully recovered upon PAH addition. The addition of PAH could potentially be used to release lysozyme in solution from PPCs immobilized on a surface.

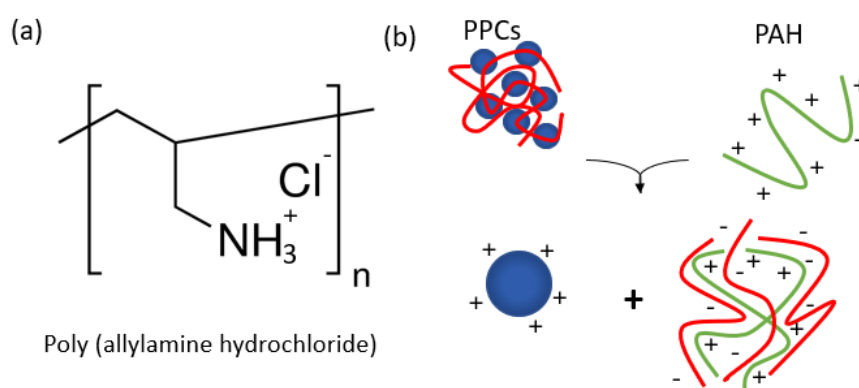


Figure 2.15: (a) Poly (allylamine hydrochloride structure); (b) purpose of the PAH addition

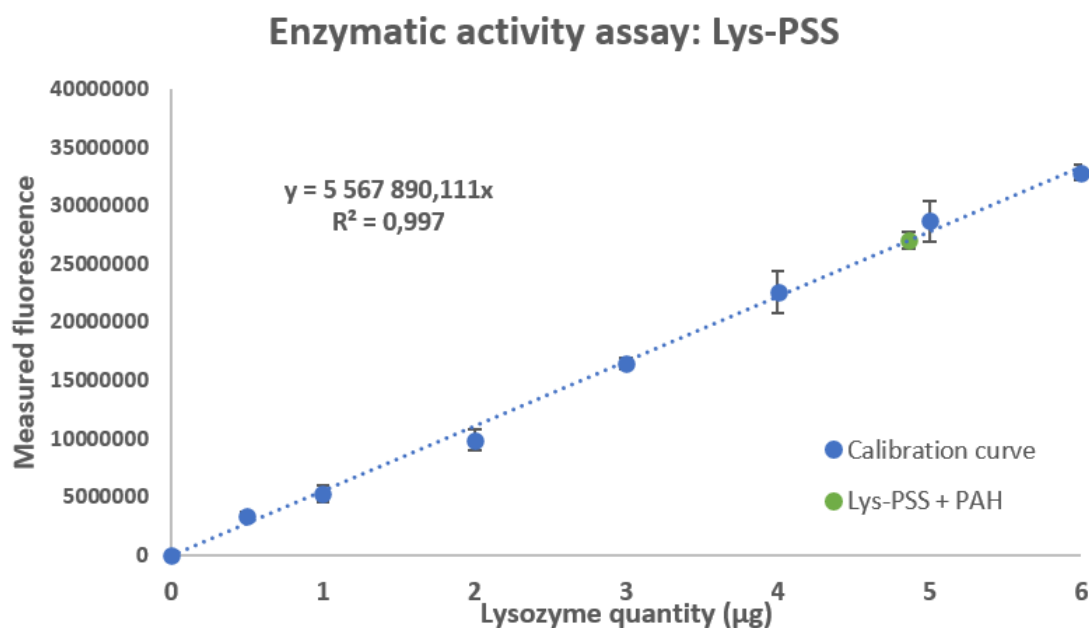


Figure 2.16: Measured fluorescence as a function of the quantity of free lysozyme

2.1.4 Summary of PPCs synthesis experiments

PPCs used in transfer experiments were produced using parameters summarized in table 2.4. Those were either found in the literature or optimized empirically *via* turbidimetric measurements and their synthesis was proven to be efficient *via* BCA supernatant assays. Enzymatic assays were performed on Lys-PSS complexes and showed that the complexation induced a decrease in the activity of lysozyme. Fortunately, it was found that the addition of another PE, *i.e.* PAH, could release lysozyme in solution and induce the recovery of the lysozyme activity.

Table 2.4: Selected PPCs synthesis conditions

Protein	PE	Medium	Prot/PE ratio
Lysozyme	PSS	Ultrapure water (pH=5,5)	1 (in charge)
Trypsin	PAA	Ultrapure water (pH=4)	2 (in mass)
Insulin	Chi	Ultrapure water (pH=5)	N.A.

2.2 Transfer of PPCs

2.2.1 Surface cleaning

Before using silicon wafers for surface experiments, those have to be cleaned either from contamination or from previous experiences. First silicon wafers are immersed in a "piranha" mixture which is a mix of concentrated sulfuric acid and hydrogen peroxide. When both reagents are put in solution together, those react to produce activated peroxymonosulfuric acid (H_2SO_5) which is a strong oxidizing agent that removes organic residues in an exothermic reaction [47].

After that, silicon wafers are rinsed with water. The rinsing water has to be removed physically rather than evaporated to avoid residues on the wafer surface. Therefore, the silicon wafer is rinsed with ethanol, which has a lower surface tension than water, and then is dried by gaseous nitrogen [48].

The last step is a UV/Ozone treatment which is used to remove the remaining organic contaminants from the surface. Short wavelength UV radiations are generated by a low pressure mercury discharge lamp. 185nm wavelength radiations are absorbed by oxygen from the ambient air leading to the formation of ozone (O_3) and atomic oxygen. 253nm UV radiations are co-produced by the lamp and react with O_3 leading to its decomposition. This cycle of production/destruction of ozone also generates atomic oxygen. The 253nm UV radiations also react with organic molecules present on the surface generating ions, free radicals and excited molecules. Those react with atomic oxygen leading to the formation of volatile molecules such as CO_2 , N_2 , H_2O (see figure 2.17). This association of UV and ozone has shown much greater cleaning efficiency than ozone or UV light alone [48].

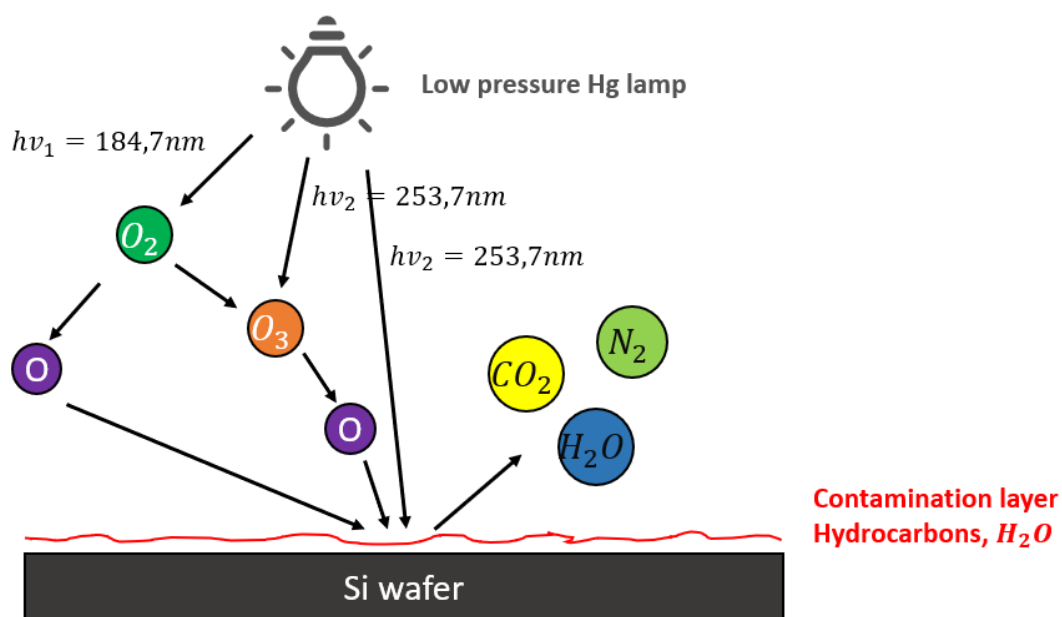


Figure 2.17: Schematic representation of UV-ozone cleaning, inspired from [48]

This cleaning procedure leads to a very clean silicon surface that is pretty reactive and will therefore attract contamination at the moment it gets exposed to air. Therefore, this procedure has to be performed every time before surface experimentation in order to limit the impact of contamination as much as possible.

2.2.2 Transfer procedure

The time of flight secondary ion mass spectrometer used in the laboratory is a *TOF.SIMS 5* (*ION-TOF GmbH, Münster, Germany*). Theoretical insights about ToF-SIMS were already discussed in section 1.2. Two different ion sources were used for the purpose of this thesis: a liquid metal ion gun (LMIG) which produces a bismuth cluster ion beam (Bi_5^+) used for surface analysis and a gas cluster ion beam (Ar-GCIB) which produces an argon cluster ion beam (Ar_n^+ with n comprised between 250 and 10 000) used for transfer experiments. Those ion guns are mounted at 45° with respect to the sample holder surface and the ToF analyzer is perpendicular to this surface.

Transfer experiments consist in bombarding a protein reservoir (target) with large argon clusters and collecting the sputtered material onto a clean silicon wafer (collector) (see figure 2.18).

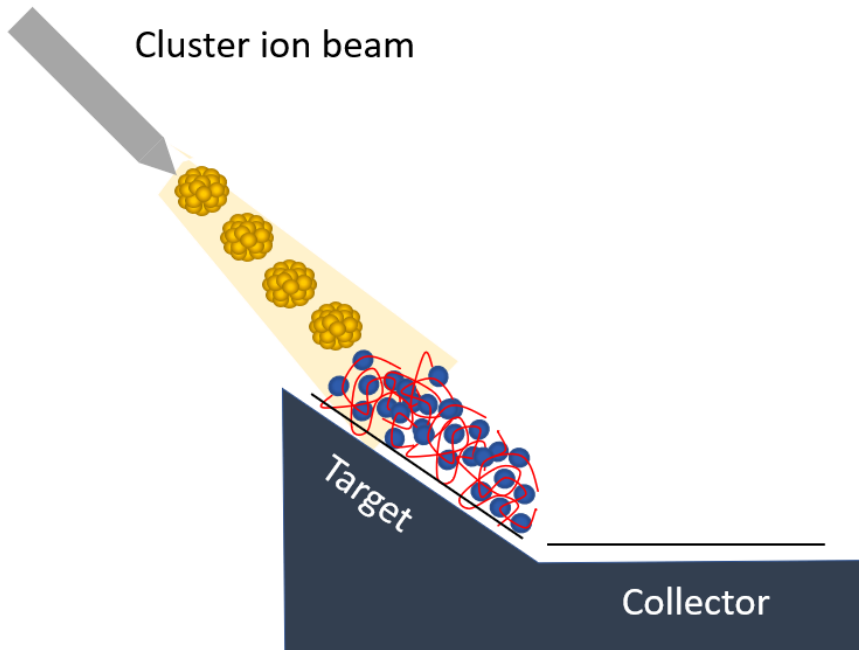


Figure 2.18: Illustration of the transfer procedure

The cluster ion beam used for the transfers is produced by the Ar-GCIB source operating at 10 kiloelectronvolt (keV) with a cluster distribution centered on Ar_{5000}^+ . The size and energy of the clusters were chosen as a compromise between minimizing the fragmentation of the proteins upon bombardment, *i.e.* minimizing the energy per atom (E/n , 2eV on average), while preserving a sufficient current intensity ($\approx 1\text{nA}$). Note that the bond dissociation energy, *i.e.* the energy required for cleaving a bond, of a typical C-C link is about

3,6 eV and about 4,2eV for a C-H link [49]. Therefore, the fragmentation of the PPCs upon cluster impact should be reduced compared to isoenergetic clusters having higher E/n ratio.

Targets are small silicon wafers ($1 \times 0,3 \text{ cm}^2$) on which $10\mu\text{L}$ of a concentrated aqueous solution of PPCs (10g/L) is drop cast thrice (see section 1.1.3). This results in a thick ($\approx 23\mu\text{m}$) dry protein layer that will act as a material reservoir for the transfers.

The collector is a thin silicon wafer ($1,4 \times 1,4 \text{ cm}^2$) that is cleaned following the procedure described in the previous section. Smaller collector ($1 \times 1 \text{ cm}^2$) were used for some experiments in order to shorten their analysis time.

The target and collector are secured with double sided tape on a sample holder specifically designed for transfer experiments. The target is mounted at 30° with respect to the collector surface. This sample holder is then introduced in the SIMS chamber. The target is then bombarded by the argon beam with a grazing incident angle of 15° for about 16 hours (see figure 2.19). The argon cluster ion beam current was measured both before and after transfer experiments in order to assess the primary ion dose, *i.e.* the quantity of ions sent to the target.

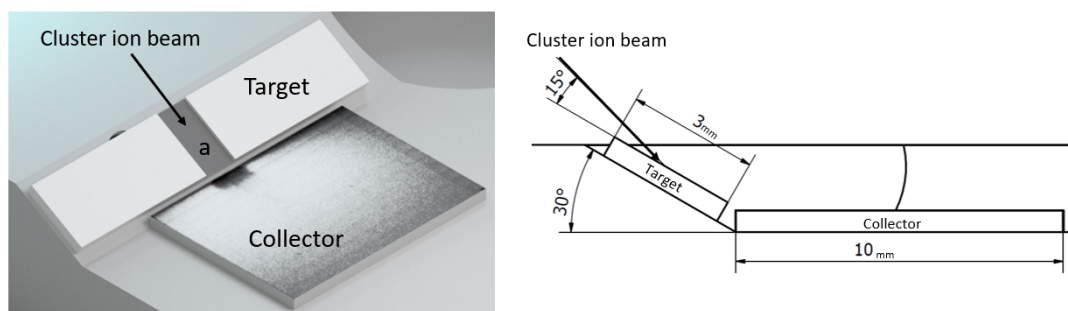


Figure 2.19: Representation of the experimental setup used for transfer of PPCs: (a) section of the target where material was removed. With the permission of K. Moshkunov

Transfers were achieved thrice for Tryp-PAA complexes and four times for Lys-PSS complexes. Unfortunately, Ins-Chi complexes could not be transferred due to time constraints.

2.3 Characterization of the transfers

Surfaces obtained by transfer experiments were compared to reference surfaces. Those were obtained by spin coating PPCs solutions on clean silicon wafers (see section 5.3.1 for practical insight).

2.3.1 2D mapping of collectors

After transfer experiments, a first analysis was performed on the collector using the 2D "mosaic" imaging mode of the SIMS instrument. In this mode, a focused Bi_5^+ ion beam scans automatically the surface taking multiple spectra on small squared patches ($500 \times 500 \mu\text{m}^2$) of the surface. Those are gathered in order to build an image of the whole collector. The mappings presented in this section show the intensities of characteristic peaks related to proteins, PE, silicon wafer and sodium. As mentioned in the introductory part of this thesis, the ion yield vary for each secondary ion, *i.e.* for each peak (see section 1.2.1). Therefore, the intensities observed in 2D mappings can only be compared analyzing the same fragments. 2D mappings were performed both in positive and negative mode since proteins are more easily seen in positive mode and PE could only be seen in negative mode.

Positive mode mapping

Unfortunately, the molecular ion peak of both lysozyme and trypsin could not be detected with this analysis. Actually, those are too large to be detected by ToF-SIMS routine analysis protocol. Therefore, the mappings were based on fragment ions characteristic of amino acid. In positive mode, fragment at $m/z=30, 44, 70, 84$ and 130 corresponding respectively to CH_4N^+ , $\text{C}_2\text{H}_6\text{N}^+$, $\text{C}_4\text{H}_8\text{N}^+$, $\text{C}_5\text{H}_{10}\text{N}^+$ and $\text{C}_9\text{H}_8\text{N}^+$ were selected for the analysis (table 2.5 shows the related amino acid).

Table 2.5: Amino acid related to positive ion chosen for 2D mapping [50]

m/z	Ion	Related amino acid
30	CH_4N^+	Many amino acids
44	$\text{C}_2\text{H}_6\text{N}^+$	Many amino acids
70	$\text{C}_4\text{H}_8\text{N}^+$	Proline
84	$\text{C}_5\text{H}_{10}\text{N}^+$	Lysine
130	$\text{C}_9\text{H}_8\text{N}^+$	Tryptophan

The silicon wafer substrate was imaged using peaks at 28 and 45, respectively corresponding to Si^+ and SiOH^+ . Figure 2.20 compares images of the collectors acquired after transfer of Tryp-PAA complexes (a and b) with reference images of a silicon wafer entirely covered by spin coated Tryp-PAA complexes (c and d).

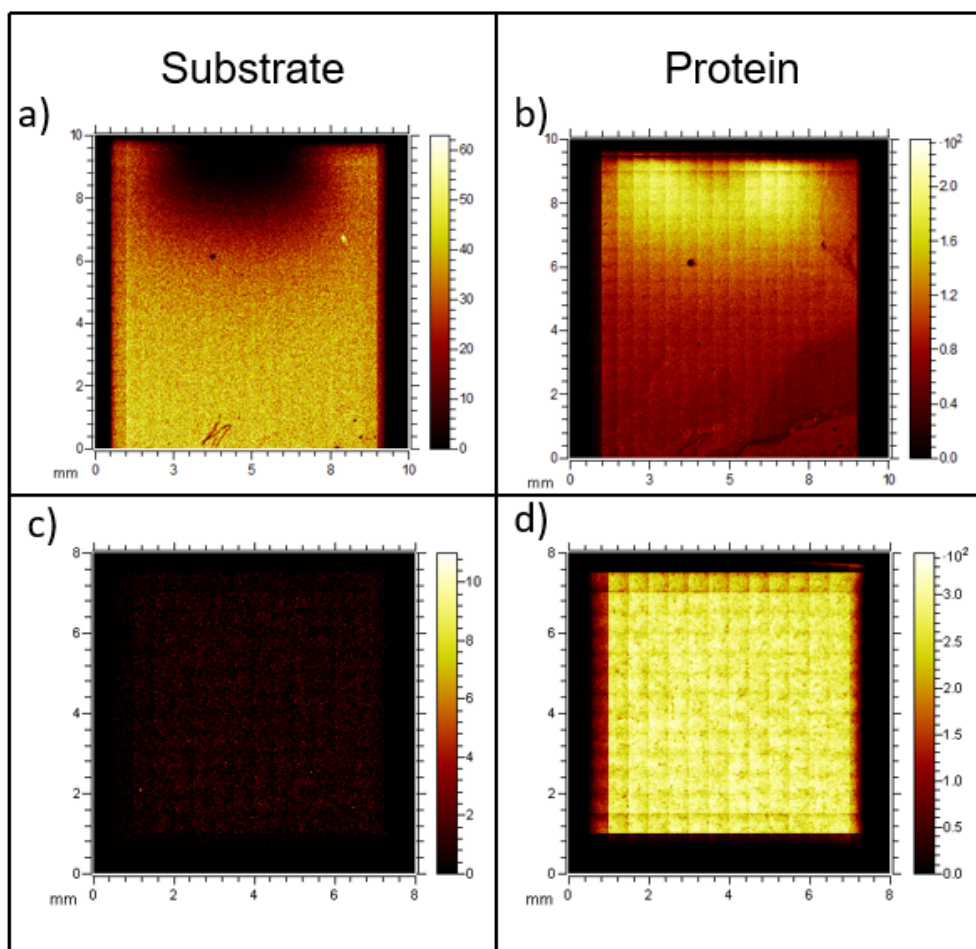


Figure 2.20: Chemical signal intensity image after deposition of Tryp-PAA complexes. a) and b) represents a collector obtained from a transfer experiment. a) displays the silicon signals as the sum of Si^+ and $SiOH^+$ intensities. b) displays the amino acid signals as the sum of CH_4N^+ , $C_2H_6N^+$, $C_4H_8N^+$, $C_5H_{10}N^+$ and $C_9H_8N^+$ intensities. c) and d) represent the same chemical signals for a silicon wafer fully covered by spin coated Tryp-PAA complexes.

This 2D mapping confirm that protein material was transferred from the target to the collector. Indeed, the substrate signal is strongly attenuated in the collector region where the amino acid peaks show a maximum intensity. This region is located near the target in the experimental setup. The pattern of deposition is a semi circular shape centered on a darker spot in the top center of the collector. This spot is often present on collector mappings (more examples are presented further in this section) and is probably due to a part of the primary Ar cluster beam impinging the collector surface during the transfer. Therefore, proteins transferred at this location will be sputtered by the Ar-GCIB. This is mainly due to the grazing incident angle of this ion beam on the target.

One can notice that the intensity of the protein characteristic peaks decreases as a function of the distance in all directions. The reverse pattern can also be observed for substrate

signals: close to zero in the region near the target and then gradually increase. It appears that there is a gradient of deposited amount of protein material across the collector surface.

Regarding the reference sample 2D mapping, the deposition of complexes seems, as expected, homogeneous along the silicon wafer surface since the amino acid signals are intense. Moreover, the substrate signal is almost non-existent.

Similar results were obtained for Lys-PSS complexes (see figure 2.21). One can observe a small circular dark patch in the middle of the collector. This artifact is most likely a contamination that landed on the surface when the sample was taken out of the instrument between the transfer and the mapping experiments.

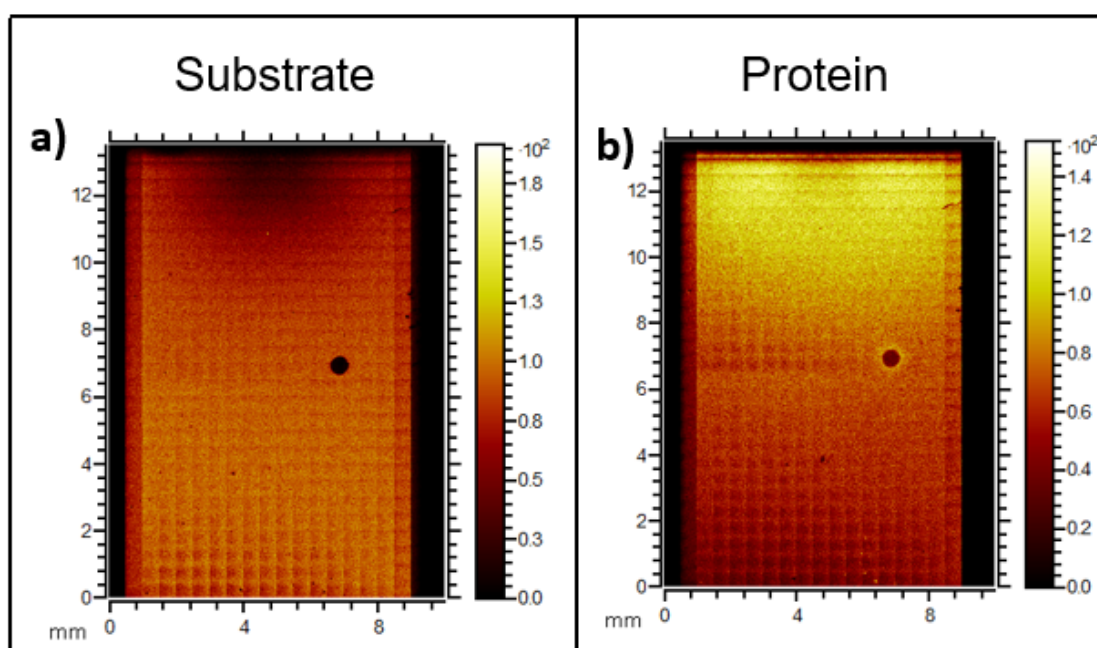


Figure 2.21: Chemical signal intensity image after transfer of Lys-PSS complexes.

a) displays the silicon signals as the sum of Si^+ and $SiOH^+$ intensities. b) displays the amino acid signals as the sum of CH_4N^+ , $C_2H_6N^+$, $C_4H_8N^+$, $C_5H_{10}N^+$ and $C_9H_8N^+$ intensities.

Negative mode mapping

The advantage of the negative mode is that it enables the analysis of both protein and PE. For proteins, the chosen fragments were CN^- and CNO^- corresponding respectively to $m/z = 26$ and 42 . For PAA, fragments at $m/z = 29$, 41 and 71 corresponding to CHO^- , C_2HO^- and $C_3H_3O_2^-$ were selected, the latter being the monomer ion. PSS was imaged using peaks at $m/z = 80$ and 180 (SO_3^- and $C_8H_7SO_3^-$, the monomer ion). The substrate signal was analyzed using the Si^- and SiO_3H^- peaks at $m/z = 28$ and 77 . Negative mode 2D mapping of both Tryp-PAA (a, b and c) and Lys-PSS (d, e and f)

collectors are shown in figure 2.22. Note that those mapping were performed on the same collector as the one presented in the positive mode section.

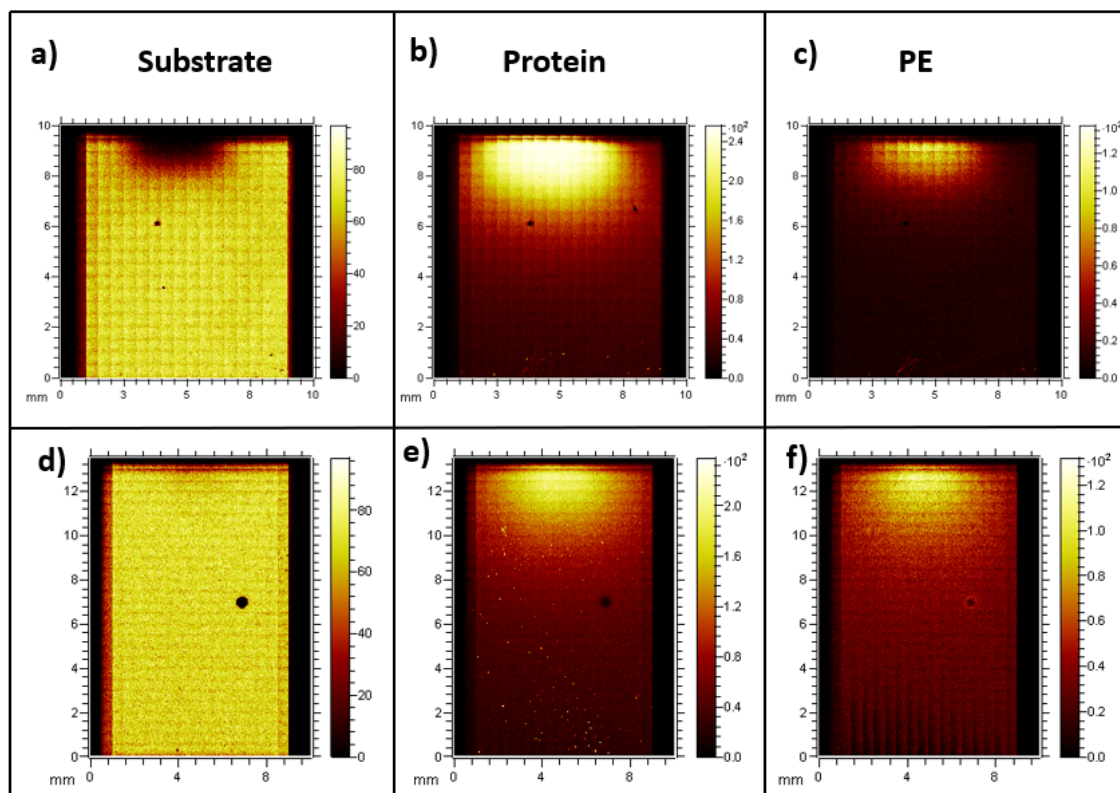


Figure 2.22: Chemical signal intensity image after transfer of PPCs. a), b) and c) represents a collector obtained from a transfer experiment of Tryp-PAA complexes. d), e) and f) represents a collector obtained from a transfer experiment of Lys-PSS complexes. a) and d) displays the substrate signal as the sum of Si^- and SiO_3H^- intensity. b) and e) displays the protein signals as the sum of CN^- and CNO^- intensities. c) displays the PAA signals as the sum of CHO^- , C_2HO^- and $C_3H_3O_2^-$ intensities. f) displays the PSS signals as the sum of SO_3^- and $C_8H_7SO_3^-$ intensities

A similar deposition pattern is observed for protein and PE characteristic peaks. The deposition might seem smaller compared to positive mode mapping but this difference can be attributed to the scale automatically assigned by the imaging program. The silicon wafer signals seem to be less attenuated for the same reason.

Ion dose effect

Providing the fact that the target is a huge PPCs reservoir, it should not become exhausted during transfer experiment. Therefore, the transferred amount is expected to be proportional to the number of clusters (ion dose) impinging on the target surface. The argon clusters are assumed to each carry one positive charge. 2D mapping of different transfer were compared as a function of the ion dose. The ion dose was calculated according to

equation 2.1:

$$\text{ion dose} = t [s] * I \left[\frac{C}{s} \right] * 6,242.10^{18} \left[\frac{\text{charge}}{C} \right] \quad (2.1)$$

Where:

- t stands for the total time of transfer
- I stand for the mean current of argon clusters during the transfer, *i.e.* the mean between the current measured before and after transfer². In general, the intensity of the current did not vary much.

Figure 2.23 shows the positive mode 2D mapping of proteins related peaks of Tryp-PAA collectors are presented in ascending order of ion dose. For information purpose, the times of transfer are also shown. The same figure was realized for Lys-PSS collectors (see figure 2.24).

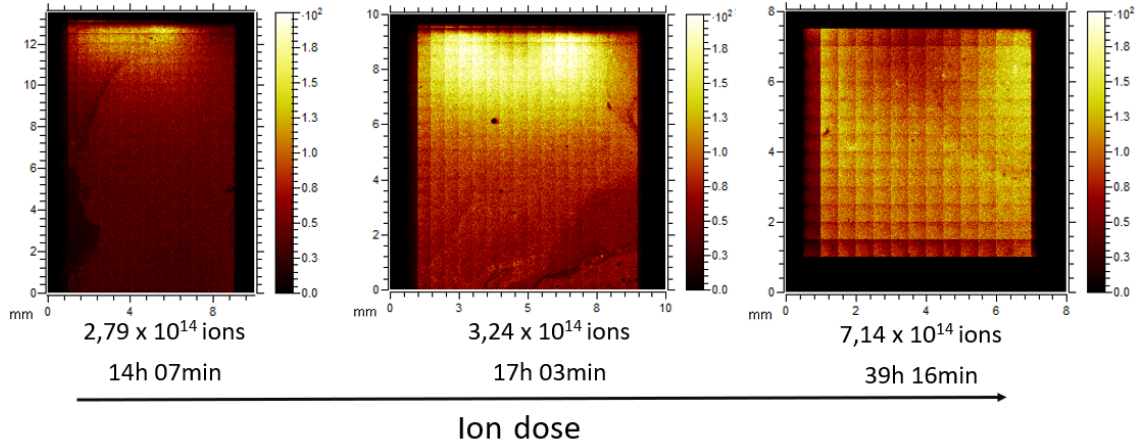


Figure 2.23: Protein characteristic peaks of Tryp-PAA collectors as a function of the ion dose

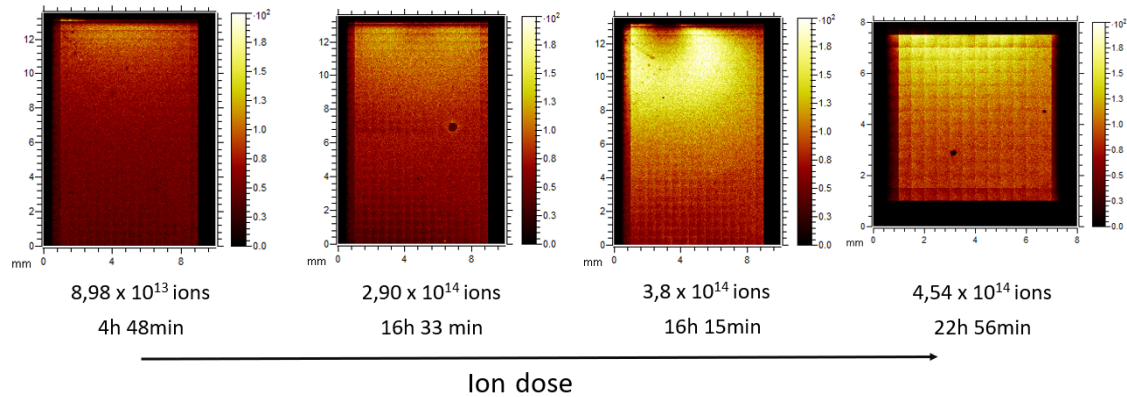


Figure 2.24: Protein characteristic peaks of Lys-PSS collectors as a function of the ion dose

²The current cannot be measured during transfer experiment

As expected, for Lys-PSS collectors, the protein related signals are more intense and cover a greater area with increasing ion dose. Nevertheless, the transfer that was performed with the biggest ion dose seem to constitute an exception to the rule. Moreover, the deposition pattern is different from other transfer experiments. The same phenomenon can be observed on Tryp-PAA collector. The intensity of protein related signals increases with the width of the analyzed layers until some point ($\approx 4\text{nm}$) where it becomes independent of the thickness. Thus, the signal stop increasing and no difference in intensity can be observed between long transfers. For high ion dose transfer, the deposited layer is maybe thick enough on a wide area so that the usual deposition pattern cannot be observed. In order to verify this hypothesis, the substrate signals (Si^+ and SiOH^+) were 2D mapped on the same collectors. Figure 2.25 and 2.26 present this mapping for respectively Tryp-PAA collectors and Lys-PSS collectors.

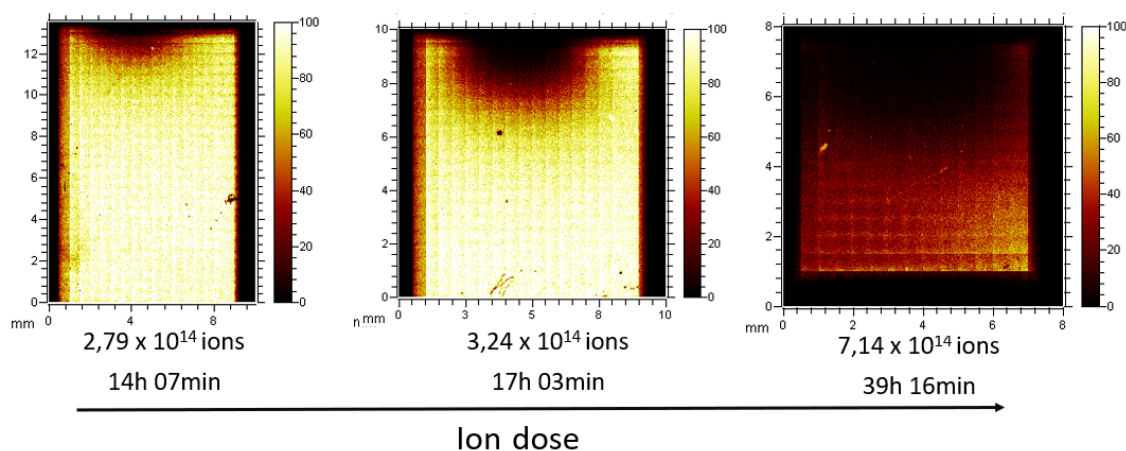


Figure 2.25: Silicon wafer characteristic peaks of Tryp-PAA collectors as a function of the ion dose

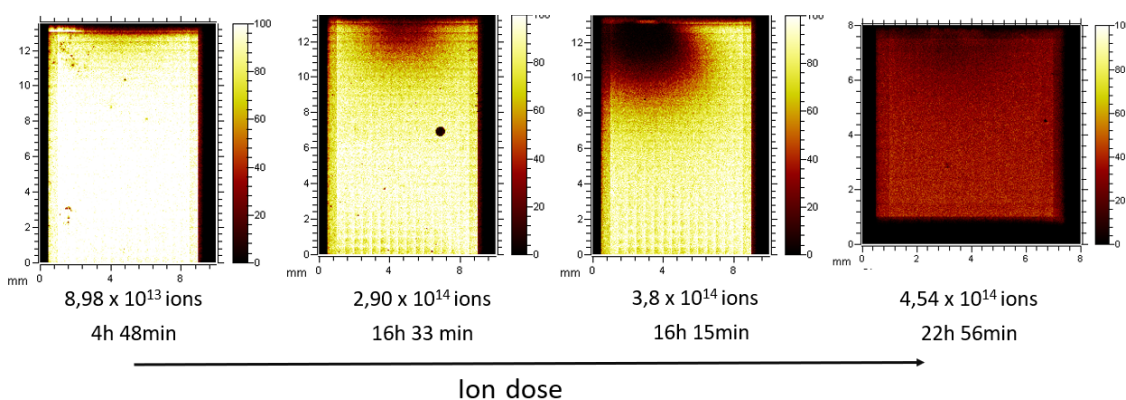


Figure 2.26: Silicon wafer characteristic peaks of Lys-PSS collectors as a function of the ion dose

One can observe that the zone where the substrate signals are low grows with increasing ion dose. For high ion dose transfers, those signals are at least attenuated all over the

collector. This means that protein material could be deposited on the whole collector surface, $\approx 64 \text{ mm}^2$ in those cases.

Sodium peaks

As sodium is the counter ion of both PSS and PAA (see figure 2.2). PPCs suspensions contains sodium. Therefore, it should also be transferred by the argon cluster ion beam. This hypothesis was assessed by mapping the Na^+ peak at $m/z=23$ on the collectors presented in the positive mode section (see figure 2.27). A Tryp-PAA collector is shown in a) and b), respectively representing sodium and proteins peaks and a Lys-PSS collector is shown in c) (sodium) and d) (protein).

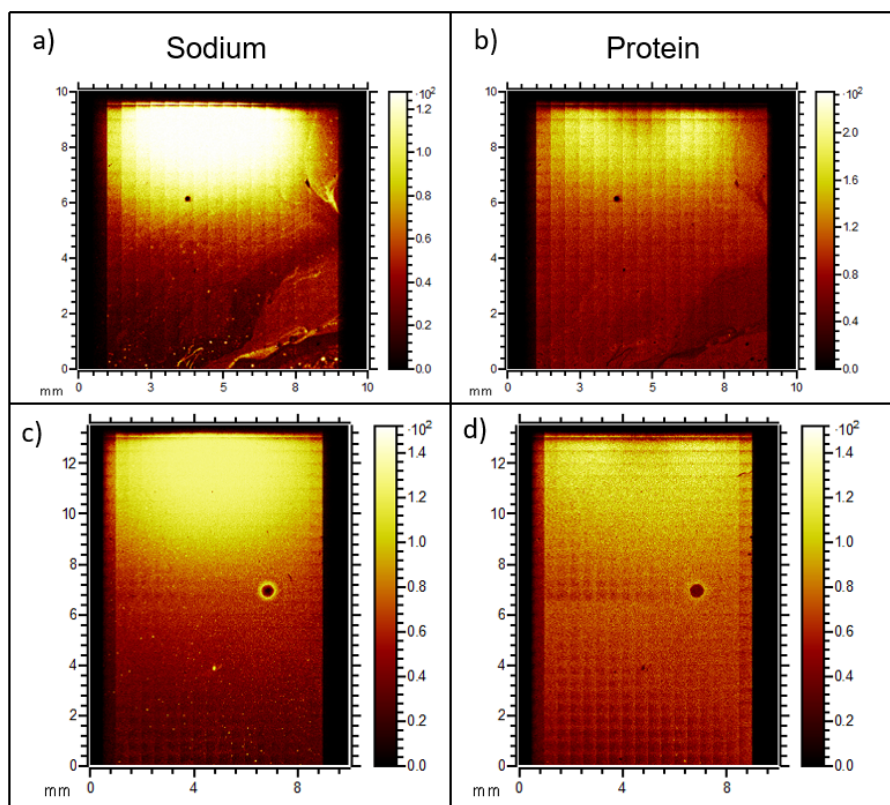


Figure 2.27: Chemical signal intensity image after transfer of PPCs. a) and b) represents a collector obtained from a transfer experiment of Tryp-PAA complexes. c) and d) represents a collector obtained from a transfer experiment of Lys-PSS complexes. a) and c) displays the ion Na^+ intensity. b) and d) displays the amino acid signals as the sum of CH_4N^+ , $\text{C}_2\text{H}_6\text{N}^+$, $\text{C}_4\text{H}_8\text{N}^+$, $\text{C}_5\text{H}_{10}\text{N}^+$ and $\text{C}_9\text{H}_8\text{N}^+$ intensities.

As expected, sodium was deposited following the same pattern as proteins and polyelectrolyte. The transfer of sodium is unavoidable. However, it should not alter the functionality of intact transferred proteins. Indeed, sodium is present in every living being and those have completely functional proteins. Moreover, sodium should be solubilized when the surface is immersed

Summary of 2D mapping of collectors

2D mapping results confirm that protein material was efficiently transferred to the collector and allowed the determination of a deposition pattern which is related to the ion dose impinging the surface. The deposited material amount is maximal near the clusters impact point, then decreases with distance in all directions. Nevertheless, the ToF-SIMS technique in this case is not successful at assessing whether proteins were transferred intact or as fragments. Moreover, it delivers no information about the 3D conformation or the functionality of transferred proteins. Therefore, other analysis were planned in order to answer those questions

2.3.2 Comparison between transferred samples and references: Principal component analysis

In order to confirm the transfer of intact PPCs, several SIMS spectra have been taken on post transfer collectors and references samples. Those were realized using the routine analysis protocol: a focused Bi_5^+ ion beam browsing randomly a small patch of $500 \times 500 \mu\text{m}^2$ during 60 seconds. As collectors present a heterogeneous surface, spectra were taken in three different zones defined by the amino acids characteristic peaks: 1) the rich zone where highest signals are observed ; 2) the intermediate zone; 3) the poor zone where the lowest signals are observed (see figure 2.28). Note that the amount of transferred material is strongly dependant on the transfer ion dose. Due to practical constrains, *i.e.* difference in argon cluster ion beam initial/final current or ToF-SIMS device reservation schedule, transfer were performed with different ion dose. This induces variability in the transferred PPCs surfaces. For both PPCs, six out of nine spectra came from transfer with ion dose approximately equal to $3\text{-}4 \times 10^{14}$ ions. The last three spectra were taken on higher ion dose collectors (see figure 2.23 and 2.24) . As the amount of transferred material decrease with the distance from the impact point, the intermediate zone spectra of those last could maybe be similar to the rich zone spectra of the others.

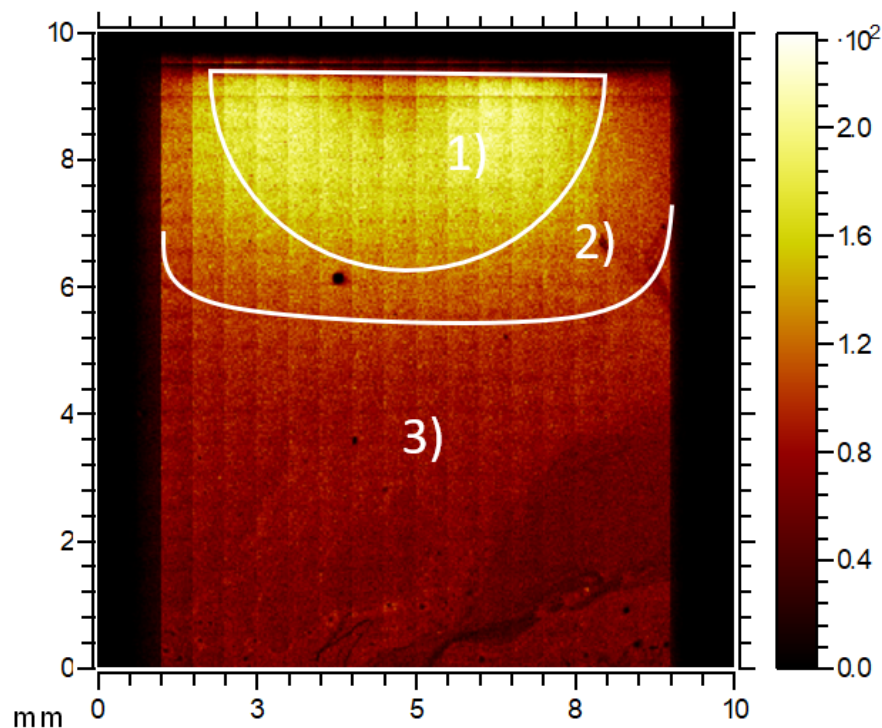


Figure 2.28: Different zones demarcation of a collector

The idea is to compare the spectra obtained from those different zones with reference spectra. Nevertheless, large amount of data is extracted from ToF-SIMS analysis. Figure 2.29 shows two overlapped spectra: in blue, the spectrum of a reference sample of Lys-PSS and in red, the spectrum of the rich zone of a Lys-PSS transfer. Useful information about the difference between those samples cannot be extracted by simply looking at those spectra. Therefore, multivariate analysis (MVA), *i.e.* statistical analysis, were performed on the

spectra data in order to reduce it. More specifically, the MVA used is principal component analysis (PCA) which is a wide spread MVA used for organic ToF-SIMS measurements [53] [51]. This section will firstly present a general overview about PCA methodology. To make this overview clear, a PCA example on pure protein surfaces will be performed. Finally, relevant PCA results on collectors and references will be presented.

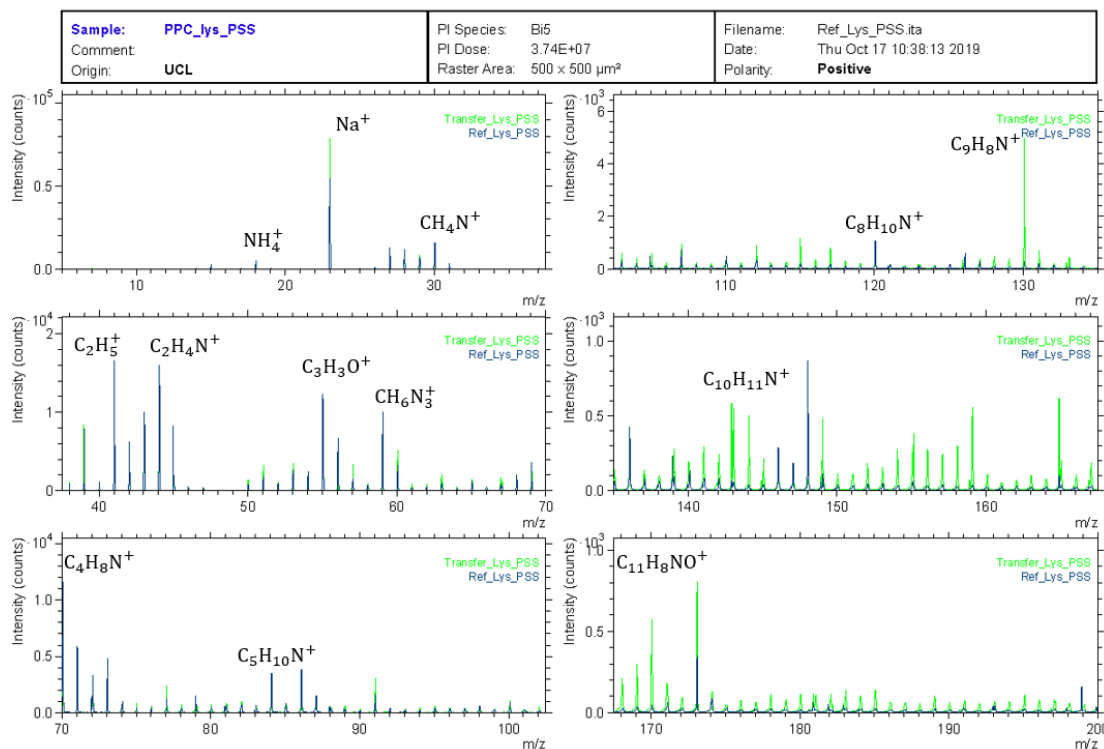


Figure 2.29: Overlap of Lys-PSS reference spectrum (in blue) and Lys-PSS collector (in green)

PCA overview

Principal component analysis is a MVA method that determines the largest directions of variance within a data set. The aim of PCA is to find the eigenvectors and eigenvalues of the variance covariance matrix of a data set. Graphically, PCA is an axis rotation that generates new axes defining the major directions of variance within a data set, *i.e.* the principal components (PC) (see figure 2.30). This axis rotation reduces the huge data set containing hundreds of variables down to a few interpretable variables, the scores and loadings. The scores describe the relationship between samples while the loadings show which variables is responsible for the differences. For ToF-SIMS data, the samples correspond to entire spectra, *i.e.* the integrated areas of all peaks for a given spectra, and the variables correspond to specific peak areas [52].

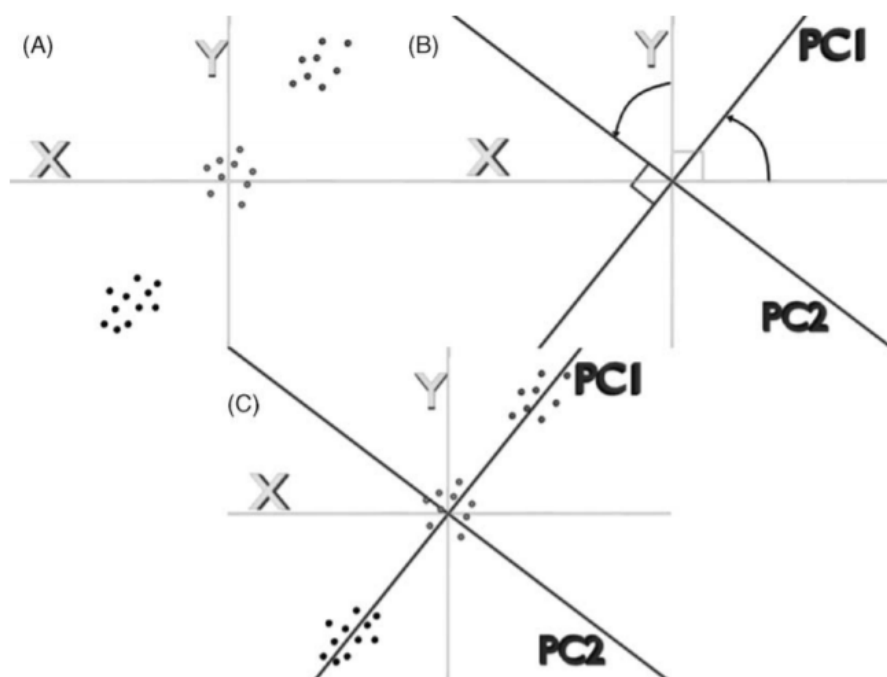


Figure 2.30: Graphical representation of PCA. The PC axes are rotated (B) to capture the greatest directions of variation in the original data containing 3 set of samples plotted in two dimensional space (A). (C) presents an overlay of the PC axes and the original axes [54]

PCA assumes a linear relationship between variables and is scale dependent. This second assumption means that variables with high relative intensities, *i.e.* large peaks, will be more highlighted by PCA than lower intensity variables. In ToF-SIMS, low mass peaks, carrying less specific chemical information, are more intense than high mass peaks, chemically specific. Therefore, those low mass ion will often have higher loadings compared to high mass ion. Finally, PCA captures the largest sources of variation within a data set, regardless of what is in those data. If the largest source of variation is coming from noise or contaminants, PCA will highlight these differences first. Therefore, it is very common to preprocess the data before running the PCA in order to highlight chemical difference between spectra rather than mathematical difference in peak intensities [52].

Data can be pretreated in many different ways, each affecting the results obtained from PCA. Most common pretreatment methods are peak selection, normalization, mean centering and transformation. (I) It is possible to run PCA on every mass channel of a ToF-SIMS spectrum. However, it is more common to run PCA on a set of selected peaks area since many mass channels contain only noise or account for contaminants. As the set of peaks affects the PCA results, peak selection is the first data processing done before running PCA. (II) Normalization of the data is performed by dividing each peak by a scalar value. This method is done to account for variations in the data acquisition that are not related to chemical differences such as variation in the detector efficiency or in the ion beam current, sample charging or instrumental conditions. Data can be normalized to the total intensity of each spectrum, to the most intense peak of a given spectrum or to the sum of selected

peaks of the spectrum [54]. (III) Mean-centering is done by subtracting the mean-value of a variable from that variable in each spectrum in the data set. Therefore, all variables vary across a common mean of zero (see figure 2.31). This allows PCA to capture difference in the relative intensities of different variables rather than difference in the means of those variables [52]. (IV) Transformation consist in modifying the data with a function such as the square root or the logarithm [51]. Note that each pretreatment method carries a set of assumptions with it. For example, if data are normalized to the total intensity of each spectrum, information about the total intensity is lost and thus assumed to contain no relevant chemical information about the samples. It is up to the researcher to validate those assumptions before applying any pretreatment method to the data [54].

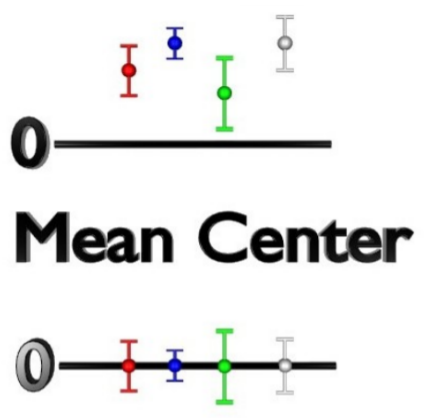


Figure 2.31: Principle of the mean centering data pretreatment [52]

PCA is calculated from the covariance matrix of a data set since it contains the variance of each peak and the covariance of each peak with all other peaks of the data set. As a reminder, variance is a measure of the spread within a given data set and the covariance is a measure of the joint variability of two variables. The covariance matrix is calculated as shown in equation 2.2 [52]:

$$cov(X) = \frac{X^T X}{m - 1} \quad (2.2)$$

Where:

- X is the data matrix where samples (spectra) are in rows and variables (peaks) are in column. Its dimensions are m x n
- m is the number of spectra
- n is the number of peaks

The PCA algorithm proceeds sequentially: the first direction of greatest variance within the data is determined (PC1). Then, this firsts PC is subtracted from the data and PC2 is determined from the greatest remaining variance in the data. Note that the variance

captured by each successive PC is decreasing. It is expressed mathematically in equation 2.3. The original data matrix X is reconstructed as the addition between the product of the scores (S) and the transpose of the loadings (L) from PC1 and a residual matrix R_1 containing the variance left after subtracting PC1 from the original data. R_1 becomes the new X matrix from which the second PC (PC2) is calculated [52].

$$X = S_1 L_1^T + R_1$$

$$\rightarrow R_1 = S_2 L_2^T + R_2 \quad (2.3)$$

The algorithm is run several (k) times until all the variance is captured. The PCA decomposition is shown on equation 2.4. The relative percentage of variance captured by each PC is also calculated by the PCA algorithm [52].

$$X = S_1 L_1^T + S_2 L_2^T + S_3 L_3^T + \dots + S_k L_k^T \quad (2.4)$$

The scores can be visualized graphically. The scores are the projection of the samples onto a given PC axis. Nine different samples are represented with the first two PC axis in a two dimensional space on figure 2.32. Those samples are divided in three categories according their color. Each sample is projected onto both PC axis by drawing a perpendicular line from the axis to the sample. As mentioned earlier, scores are used to investigate the relationship between samples. On PC1, the different categories of samples are clearly separated while those overlap on PC2. This suggest that PC1 captures the difference between the samples and PC2 captures random variation in the data [52].

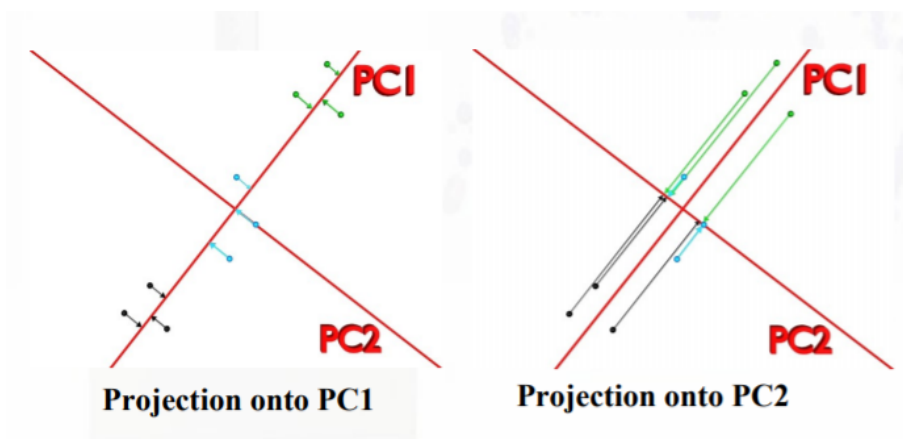


Figure 2.32: Graphical representation of the scores [52]

Loadings are the impact factors of the different variables in the PCA separation. A high loading value means that this variable had high influence on the separation. In other word, the loadings show which peak areas contributed to the separation observed in the scores [52].

Since PCA is based on statistics, it is important to collect enough data to have statistically relevant results. Therefore, it was really important to design the experimental plan for PCA

and not thinking of performing PCA after experimentation. Spectra were collected from three spots per samples across three different samples for each type of analyzed surface (reference/transfer) [51].

Example: PCA of spin coated proteins

An example of PCA is presented in this section in order to make it clear for the next section. PCA was performed on data coming from spin coated protein surfaces. Three different proteins were analyzed: lysozyme, trypsin and insulin. All three were spin coated from 5g/L solutions of proteins, three surfaces were made for insulin and trypsin and three spectra per surfaces were taken. For lysozyme, only two surfaces were made and two spectra per surface were taken. Actually, this reference was made at the beginning of the thesis and was not made for the purpose of PCA. It is not an issue because this PCA is an example made to clarify how it works and no important results will be extracted from this analysis.

Data preprocessing included the selection of 60 peaks related to protein fragments (see material and methods (M & M)), normalization to the total intensity of each spectra and mean centering. Those were chosen because those are the most commonly used for the PCA of ToF-SIMS data [51]. PCA was performed using the program "Spectragui" from the Nesac/bio toolbox software developed by Dr. Daniel J. Graham (Washington University, USA).

The scores of PC1 are shown on figure 2.35. PC1 captures 96% of the total variance and discriminates insulin samples from lysozyme and trypsin samples. Indeed, insulin presents positive scores while the others present negative scores.

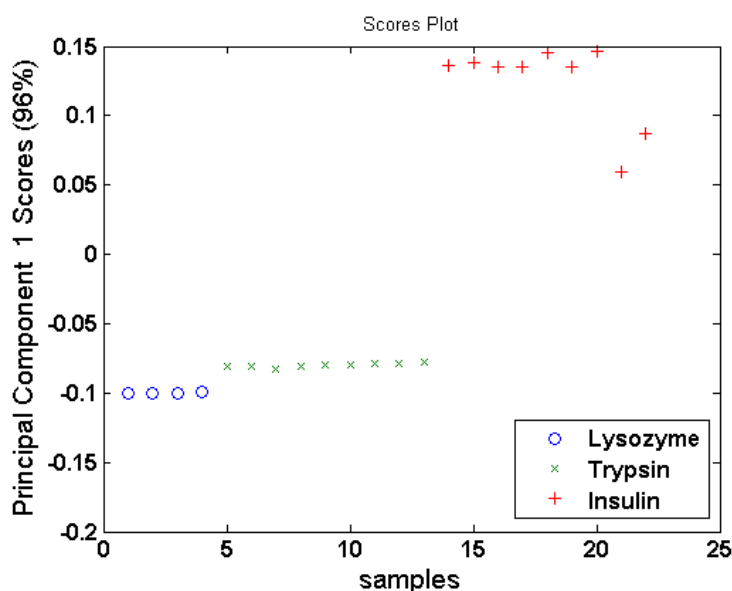


Figure 2.33: PCA of spin coated proteins: protein samples as a function of the PC1 scores

The loading of PC1 are shown in figure 2.34. It appears that the sodium peak ($m/z=23$)

contributes to the positive score of insulin. This means that the sodium peak is very different for insulin samples compared to other proteins. This might be explained by the fact that sodium is present in pure protein powder for conservation purposes. It seems that there is a higher sodium content in insulin compared to other protein. Other peaks, notably $m/z=30, 44, 70, 72, 86$, contribute to the negative separation of lysozyme and trypsin. It means that those peaks are more intense for those proteins compared to insulin.

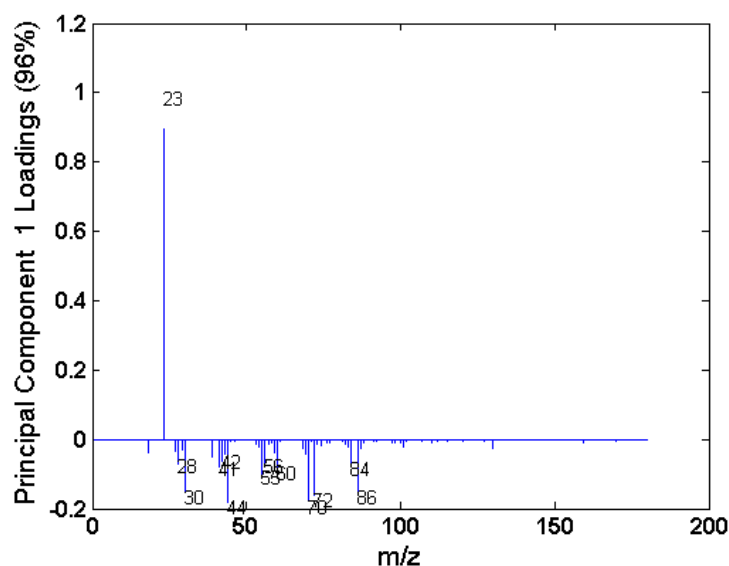


Figure 2.34: PCA of spin coated proteins: peaks as a function of the PC1 loading

The scores of PC2 are shown on figure 2.35. PC2 captures 4% of the total variance and discriminates lysozyme from trypsin samples. Indeed, trypsin samples present positive scores while lysozyme samples have negative scores. Note that insulin samples present scores around zero, it is explained by the fact that all variance related to insulin was captured by PC1.

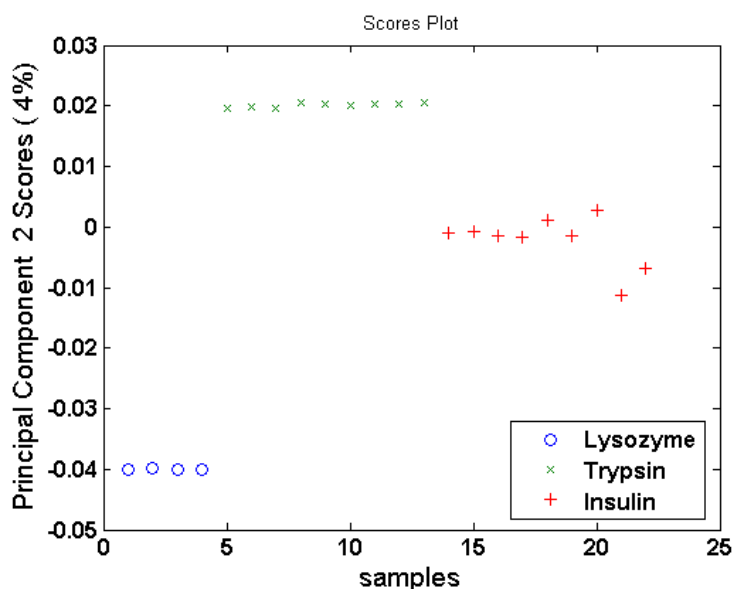


Figure 2.35: PCA of spin coated proteins: protein samples as a function of the PC2 scores

The loading of PC2 are shown in figure 2.36. The peaks with positive loadings are more intense in trypsin samples and the peaks with negative loadings are more intense in lysozyme samples. This is probably due to the fact that both proteins present different content in each amino acid, the signal associated to each of those thus vary and this is noticed by the PCA algorithm. Note that the sodium peak is more intense in trypsin (positive loading). Therefore, the pure proteins powder might be classified as a function of their sodium content (insulin > trypsin > lysozyme).

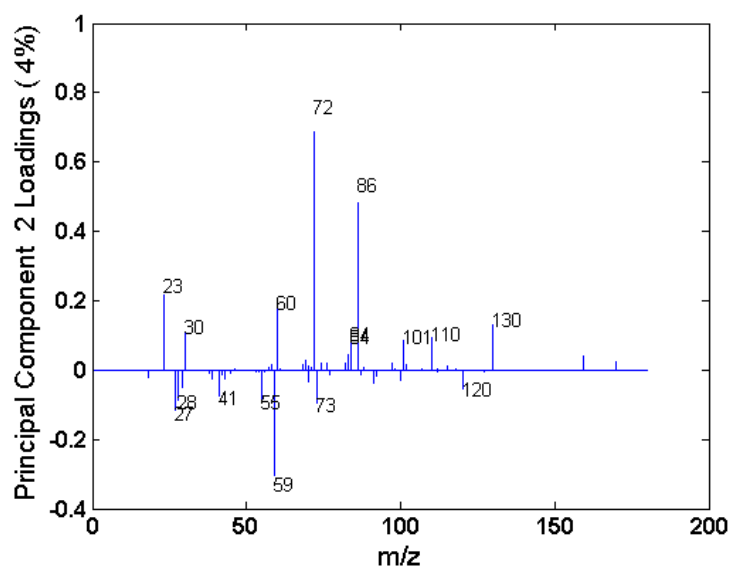


Figure 2.36: PCA of spin coated proteins: peaks as a function of the PC2 loading

Figure 2.37 summarizes the PCA of spin coated proteins. The scores of PC1 and PC2 are plotted in a), the circles represent a 95% confidence interval. The loadings of PC1 and PC2 are plotted in b). This type of display enables faster results processing since two PC

can be taken into account at the same time.

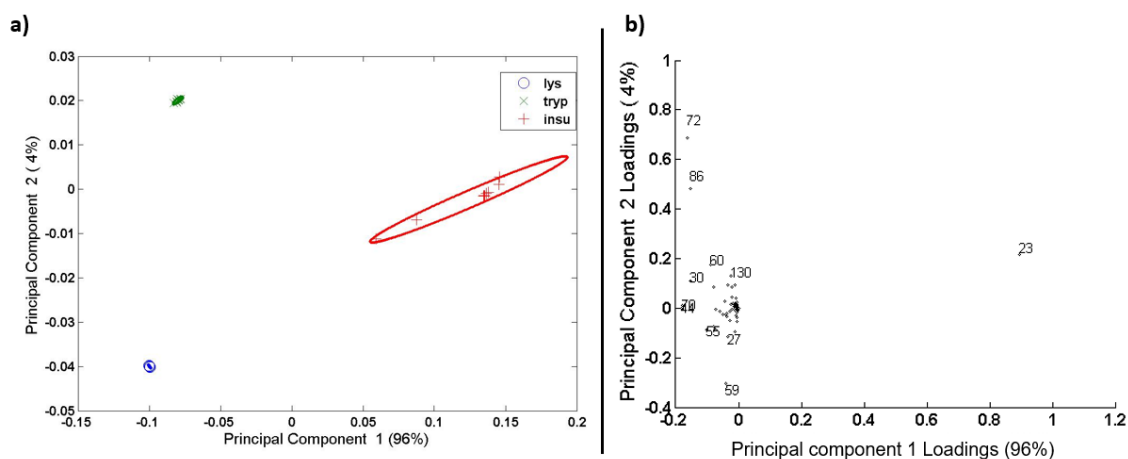


Figure 2.37: PCA of spin coated proteins: a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

The sodium peak has a major influence on the PC1. The separation between proteins was thus mainly based on a contamination. Therefore, PCA was performed on the same data set from which the sodium peaks have been removed. The goal was to see if the discrimination between proteins could be realized by taking into account only protein related peaks. Figure 2.38 presents the scores (a) and loadings obtained by the first two PC. The amount of variance captured by PC1 (81%) was lowered to the benefit of PC2 (18%). This time, PCA separate lysozyme from other proteins in PC1 and insulin and trypsin are discriminated in PC2.

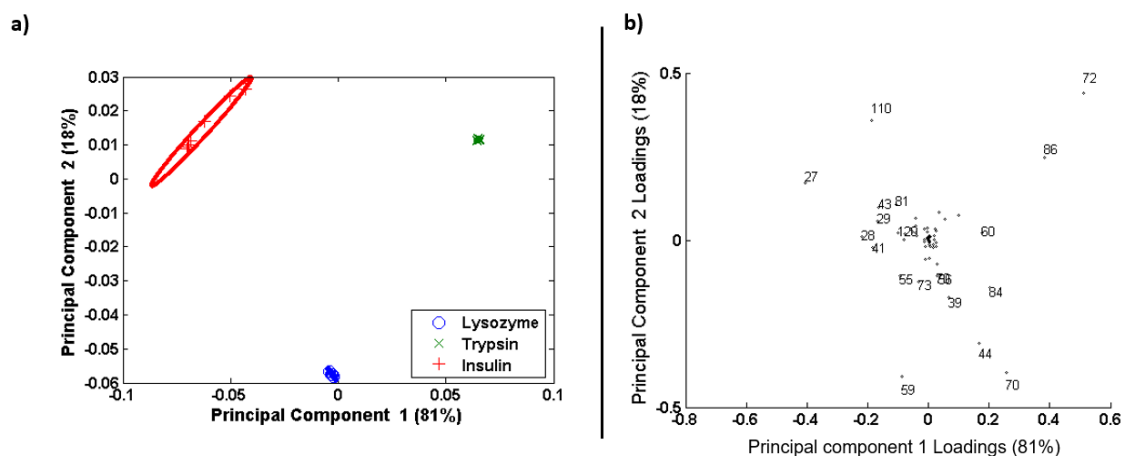


Figure 2.38: PCA of spin coated proteins without sodium peak: a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

Note that data were normalized to the intensity of the sum of selected peak. Indeed, the sodium peak in insulin samples contributes a lot to the total intensity of the spectrum. Therefore, when normalizing data by the total intensity of each spectra, all other peaks in

the insulin spectra are flattened by the sodium peak in a bigger way compared to peaks in lysozyme or trypsin spectra. As this PCA does not take sodium into account, the influence of this peak on the results was removed by changing the normalization method. This example highlights how important the choice of data pretreatment is.

PCA of transferred samples and references

In the context of this thesis, PCA was used to compare Lys-PSS and Tryp-PAA collectors with their respective references. The idea of this analysis is that there should be a greater variability between spectra of surfaces where different PPCs were deposited and a lower variability between the spectra from the rich zone and the reference spectra of the same PPCs. Note that the mass spectra can be influenced by other factors than the chemical identity of surface molecules: (I) As mentioned earlier, the thickness of very thin layer ($<4\text{nm}$) influence the shape of the spectra. (II) A difference in the layout of molecules in the deposited PPCs layer can induce a matrix effect. Therefore, if the PCA separate the spectra as expected, it would confirm that the transferred and reference surfaces are chemically similar. If the PCA does not, the difference between spectra would not especially be related to chemical difference between the deposited layers.

PCA was performed on both positive and negative spectra. Many PCA analysis were performed in order to test different normalization methods and different peak list accounting or not to sodium or substrate peaks. Presenting all PC of those would be long and pointless because some PC did not separate anything. Therefore, if nothing can be interpreted from some PC graphs, those will not be presented.

For positive spectra, data preprocessing included the selection of 60 peaks (see M & M chapter) related to proteins, substrate and sodium, normalization to the total intensity of each spectra and mean centering. PCA results are shown in figure 2.39. 93% of the variance is captured in PC1 and PC2. The separation in PC1 is mainly due to the sodium peak since this peak has a higher loading than any other peak. The presence of sodium in both reference and transferred samples is unavoidable since sodium is the counter ion of both PPC and, in a lesser extent, is present in protein powders for conservation. ToF-SIMS measurements are particularly sensitive to the ion Na^+ because of its low mass and ionic nature. The separation in this PCA is mainly due to this sodium peak and does not really separate the different samples. Therefore, the sodium peak was removed from every data sent to the PCA algorithm and a normalization to the sum of selected peaks was chosen for the same reasons as the ones presented at the end of the last section.

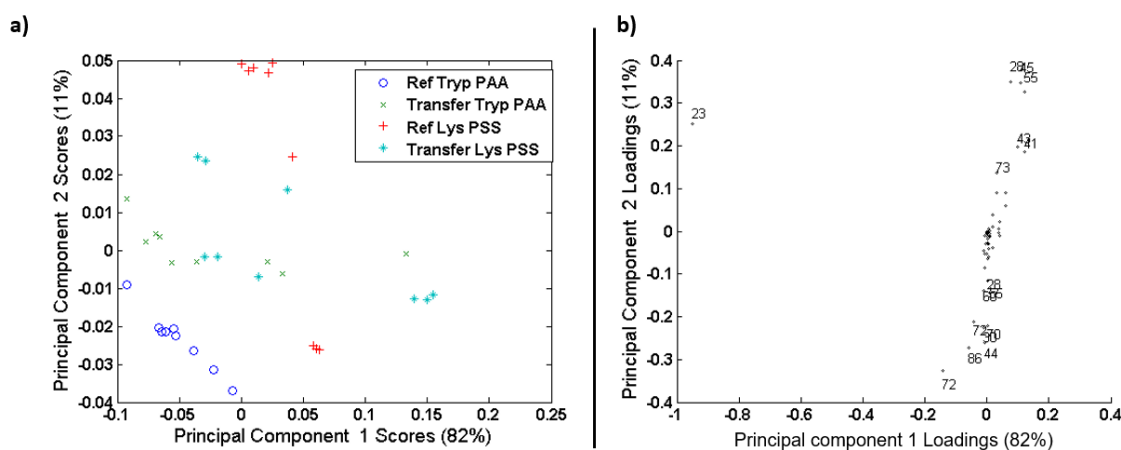


Figure 2.39: PCA of reference and transferred surface: a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

Figure 2.40 presents PC1 and PC2 of a PCA performed on the same data from which the sodium peak has been removed. In PC1, which account for 71% of the total variance, references and some transfer of Tryp-PAA present a negative score mostly due to the $m/z=72$ peak which is related to an amino acid (valine). On the other hand, some references and transfers of Lys-PSS present a positive score mostly due to peaks related to the substrate or substrate contamination, *i.e.* small aliphatic chains (see table 2.6). Even though a trend that separate both PPCs surfaces can be identify, this one is not observed for all spectra and Lys-PSS samples are separated by substrate related signals rather than protein peaks. Therefore, no conclusion will be drawn from this PC1. As PC2 discriminate the samples within the same category, nothing can be interpreted from it.

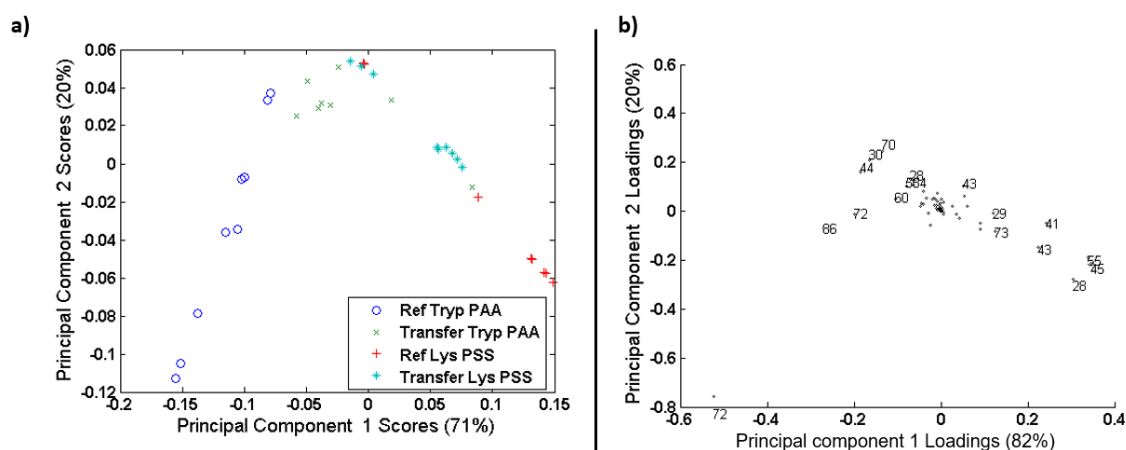


Figure 2.40: PCA of reference and transferred surface without Na peak: a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

Table 2.6: Peaks and their origins responsible of the positive scores observed in PC1 for Lys-PSS samples

m/z	ion	Origin
28	Si ⁺	Substrate
41	C ₃ H ₅ ⁺	Contamination
43	C ₃ H ₇ ⁺	Contamination
45	SiOH ⁺	Substrate
55	C ₄ H ₇ ⁺	Contamination

For negative spectra, data preprocessing included the selection of 48 peaks (see M & M chapter) related to proteins, PE and substrate, normalization to the total intensity of each spectra and mean centering. The PCA results are shown in figure 2.41. The same tendency as the one observed in positive PCA is observed: Tryp-PAA surfaces have a negative score mainly due to protein and PAA related peaks (see table 2.7). Lys-PSS samples tends to show a positive score due to both PSS and substrate related peaks (see table 2.8)

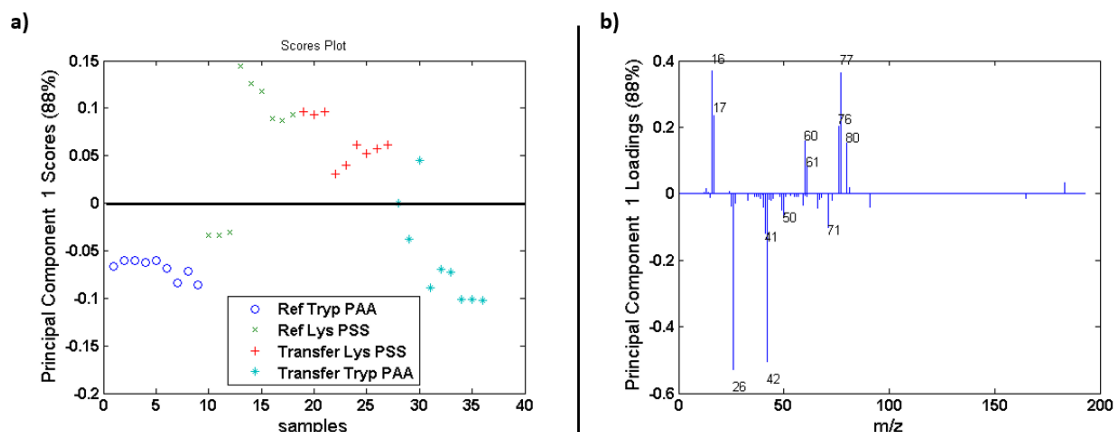


Figure 2.41: PCA of reference and transferred surface in negative mode: a) samples as a function of their scores in PC1; b) loadings of peaks in PC1

Table 2.7: Peaks and their origins responsible of the negative scores observed in PC1 for Tryp-PAA samples

m/z	ion	Origin
26	CN ⁻	Protein
41	C ₂ OH ⁻	PAA
42	CNO ⁻	Protein
50	C ₃ N ⁻	Protein
71	C ₃ H ₃ O ₂ ⁻	PAA monomer

Table 2.8: Peaks and their origins responsible of the positive scores observed in PC1 for Lys-PSS samples

m/z	ion	Origin
16	O^-	Substrate
17	OH^-	Substrate
61	CHSO^-	PSS
77	CHSO_2^-	PSS
80	SO_3^-	PSS
183	$\text{C}_8\text{H}_7\text{SO}_3^-$	PSS monomer

One can notice that the references of Lys-PSS (red cross on positive PCA and green cross on negative PCA), which are by nature supposed to be all the same, are separated in two groups. Six spectra are separated on PC1 because of substrate peaks while the three others are not. As three spectra were taken by reference, it appears that two references are different from the last one. This difference might be originated from the manufacturing of those references. Indeed, spin coating is supposed to produce homogeneous layers but it seems that it was only the case for one. As PPCs tends to form solid colloidal particles, those might stick together upon spin coating to form an heterogeneous surface where PPCs particles are deposited in different spot of the surface. The 2D mapping of one reference surface with intense substrate signal confirms this hypothesis (see figure 2.42). As the spectra coming from one reference surface seems not to be separated by substrate signals, this surface is supposed to be homogeneous.

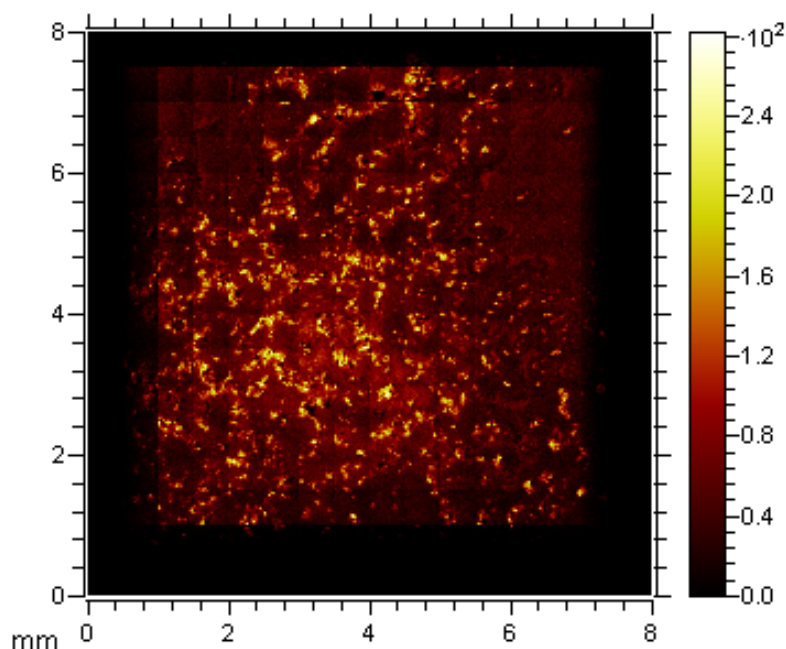


Figure 2.42: 2D mapping of protein related peaks of a Lys-PSS reference surface

Therefore, spectra originated from the two heterogeneous Lys-PSS reference surface were discarded from further PCA. By doing so, the statistical analysis becomes statistically not

significant but is expected to show a global tendency. The idea was to see if a separation could be obtained without accounting for the heterogeneity observed for those references.

PCA was performed both on positive (figure 2.43) and negative (figure 2.44) mode spectra using the same data pretreatment as presented earlier in this section. A separation within sample categories can be observed on those PCA graphs. Samples on which the same PPCs have been deposited are separated in both analysis mode. However, it seems that three Lys-PSS transfer spectra were separated close to their reference spectra in positive mode. As those three spectra comes from the same collector, this means that this surface have pretty similar spectra compared to the reference. This could mean that the chemical identity of the material present on both surface is very similar. Moreover, the collector is the one for which the transfer ion dose was the highest. Furthermore, the other Lys-PSS transfer spectra are separated in the bottom right corner of the PC1-PC2 graph due to substrate signals (28, 41, 43, 45 and 55) which goes in favor of the above mentioned hypothesis. Of course, this theory should be verified by running PCA on more samples of high ion dose transfer and references.

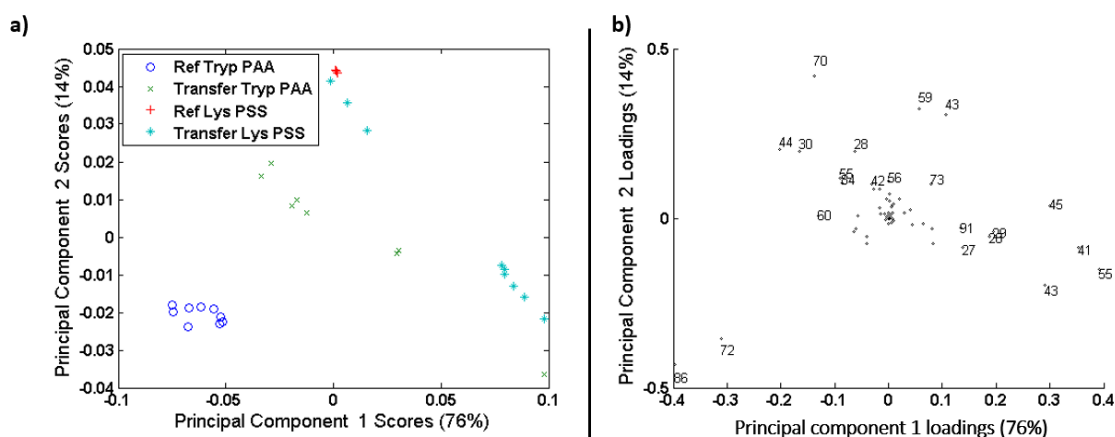


Figure 2.43: PCA of positive spectra from reference and transferred surfaces without heterogeneous Lys-PSS reference surfaces and Na peak: a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

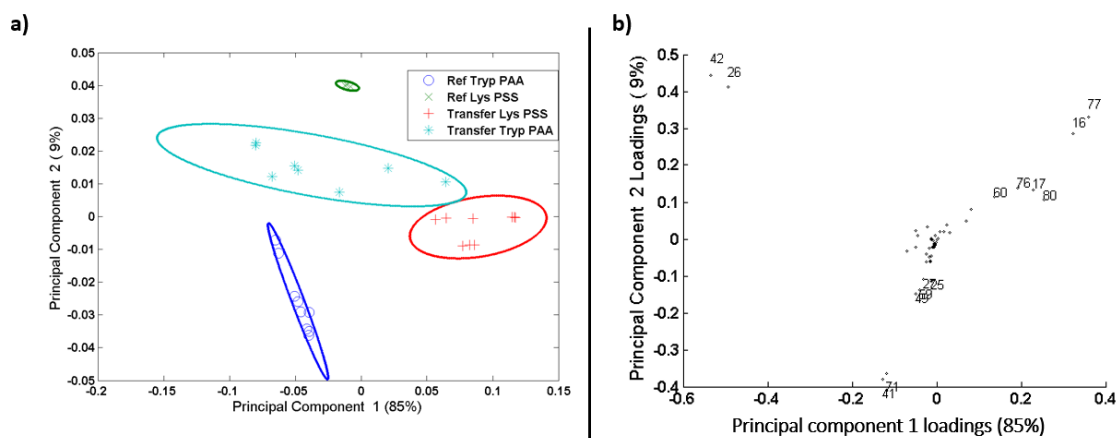


Figure 2.44: PCA of negative spectra from reference and transferred surfaces without heterogeneous Lys-PSS reference surfaces: a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

PCA were performed with peak lists that does not account for substrate signals but no clear separation within samples categories could be observed neither in positive (figure 2.45) nor in negative mode (figure 2.46).

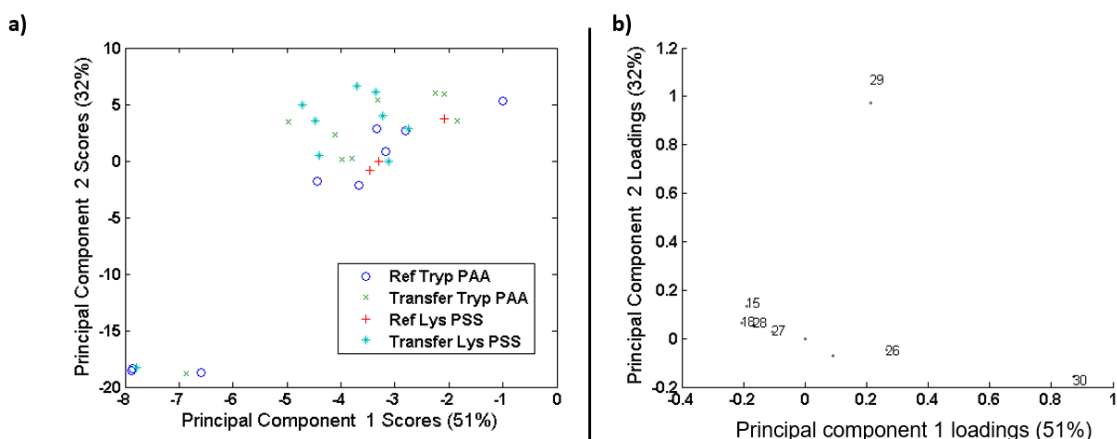


Figure 2.45: PCA of positive spectra from reference and transferred surfaces without Na or substrate related peaks : a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

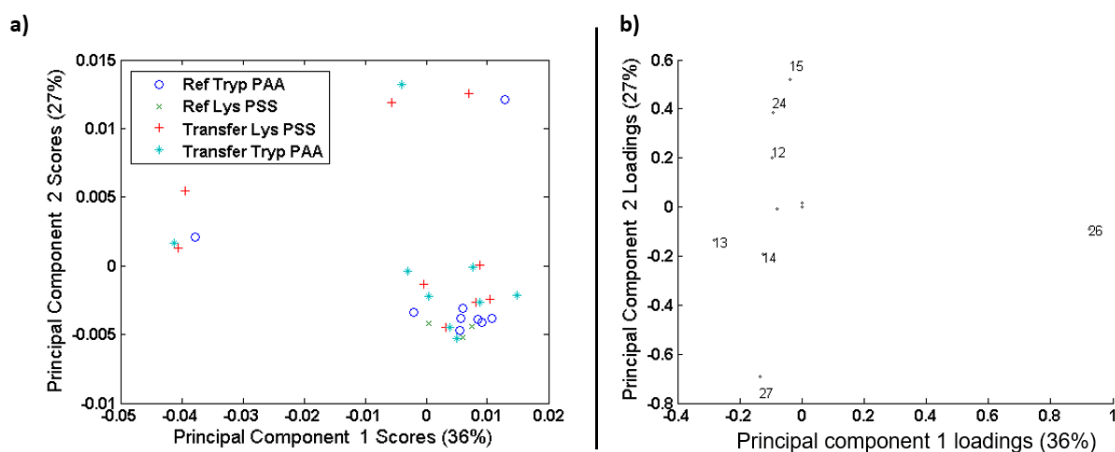


Figure 2.46: PCA of negative spectra from reference and transferred surfaces without substrate related peaks : a) scores of PC2 as a function of scores of PC1; b) loadings of PC2 as a function of loadings of PC1

Other PCA were achieved with peak list accounting only for protein/PE related peaks but no clear separation could be observed either.

PCA of different collector zones

PCA were performed in order to compare the spectra obtained from different collector zones for both PPCs transfers with their respective references. The rich zone should be more similar to the reference than the poor zone since the references are supposed to be homogeneous multi-layer PPCs interfaces while the poor zone spectra should be separated due to substrate related peaks.

PCA results for Lys-PSS spectra in positive mode are shown in figure 2.47. Note that the spectra from heterogeneous samples were discarded. As expected rich zone spectra are separated with reference spectra from intermediate and poor zone. The negative PC1 scores are originated from protein peaks (see table 2.9) while the positive PC1 scores are originated from substrate peaks (see table 2.10). Note that the rich zones spectra that are the closest to the reference were taken on the collector for which the ion dose was the greatest. This goes in favor of the hypothesis aforementioned. Other rich zone and the intermediate zone associated to this high ion dose transfer have a score close to zero. Their deposited protein layers seem therefore to be thinner than the reference surface. This intermediate zone would thus have deposited PPCs layers having the same thickness as the rich zone of collector made by lower ion dose transfer. As reference samples only comes from the only spin coated surface that is supposed to be homogeneous, these results have to be taken with caution.

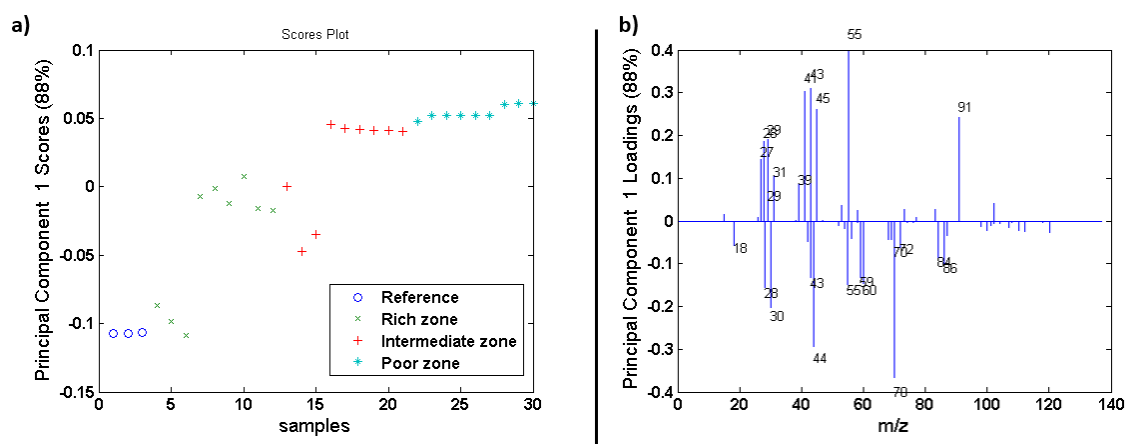


Figure 2.47: PCA of spectra from different zone collectors and reference of Lys-PSS in positive mode: a) samples as a function of their scores in PC1; b) loadings of peaks in PC1

Table 2.9: Peaks and their origins responsible of the negative scores observed in PC1 for Lys-PSS samples

m/z	ion	Origin
18	NH_4^+	Protein
30	CH_4N^+	Protein
44	$\text{C}_3\text{H}_4\text{N}^+$	Protein
60	$\text{C}_2\text{H}_6\text{NO}^+$	Protein
70	$\text{C}_4\text{H}_8\text{N}^+$	Protein
86	$\text{C}_5\text{H}_{12}\text{N}^+$	Protein

Table 2.10: Peaks and their origins responsible of the positive scores observed in PC1 for Lys-PSS samples

m/z	ion	Origin
28	Si^+	Substrate
29	C_2H_5^+	Contamination
41	C_3H_5^+	Contamination
43	C_3H_7^+	Contamination
45	SiOH^+	Substrate
55	C_4H_7^+	Contamination
91	C_7H_7^+	Contamination

Results for Tryp-PAA spectra in negative mode are shown in figure 2.48. Reference and 6 rich zones spectra have a negative score in PC1 originated from protein and PAA related peaks (see table 2.12). On the other hand, positive score in PC1 are originated from substrate signals (see table 2.11). Peaks related to suffer are most probably caused by

traces of piranha mixture left on the silicon wafer surface.

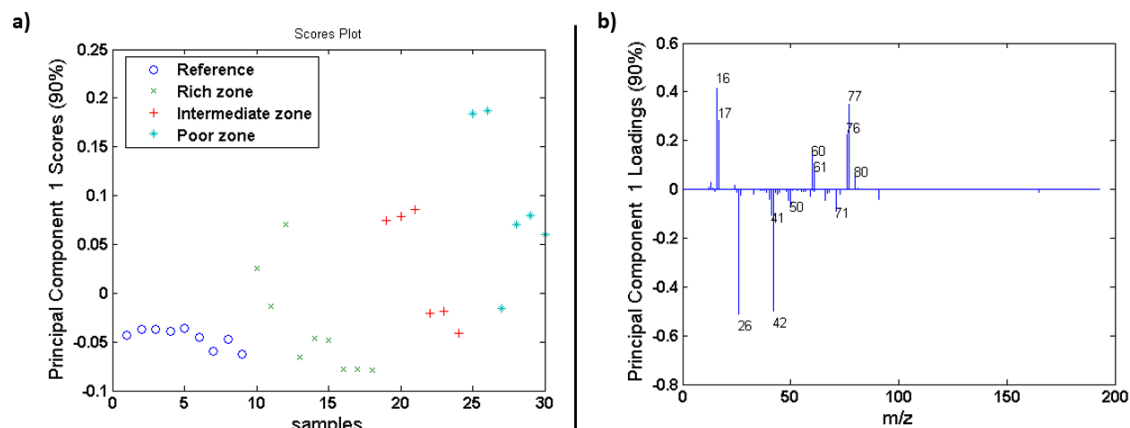


Figure 2.48: PCA of spectra from different zone collectors and reference of Tryp PAA in negative mode: a) samples as a function of their scores in PC1; b) loadings of peaks in PC1

Table 2.11: Peaks and their origins responsible of the positive scores observed in PC1 for Tryp-PAA samples

m/z	ion	Origin
16	O^-	Substrate
17	OH^-	Substrate
60	C_5^-	Contamination
61	$CHSO^-$	Piranha residue
76	CSO_2^-	Piranha residue
77	$CHSO_2^-$	Piranha residue
80	SO_3^-	Piranha residue

Table 2.12: Peaks and their origins responsible of the negative scores observed in PC1 for Tryp-PAA samples

m/z	ion	Origin
26	CN^-	Protein
41	C_2HO^-	Protein or PAA
42	CNO^-	Protein
50	C_3N^-	Protein
71	$C_3H_3O_2^-$	PAA

Summary of PCA results

Numerous PCA were performed on spectra obtained on both collector and reference PPCs surfaces. It was shown that Tryp-PAA and Lys-PSS interfaces tends to be separated by

PCA accounting for substrate signal which is more intense for Lys-PSS. Therefore transfer of Lys-PSS seems to present either a thinner or a more heterogeneous interface compared to Tryp-PAA transfer.

When the heterogeneous references of Lys-PSS were discarded, it seemed to appear that high ion dose transfer of Lys-PSS are similar to spin coated references.

PCA seems also to confirm that the deposited amount of PPCs decreases with the distance from the cluster ion beam impact point since poor zone spectra were separated due to substrate signals.

3 Conclusion

This master thesis falls within a project that, amongst others, aims at the development of a new solvent-free approach for the immobilization of proteins at interfaces, *i.e.* biofunctionalization of surfaces. The main objective of this work was to investigate the transfer of PPCs from one surface to another using large argon clusters (*in situ* a ToF-SIMS instrument). Three different protein-polyelectrolyte couples were used for this purpose: Ins-Chi, Lys-PSS and Tryp-PAA complexes

First, the synthesis of these complexes was investigated. Turbidimetry measurements were performed to optimize the protein/PE ratio. BCA supernatant test assessed that the complexation is efficient since more than 80% of the proteins complexed with their corresponding PE. The biological activity of Lys-PSS complexes was measured by enzymatic activity assays. It was shown that a part of the activity of lysozyme is lost upon complexation but can be recovered in solution by adding another positively-charged PE, PAH.

Second, Lys-PSS and Tryp-PAA complexes were transferred using a 10 keV Ar_{5000}^+ cluster ion beam. Targets were prepared by drop casting a PPCs suspension and collector were cleaned following a rigorous procedure.

After transfer experiments, collectors were characterized. ToF-SIMS 2D mapping revealed that protein material was successfully deposited in multilayers in the region of the collector close to the target. It was observed that the amount of protein material decreases with the distance from the target in all directions. It was also shown that the total quantity of transferred material and the covered area increase with increasing the primary ion dose. ToF-SIMS spectra were taken on different regions of the collector surfaces and on reference surfaces of PPCs. These different surfaces were compared by principal component analysis. It appeared that spectra taken in the region near the argon clusters impact point are more similar to their references than spectra taken further away from it, confirming the deposition gradient observed in 2D mapping. Moreover, spectra of high ion dose transfer of Lys-PSS seemed to be similar to their reference, meaning that both surfaces are chemically similar. This should be confirmed by repeating the analysis on more samples (references and transfers).

The following chapter will present some leads to continue the work done during this academic year.

4 Perspectives

This chapter will present some trails that could be explored following this thesis. Some of those were supposed to be investigated and experiments were already planned for this purpose. However, fate decided otherwise and those were cancelled. Some other could answer questions raised during the redaction of this thesis. Finally, some ideas that step outside of the framework of this thesis will be presented.

4.1 PPCs synthesis

Turbidimetry measurement: Ins-Chi at pH=5

The BCA supernatant assay showed that insulin and Chi complex at pH=5. However, as the protein/PE ratio was not optimized for those complexes, an arbitrary mass ratio of one was chosen. Therefore, it should be optimized to ensure a greater complexation.

Biological activity of complexed trypsin

The biological activity of trypsin could not be assessed during this thesis. Therefore, an activity test should be performed. However, it was found in the literature that trypsin is a pancreatic enzyme implied in the digestion of proteins that is most active in the pH range between 7 and 9 [55]. This is a problem since the complexation of trypsin with PAA happens at pH=4. By modifying the pH for the test, trypsin would be released from the complexes. Therefore, no complexation effect on the activity of trypsin should be observed. However, this would mean that Tryp-PAA complexes immobilized on a surface could potentially be released in solution by setting the pH at 7-9. This could be useful in order to quantify the amount of intact trypsin deposited during the transfer experiments. This idea is illustrated on figure 4.1.

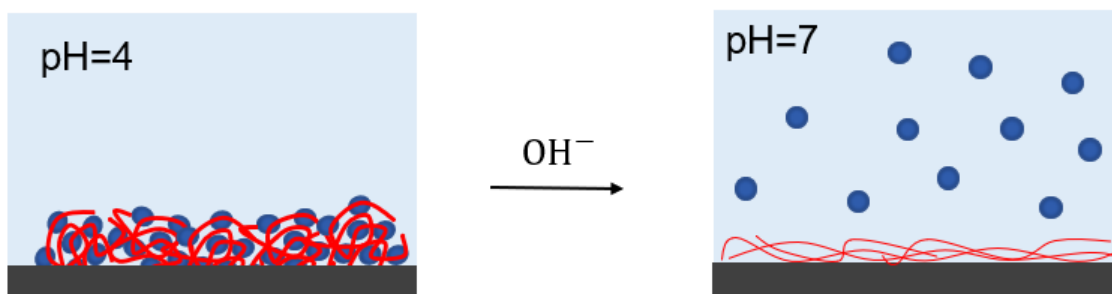


Figure 4.1: Releasing trypsin from deposited complex

Practically, the release of immobilized trypsin should first be confirmed using surface with known amount of trypsin. Those surfaces could be manufactured by dry dropping or spin coating Tryp-PAA suspension.

4.2 Transfer of PPCs

4.2.1 Optimization of the experimental set-up

Sample holder

The size of the impinging cluster ion beam has already been optimized before the start of this thesis as a compromise between current intensity and fragmentation (see section 2.2.2). However, other experimental parameters such as the impact angle of clusters or the placement of the collector, *i.e.* the range of angle collecting transferred molecules, did not follow an optimization process. Those are indeed imposed by the manufactured sample holder. Therefore, a new sample holder could be designed to assess what are the parameters that ensure the best transfer of intact proteins. A personal proposition for the design of a new sample holder is represented on figure 4.2.

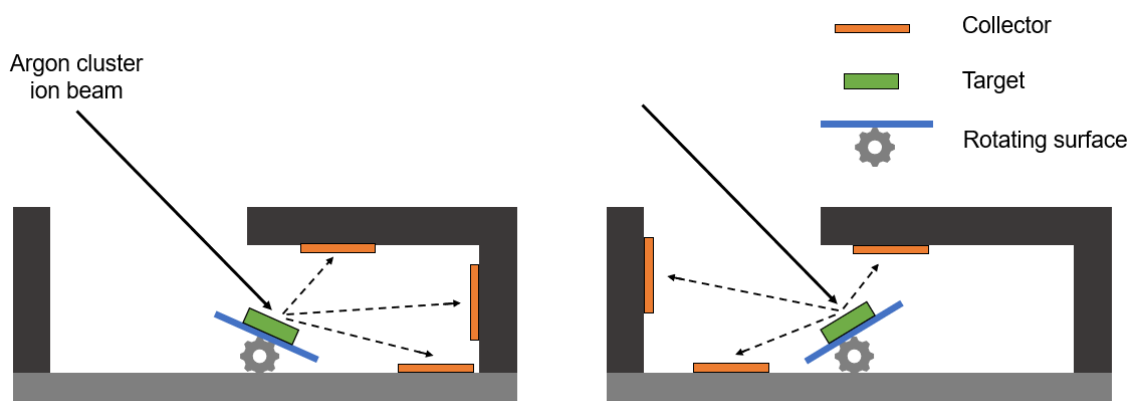


Figure 4.2: Idea of a new sample holder for transfer experiments

The sample holder is divided in two parts (light grey and dark grey). The first part is a flat

surface on which a rotating device is fixed. Target could be settled on it in order to test multiple impinging angle. The second part is kind of an half box with an hole for cluster ion beam to pass through. It can be removed in order to place collector on different spot. Sputtered material could thus be collected from a wide range of angles.

Ar-GCIB parameters

It was shown that the transferred amount of protein material is related to the ion dose impinging the target during transfer experiments. Therefore, if the current of argon clusters was increased, more protein could be transferred in shorter time. This could be done either by optimizing the Ar-GCIB parameters or by replacing the cluster sources with another one with higher current intensity for the same energy per atom ratio. Note that the current intensity should neither be too high in order to avoid surface charging issue.

4.2.2 Target manufacturing

From the start, PPC synthesis was optimized to ensure the greatest complexation possible in order to give the best protection for proteins. However, this assumption should be verified. There is a possibility that proteins cannot be transferred intact from the collector because those are too strongly anchored to the PPCs network. This could be verified by using targets made from PPCs solutions containing less PE than the optimized ratio. The resulting PPCs network on the targets would therefore be less dense and could potentially allow more transfer of intact proteins.

4.2.3 Transfer of Ins-Chi complexes

Once the optimization of the protein-PE ratio of Ins-Chi is achieved, those should be transferred. The main advantage transferring insulin compared to both lysozyme and trypsin is that its molecular ion, *i.e.* the whole insulin molecule, can be seen in ToF-SIMS measurement due to its lower mass. The success of the transfer could thus directly be assessed and fragmentation could easily be compared with that observed when transferring pure proteins.

4.3 Characterization of the transfers

Following transfer experiments, collectors were only analyzed by ToF-SIMS measurements mostly because it could be performed right after transfer experiments. However, other characterization methods were considered to confirm that intact proteins were transferred, to quantify those and assess the topography of transferred layer. Those would also enable the comparison between transfers of pure proteins and transfers of complexed proteins. In this section, a short description of those methods and their purpose is presented. Note that for each presented method, transfer experiments should be performed for the three PPCs of this thesis. As each transfer experiment take one day and the lab dispose of only one ToF-SIMS device for all searchers, the sample preparation for those experiment would take a lot of time.

4.3.1 Verify PCA results

PCA seemed to reveal that high ion dose transfer of Lys-PSS spectra are similar to spin coated reference spectra. Meaning that both surface are chemically similar. However, this result is based on spectra coming from only two surfaces and thus cannot really be interpreted since it is not statistically significant. Spectra taken from homogeneous references should be obtained. Spin coating less concentrated Lys-PSS suspensions, *e.g.* 1g/L instead of 5g/L, could potentially lead to homogeneous surface. Since spin coating aqueous suspensions is challenging, other immobilization methods, *e.g.* dry drop, could also be tested. Moreover, more high ion dose transfer of Lys-PSS should be performed.

4.3.2 Confirm the success of transfer experiments

Electrospray ionization mass spectrometry (ESI)

ESI is a soft ionization mass spectrometry method that uses electric energy to convert molecules in solution into ions in gaseous phase that are then pass through a mass analyzer. Compared to SIMS, the mass range of ions produced and detected is greater [56]. Therefore, the molecular ion of lysozyme and trypsin could be seen. Note that transferred proteins have to be released in a solution which could be tricky to realize.

Matrix assisted laser desorption ionization mass spectrometry (MALDI)

MALDI mass spectrometry is a mass spectrometry method that uses a laser to desorb molecules from a matrix that is sensitive to this laser. This method induces low fragmentation of large molecules [57]. The molecular ion of lysozyme and trypsin could thus be seen. Note that transferred proteins have to be mixed in a matrix before experimentation which could be a tricky step.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE is one of the most widespread technique used for protein analysis. Protein are reacted with sodium dodecyl sulfate (SDS), *i.e.* a negatively charged detergent, producing negatively charged complexes. The amount of SDS bound to proteins is roughly proportional to the protein size. Therefore, the overall charge of the complexes can be related to the protein molecular mass. Complexes are then separated on the basis of their charge and thus their size in a polyacrylamide gel on which an electrical field is applied. This method presents the advantages to be very sensitive and the use of detergent facilitate the desorption of immobilized proteins [58].

The success of the transfer could therefore be confirmed if the resulting polyacrylamide gel shows traces at mass corresponding to the molecular weight of transferred proteins.

4.3.3 Assess the morphology of the transferred layers: atomic force microscopy (AFM)

AFM is a microscopy method that uses a cantilever with a very small and acute tip to scan over a surface. A laser beam focused on the cantilever is reflecting to a detector. As it approaches the surface, the tip is deflected inducing slight deviation in the direction of the laser. This deviation is measured by the detector. As one of many modes of analysis available using AFM, the tip can be browsed over a surface in order to assess its topography. This method presents many advantages such as its nanoscale vertical resolution and the ability to work in air [59].

In the framework of this thesis, AFM could be used to assess the topography of transferred layer. Moreover, the thickness of the deposited layer could be assessed using the scratching mode of AFM. In this mode, the tip gently scratches in a soft layer (transferred layers) until it reaches a more rigid surface (silicon wafer) and then assess the depth of the hole dug.

4.3.4 Quantification of transferred material

Protein quantification assay

The amount of transferred protein material, *i.e.* intact and fragmented protein, could be assessed by using protein quantification assay. Unfortunately, the BCA assay is not suitable with silicon wafer. Fortunately, plenty protein quantification assays are available. Note that the protein material should first be released in solution since those tests are optimized for solutions.

Quartz crystal microbalance measurements (QCM)

A QCM measures the change in resonance frequency of a quartz crystal which varies with the addition/removal of small mass on its surface [60]. Transfer experiment could thus be performed using quartz crystal as a collector. The amount of transferred proteins could therefore be assessed by measuring the change of resonance frequency of the quartz crystal before and after transfer experiment.

Enzymatic assays

Contrary to previously mentioned method, enzymatic assays are specific to intact proteins having the right conformation. Therefore, the amount of intact transferred proteins from PPCs target could be compared the amount transferred from pure proteins target. This would prove, or not, that the use of PPCs is beneficial to transfer experiments.

By desorbing the immobilized PPCs layer, it could be possible to compare the number of intact proteins that are active with the total number of intact proteins transferred. It could also be interesting to compare the amount of intact proteins transferred with the total mass of protein material transferred.

4.3.5 Releasing immobilized proteins in solution

For some experimentation presented in this section, proteins need to be released in solution. This step might be difficult for methods that does not allow the use of detergent since those denature the proteins and thus interfere with some assays. It was proved that the addition of PAH release lysozyme from Lys-PSS complexes in solution. For tryp-PAA, modifying the surrounding pH should free trypsin in solution. The applicability of those ideas should first be assessed on known immobilized protein amount samples.

Another way to release proteins in solution would be to coat the silicon collectors with a flat water soluble layer, sugar for example. Transferred molecules would thus be deposited on a substrate that could easily be removed by placing it into water. Note that the choice of soluble layer should be made carefully in order to avoid interference with the tests. The idea is illustrated on figure 4.3.

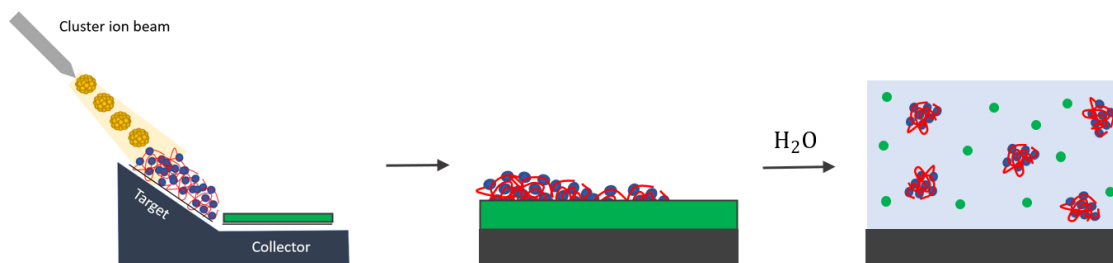


Figure 4.3: Transfer experiment on soluble layer

5 Materials and methods

This chapter presents a practical insight on the material and methods used during this thesis.

5.1 PPCs synthesis

5.1.1 Chemicals

Proteins

- **Lysozyme** : Lysozyme from chicken egg white purchased from *Sigma-Aldrich* (L6876)
 $M_w=14,3$ kDa
- **Trypsin** : Trypsin from bovine pancreas purchased from *Sigma-Aldrich* (T1426)
 $M_w=23,8$ kDa
- **Insulin** : Insulin human purchased from *Sigma-Aldrich* (I2643)
 $M_w=5,807$ kDa

Polyelectrolyte

- **Polystyren sulfonate** : Sodium polystyrene sulfonate standard purchased from *Scientific Polymer product* (CAT#620)
 $M_w=8$ kDa
- **Poly (acrylic acid)** : Poly (acrylic acid) (PAA) purchased from *Sigma-Aldrich* (323667)
 $M_w=1,8$ kDa
- **Deacetylated Chitosan**: Chitosan 95/5 purchased from *Heppe medical chitosan* (24700)
 $M_w=10-50$ kDa

5.1.2 Turbidimetry measurement

Proteins and PE solutions were mixed in different ratio in a 96 multi-well plate then the absorbance of those suspension was assessed in the 420-450nm wavelength range using a spectrophotometer SpectraMax iD3 (Molecular devices, Wokingham, UK). Measurements were performed in triplicate and basic statistical analysis were achieved (standard deviation and confidence interval).

5.1.3 BCA assay

Protein amount was assessed by bicinchoninic acid assay (BCA, Thermo scientific, Rockford, USA). A 96-well plate was loaded with 20 μL either of protein solutions in different concentration for the calibration curve or the supernatant from centrifuged PPCs suspension. Afterwards, 200 μL of BCA working reagent was added in each well. Then the plate was then incubated for 1 hour at 60°C before absorbance was measured at 562nm using a spectrophotometer SpectraMax iD3 (Molecular devices, Wokingham, UK). Measurements were performed in triplicate and basic statistical analysis were performed (standard deviation and confidence interval).

5.1.4 Enzymatic activity assay of Lys-PSS complexes

A 96 well plate was loaded with 20 μL of either lysozyme in different concentration for the calibration curve or Lys-PSS complexes. Then the volume of the well is adjusted to 180 μL by adding an acetate buffer (10mM) at pH 5 and ionic strength adjusted to 10mM with NaCl. After that, each well are filled with 20 μL of 4-methylumbelliferyl β -D-N,N,N-triacetylchitotrioside (0.1 g/L, Steinheim, Germany) in dimethyl sulfoxide /water 2:1 (v:v) solution. The multi well plate is then incubated 24h at 40°C. Afterwards, 180 μL are moved to a black 96 well plate and mixed with 50 μL of glycine buffer (50mM) at pH 10,6 (adjusted with NaOH) in order to quench the reaction. Ultimately, fluorescence measurements (SpectraMax iD3 (Molecular devices, Wokingham, UK)) are performed with excitation/emission wavelengths set at 360/450 nm respectively. Measurements were performed in triplicate and basic statistical analysis were achieved (standard deviation and confidence interval).

5.2 PPCs transfer

5.2.1 Surface cleaning procedure

Piranha mixture

Silicon wafers were immersed 15 minutes in a 3:1 (v:v) mixture of concentrated sulfuric acid and 30% hydrogen peroxide. Wafers were then rinse 10 times with ultra pure water.

Uv/ozone

Silicon wafers were exposed 15 minutes to UV-ozone using a UVOcleaner Model 42 Series(Jelight Company Inc, California, USA).

5.2.2 Transfer procedure

The cluster ion beam used for the transfers is produced by the Ar-GCIB source operating at 10keV with a cluster distribution centered on Ar_{5000}^+ . The current intensity was measured before and after transfer experiment and was always lightly oscillating around 1 nA. The beam raster was fixed at 128 x 128 data points over an area of 700 x 700 μm^2 .

5.3 Transfer characterization

5.3.1 Reference surface manufacturing

PPCs suspensions were made with 5g/L of proteins and the corresponding optimized amount of PE. Those solutions were spin coated 60 seconds at 3000 rpm using a Model WS-400B6NPP/LITE (Laurell technologies corporation, Pennsylvania, USA).

5.3.2 ToF-SIMS 2D mapping analysis

In this mode, the ion beam browses automatically the surface taking multiple squared spectrum on the surface. Those are gathered in order to build an image of the whole collector. A Bi_5^+ 30keV ion beam having a current intensity of about 0,1pA, with a raster of 128 x 128 data points over $500 \times 500 \mu\text{m}^2$ was used. A single scan was performed, but 2 frames were taken on every $500 \times 500 \mu\text{m}^2$ patch to increase signal intensity. Both positive and negative spectrum were analyzed. Mass resolution $\frac{m}{\Delta m} \approx 4300$ at 30 m/z (corresponding to CH_4N^+) was maintained for spectra acquisition.

5.3.3 Spectra acquisition for PCA

60 seconds spectra of $500 \times 500 \mu\text{m}^2$ patches were taken on different region of the collector and on reference samples using a Bi_5^+ 30keV ion beam having a current intensity of about 0,1pA, with a raster of 128 x 128 data points over $500 \times 500 \mu\text{m}^2$. Both positive and negative spectrum were analyzed. Mass resolution $\frac{m}{\Delta m} \approx 4300$ at 30 m/z (corresponding to CH_4N^+) was maintained for spectra acquisition.

5.3.4 Principal component analysis

This section presents the different peak list used in PCA.

Table 5.1: Peak list used in PCA of protein surfaces

m/z	ion	m/z	ion	m/z	ion
18,0348	NH_3^+	61,0115	$\text{C}_2\text{H}_5\text{S}^+$	91,0529	C_7H_7^+
22,9914	Na^+	68,0508	$\text{C}_4\text{H}_6\text{N}^+$	92,0504	$\text{C}_6\text{H}_6\text{N}^+$
27,0229	C_2H_3^+	69,0357	$\text{C}_4\text{H}_5\text{O}^+$	97,0691	$\text{C}_6\text{H}_9\text{O}^+$
28,0189	CH_2N^+	70,0714	$\text{C}_4\text{H}_8\text{N}^+$	98,024	$\text{C}_4\text{H}_4\text{NO}_2^+$
29,0385	C_2H_5^+	70,0302	$\text{C}_3\text{H}_4\text{NO}^+$	98,0633	$\text{C}_5\text{H}_8\text{NO}^+$
30,0355	CH_4N^+	71,0126	$\text{C}_3\text{H}_3\text{O}_2^+$	100,0835	$\text{C}_4\text{H}_{10}\text{N}_3^+$
39,0225	C_3H_3^+	72,0874	$\text{C}_4\text{H}_{10}\text{N}^+$	101,0812	$\text{C}_4\text{H}_{11}\text{N}_3^+$
41,0386	C_3H_5^+	73,0635	$\text{C}_2\text{H}_7\text{N}_3^+$	102,062	$\text{C}_4\text{H}_8\text{NO}_2^+$
42,0349	$\text{C}_2\text{H}_4\text{N}^+$	74,0645	$\text{C}_3\text{H}_8\text{NO}^+$	107,0509	$\text{C}_7\text{H}_7\text{O}^+$
43,054	$\text{C}_2\text{H}_3\text{O}^+$	76,0242	$\text{C}_2\text{H}_6\text{SN}^+$	110,0819	$\text{C}_5\text{H}_8\text{N}_3^+$
53,0383	C_4H_5^+	77,0366	C_6H_5^+	112,0797	$\text{C}_6\text{H}_{10}\text{NO}^+$
54,0346	$\text{C}_3\text{H}_4\text{N}^+$	81,041	$\text{C}_4\text{H}_5\text{N}_2^+$	112,0401	$\text{C}_5\text{H}_6\text{NO}_2^+$
55,0194	$\text{C}_3\text{H}_3\text{O}^+$	82,0608	$\text{C}_4\text{H}_6\text{N}_2^+$	115,0537	$\text{C}_4\text{H}_7\text{N}_2\text{O}_2^+$
56,0511	$\text{C}_3\text{H}_6\text{N}^+$	83,0543	$\text{C}_5\text{H}_7\text{O}^+$	118,0649	$\text{C}_8\text{H}_8\text{N}^+$
57,0558	$\text{C}_3\text{H}_7\text{N}^+$	84,0462	$\text{C}_4\text{H}_6\text{NO}^+$	120,0856	$\text{C}_8\text{H}_{10}\text{N}^+$
58,0654	$\text{C}_3\text{H}_8\text{N}^+$	84,0876	$\text{C}_5\text{H}_{10}\text{N}^+$	127,0985	$\text{C}_5\text{H}_{11}\text{N}_4^+$
58,0294	$\text{C}_2\text{H}_4\text{NO}^+$	86,1072	$\text{C}_3\text{H}_4\text{NO}_2^+$	130,0676	$\text{C}_9\text{H}_8\text{N}^+$
59,0501	$\text{C}_3\text{H}_7\text{O}^+$	87,0613	$\text{C}_3\text{H}_7\text{N}_2\text{O}^+$	159,1011	$\text{C}_{10}\text{H}_{11}\text{N}_2^+$
60,0491	$\text{C}_2\text{H}_6\text{NO}^+$	88,0449	$\text{C}_3\text{H}_6\text{NO}_2^+$	170,0696	$\text{C}_{11}\text{H}_8\text{NO}^+$

Table 5.2: Peak list used in PCA of PPCs positive spectra

m/z	ion	m/z	ion	m/z	ion
15.0231	CH_3^+	46.9876	CH_3S^+	77.0367	$C_6H_5^+$
18.035	NH_4^+	51.0222	$C_4H_3^+$	83.0524	$C_5H_7O^+$
22.9924	Na^+	52.0167	$C_3H_2N^+$	84.0461	$C_4H_6NO^+$
26.0144	$C_2H_2^+$	53.0383	$C_4H_5^+$	84.0855	$C_5H_{10}N^+$
27.023	$C_2H_3^+$	54.035	$C_3H_4N^+$	86.1036	$C_5H_{12}N^+$
28.0186	CH_2N^+	55.0186	$C_3H_3O^+$	87.0593	$C_3H_7N_2O^+$
29.0021	CHO^+	55.0543	$C_4H_7^+$	91.0527	$C_7H_7^+$
29.0386	$C_2H_5^+$	56.0509	$C_3H_6N^+$	98.0229	$C_4H_4NO_2^+$
30.035	CH_4N^+	58.0297	$C_2H_4NO^+$	100.0903	$C_4H_{10}N_3^+$
31.0179	CH_3O^+	58.0656	$C_3H_8N^+$	101.0785	$C_4H_{11}N_3^+$
38.0143	CH_3Na^+	59.0499	$CH_5N_3^+$	102.0453	$C_4H_8SN^+$
39.0225	$C_3H_3^+$	60.0473	$C_2H_6NO^+$	102.129	$C_6H_{16}N^+$
27.9755	Si^+	68.0507	$C_4H_6N^+$	104.0553	$C_4H_{10}SN^+$
41.0387	$C_3H_5^+$	69.0349	$C_4H_5O^+$	107.0501	$C_7H_7O^+$
42.0339	$C_2H_4N^+$	70.0306	$C_3H_4NO^+$	108.0474	$C_5H_6N_3^+$
43.0543	$C_3H_7^+$	70.0687	$C_4H_8N^+$	110.0792	$C_5H_8N_3^+$
43.029	$C_2H_3O^+$	72.0824	$C_4H_{10}N^+$	112.0782	$C_6H_{10}NO^+$
44.0508	$C_2H_6N^+$	73.0642	$C_2H_7N_3^+$	118.0651	$C_8H_8N^+$
44.0129	CH_2NO^+	74.0631	$C_3H_8NO^+$	120.0842	$C_8H_{10}N^+$
44.9787	$SiOH^+$	76.0237	$C_2H_6SN^+$	127.0962	$C_5H_{11}N_4^+$

Table 5.3: Peak list used in PCA of PPCs negative spectra

m/z	ion	m/z	ion	m/z	ion
12.0006	C^-	39.9952	C_2O^-	60.0006	C_5^-
13.0084	CH^-	40.0205	$C_2H_2N^-$	59.9706	SiO_2^-
14.016	CH_2^-	41.0066	C_2HO^-	60.9763	$CHSO^-$
15.0255	CH_3^-	42.0031	CNO^-	61.0085	C_5H^-
15.9958	O^-	43.02	$C_2H_3O^-$	65.9999	C_3NO^-
17.0036	OH^-	44.0156	CH_2NO^-	67.0221	$C_4H_3O^-$
24.0006	C_2^-	44.9987	CHO_2^-	68.0162	$C_3H_2NO^-$
25.0091	C_2H^-	48.0005	C_4^-	71.0192	$C_3H_3O_2^-$
26.0065	CN^-	49.0086	C_4H^-	73.0082	C_6H^-
27.0247	$C_2H_3^-$	50.0048	C_3N^-	76.9737	$CHSO_2^-$
32.9819	HS^-	51.0245	$C_4H_3^-$	75.9682	CSO_2^-
36.0006	C_3^-	53.0046	C_3HO^-	79.9606	SO_3^-
37.0083	C_3H^-	55.0201	$C_3H_3O^-$	80.9633	SO_3H^-
38.0036	C_2N^-	56.0163	$C_2H_2NO^-$	90.9924	$C_2H_3SO_2^-$
38.016	$C_3H_2^-$	56.9812	C_2HS^-	165.0254	$C_8H_5O_4^-$
39.0115	C_2HN^-	59.018	$C_2H_3O_2^-$	183.0149	$C_8H_7SO_3^-$

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Tailoring surfaces using protein-polyelectrolyte complexes and cluster ion beams

By Thomas Daphnis

The biofunctionalization of surfaces, i.e. immobilization of proteins at interfaces, is a cornerstone technology in diverse fields of application. Deposition from aqueous protein solutions has proven efficient for some utilizations. However, such methods suffer from several drawbacks, reducing the range of achievable functions. Among those disadvantages, the inherent drying step often causes problems such as the loss of 3D conformation of immobilized proteins, a lack of control on the spatial distribution of the engineered layer, etc. Therefore, recent research aims at the development of new methods outpacing the drawbacks of current ones.

This master thesis investigates the elaboration of a new protein immobilization technique that uses large argon clusters produced by a Time-of-Flight Secondary-Ion Mass Spectrometer (ToF-SIMS) in order to transfer proteins from one surface rich in proteins to another one clean. The idea is to take advantage of the low energy per atom of such clusters to induce the sputtering of intact proteins under ultra-high vacuum. For the purpose of granting proteins with an extra protection, those were complexed with oppositely-charged polyelectrolytes (PE) resulting in protein-PE complexes (PPCs). Three different PPCs containing protein of different sizes were considered during this thesis.

First, the synthesis of the different PPCs was investigated with the aim of optimizing the different parameters of the complexation. Moreover, the efficiency of the PPCs production method and the activity of complexed proteins were assessed. Using optimized parameters, more than 80% of the proteins were found to be complexed with their corresponding PE. The complexed proteins tend to lose part of their activity, but it was shown that this one is retrievable. Second, PPCs were transferred with a 10keV Ar_{5000}^+ cluster ion beam inside the ToF-SIMS chamber. Finally, the collecting surface was characterized by ToF-SIMS and multivariate analysis. It appeared that protein material was successfully transferred with a semi-circular deposition pattern. Other characterization methods that aim to determine whether proteins were transferred intact and to quantify their amount and biological activity are presented.

