WORK END OF MASTER

MASTER IN MATERIAL SCIENCE

PRODUCTION OF MULTIFUNCTIONAL SURFACES WITH ANTIBACTERIAL AND CELL ADHESIVE PROPERTIES

Report

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Abstract

Nowadays, titanium implants are commonly used for dental and orthopaedic repair or to replace bone defects due to aging or diseases. Despite the good biocompatibility of titanium, low osseointegration of the implant with the surrounding tissues and bacterial infections may cause the rejection and failure of the prosthesis.

To address this problem, this research project is focused on the biofunctionalization of titanium surfaces with a multifunctional approach: the deposition of polyethylene glycol to achieve an antifouling effect (which inhibits bacterial adhesion but also cell attachment) and the immobilization of a peptidic platform. This peptidic platform is composed of the RGD sequence (Arg-Gly-Asp), which has cell adhesion properties, and the hLF1-11 sequence that has bactericidal potential.

The central theme of this research is to characterize physicochemically the functionalized surface, and evaluate its performance in terms of cell adhesion and antibacterial efficiency. Thus, the surface will be characterized by contact angle, SEM, interferometry and FTIR measurements. The efficiency of the cell adhesion will be evaluated with LDH and immunofluorescence assays. The antibacterial effect will be evaluated by counting bacterial colonies on agar and Alamar Blue assays.

The physicochemical characterization of the samples proved that the deposition of PEG and the functionalization were correctly done on the titanium. The LDH and immunofluorescence assays showed an important improvement in cell adhesion for the samples functionalized with the peptidic platform compared to control titanium. As expected, no cell adhesion was observed on the samples only coated with PEG.

The antibacterial activity of the coatings gave interesting results with the agar assay. Moreover, the Alamar Blue assay gave encouraging results, with a reduction on the attachment of the bacterial strain *E. coli* on the surface of functionalized titanium.

Summary

1. Abbreviations

Ti: Titanium

PEG: Poly(ethylene Glycol) (In the project the PEG used is bis(3-aminopropyl) terminated)

PTF: peptidic platform with RGD and hLF1-11

RGD: Sequence of amino acids L-arginine, L-glycine, L-aspartic acid

hLF1-11: Human lactoferrin-derived (antibacterial peptide)

CL: crosslinker 3-Maleimidopropionic acid N-hydroxysuccinimide ester 99% between PEG and PTF

FN: Fibronectin

SEM: Scanning electron microscopy

FIB: Focused ion beam

FTIR: Fourier transform infrared spectroscopy

Ra: Average roughness

Rsk: Roughness skewness

Rku: Roughness kurtosis

BSA: Bovine serum albumin

FBS: Foetal bovine serum

PBS: Phosphate buffered saline

LDH: D-Lactate dehydrogenase

SAOS-2: Sarcoma osteogenic cells

M-PER: Mammalian protein extraction reagent

Gly: Glycine

DAPI: 4,6-diamidino-2-phenylindole

AB: Alamar Blue

PFA: Paraformaldehyde

DMSO: dimethylsulfoxyde

On the graphs, **PEG** means **Ti+PEG**, **CL** means **Ti+PEG+CL,** and **PTF** means **Ti+PEG+CL+PTF.**

2. Introduction

2.1 State of the art

Nowadays, it is common to implant metal devices in the human body for medical issues. However, the biocompatibility of these implants does not exclude the risk of infection and rejection of the implant by the body. In particular, bacterial infection represents one of the main causes of implant failure. Those infections are due to bacteria agglomerating on the prosthesis and creating a biofilm.

Several possibilities are available to reduce or inhibit bacterial adhesion and proliferation on the substrate, representative examples are shown below:

- Deposition on the substrate of polymeric systems, such as capsules, containing antibiotics. The antibiotic will then diffuse to kill the bacteria. This process is commonly used nowadays. However bacteria can develop resistance to the antibiotic, thus reducing its efficiency. **[1]**

- Biofunctionalization with a bactericidal substance, such as the antimicrobial peptide hLF 1-11, a peptide derived from the antibacterial protein lactoferrin. This sequence can be synthesized and retains the antibacterial properties of the whole protein. It causes a disruption of the bacterial membrane, thereby increasing the permeability of the bacteria, which is fatal for the bacteria.
  This option has the advantage that bacteria do not develop resistance to antimicrobial peptides.**[2][3]**

- Coating with an anti-adhesive substance like Polyethylene Glycol (PEG). If the substrate is coated with this polymer, bacteria will not be able to adhere on it, thus the formation of a biofilm is not possible and the infections due to bacteria will be avoided.

However, bacterial infections are not the only problem involved in medical implants. Indeed, a poor osseointegration of the implant with bone may result in poor mechanical fixation and loosening of the implant, which can lead to hyper sensibility, internal bleedings, or other issues that need the intervention of a surgeon. **[4]**

To improve the integration and functionality of the implant, the use of biocompatible and bioactive materials that facilitate the adhesion and growth of bone-forming cells is an emerging and promising approach.

Bone formation begins with the apparition of an ossification center in the fibrous tissue membrane (Fig(1)). This center is composed of mesenchymal cells, which differentiate into osteoblasts.

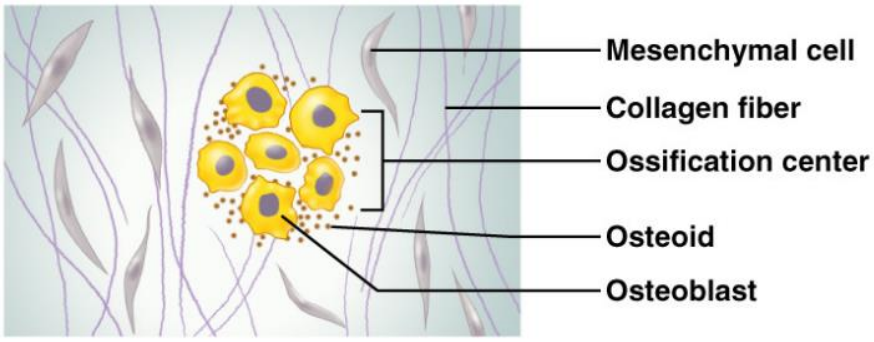

Figure 1: Scheme showing the first step of intramembranous ossification: differentiation in osteoblasts and osteoid production

Those osteoblasts will produce osteoids, which will mineralize, forming the bone matrix. The trapped osteoblasts in the matrix differentiate into osteocytes (fig(2)). They regulate the mineralization and produce collagen fiber, improving the mechanical resistance of the bone.**[6][7]**

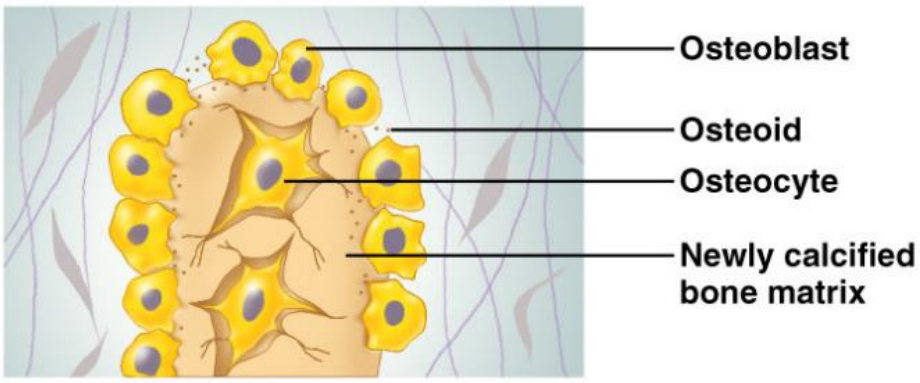

Figure 2: Scheme showing the first step of intramembranous ossification: mineralization of osteoid in bone matrix, and osteocytes differentiation [5]

Thus, the best way to have a good osseointegration of implants is to improve the adhesion of osteoblasts on the surface of implant.

Several materials are commonly used for bone implants, such as stainless steel, cobalt based alloys and commercially pure titanium and titanium-based alloys. These materials must be not only biocompatible but also have an elasticity (Young modulus) similar to that of bone and good mechanical properties. Most metals can be used in the human body but only in small amounts. Indeed, the corrosion of metals and their disintegration will affect the mechanical properties of the implant. Moreover the corrosion is harmful for the surrounding tissues. That is why the titanium, which has a passive oxide layer, is a good biocompatible material. It is the most commonly used for implants.

The inert behavior (biocompatibility) of titanium is a major reason of its common use in prosthesis. However it is not the only advantage of titanium for this application. In the following table some mechanical properties of materials in comparison to bone can be seen:

Property/material	Stainless steel	Cobalt-Chrome alloy	Titanium	Average bone
Elastic modulus (GPa)	220	220	110	15
Density (kg/m^3)	7850	8800	4500	1500

Table 1: Mechanical properties of materials [8][9][10]

On this table can be seen that in spite of being almost 10 times higher than the bone's modulus, the elastic modulus of titanium is still the most corresponding to the bone, in comparison with stainless steel. Another factor to take into account for bone replacement is the density. Indeed, bones have a low density, which permits to lower the energy consumed by the body when it is in movement. A replacement with a material too heavy would cause an inefficient use of energy for the body. On the contrary, a material like titanium is lighter than stainless steel thus it is more accurate for bone replacement applications.

Although materials like stainless steel have a good anticorrosion behavior, the material with the closest mechanical properties with bone is titanium. However, titanium alloys have a relatively poor shear strength, thus it is not the best material for bone screws and plates applications which are submitted to high shear stresses

To improve the osseointegration of implant materials, several options are available:

1) Modification of the surface morphology. For example, increasing the roughness of the implant to a certain level improves the differentiation of osteoblasts and bone matrix formation. It can be done by acid etching, sandblasting, etc.**[11]**

2) Biofunctionalization of implant's surface with cell adhesive molecules, which will improve the adhesion of cells on the surface.

- Hydroxyapatite is a calcium phosphate that constitutes the mineral phase of bone, and thus osteoblasts have a good adhesion on it. A coating of hydroxyapatite can improve the adhesion of cells on the implant.

- Another possibility is to deposit by physisorption or covalent attachment an extracellular matrix (ECM) protein with cell adhesive potential like fibronectin.

- It is also possible to synthetize peptides derived from ECM proteins, like the RGD sequence, which have the capacity to promote cell adhesion and attach them covalently to the substrate. The RGD peptide (Arg-Gly-Asp sequence as shown in fig[3]) is present in fibronectin and many other ECM proteins. This sequence is recognized by integrins and can interact with them. Integrins are a family of transmembrane cell adhesive protein, which link the cell to the ECM. Thus, if the RGD peptide is immobilized on the substrate, the integrin (which is on the cell surface) will interact with it and mediate cell adhesion on the substrate.**[12]**

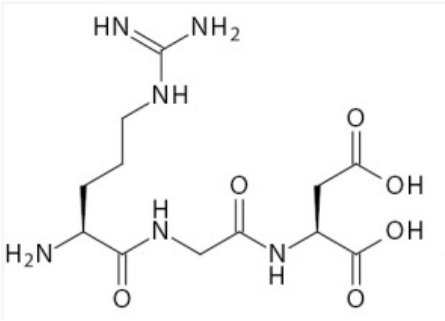

Figure 3: RGD molecule [13]

Recently, some studies have been done studying combinations of peptides (bioactive sequences) with different biological functions in order to obtain multifunctional surfaces. For example, a peptide-based platform with the capacity to present two distinct peptide sequences (RGD and PHSRN motifs) was recently described to improve osteoblast adhesion on titanium.

The structure of the platform is the following:

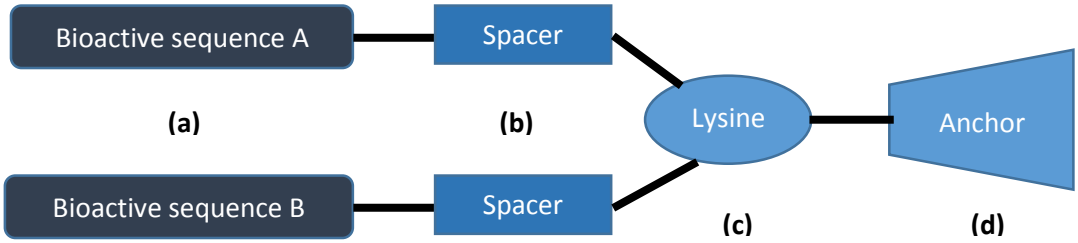

Figure 4: Scheme of peptidic platform structure

The bioactive sequences **(a)** are linked to a spacer **(b)**. This spacer is a molecule, which will give the optimal spacing and orientation of the peptide sequences. Then a lysine **(c)**, which is a branched amino acid, links the spacers with an anchoring group **(d)**. This anchor should provide a chemo-selective and stable binding of the molecule to the substrate.**[14]**

2.2 Objectives of the project

The aim of this project is to produce, characterize and study the efficiency of a multifunctional coating to functionalize titanium. This coating aims to improve its cell adhesive properties and simultaneously inhibit bacterial infection. The coating is based on the following parts:

1) A PEG coating for an anti-adhesive (anti-fouling) effect. The coating will avoid the adhesion of bacteria on the substrate. However, neither osteoblast cells will be able to adhere on it.

2) Functionalization with a multifunctional peptidic platform with hLF 1-11 and RGD. The hLF 1-11 peptide will kill the bacteria left on the substrate, and the RGD will rescue the cell adhesion properties to the surface. The fig (5) schematically shows the multifunctionality of the coating.

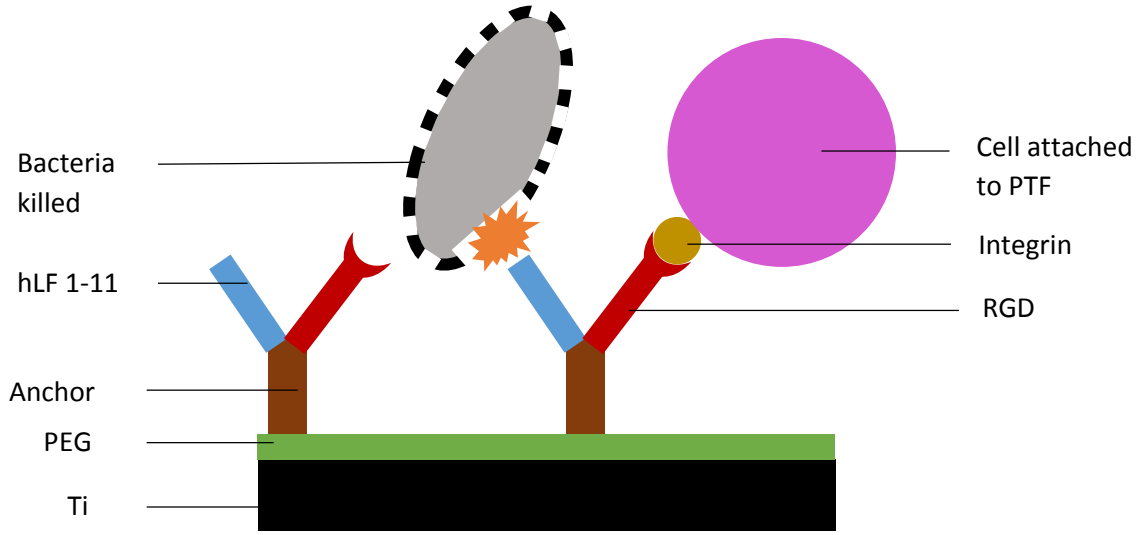

Figure 5: Scheme of the multifunctional surface with PTF and PEG on Ti

The structure of the peptidic platform used in this study is the following:

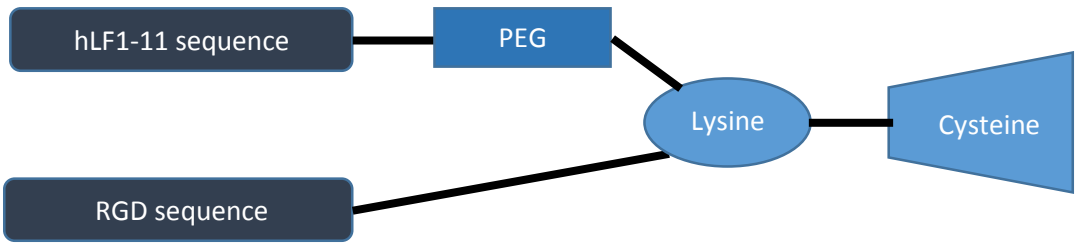

Figure 6: Scheme of peptidic platform structure used in the project

The spacer between the RGD and the lysine is a PEG unit, and the anchor is a cysteine, which will be used to anchor the platform to the PEG coating present on the surface of Ti. The PEG spacer has in this

case both an antifouling role (antibacterial) and gives the optimal vertical distance between the RGD and the material. Indeed, if the RGD is too close to the material, it will not be accessible to the cells. **[15]**

The detailed chemical structure of the platform used in this project is shown in Figure (7).

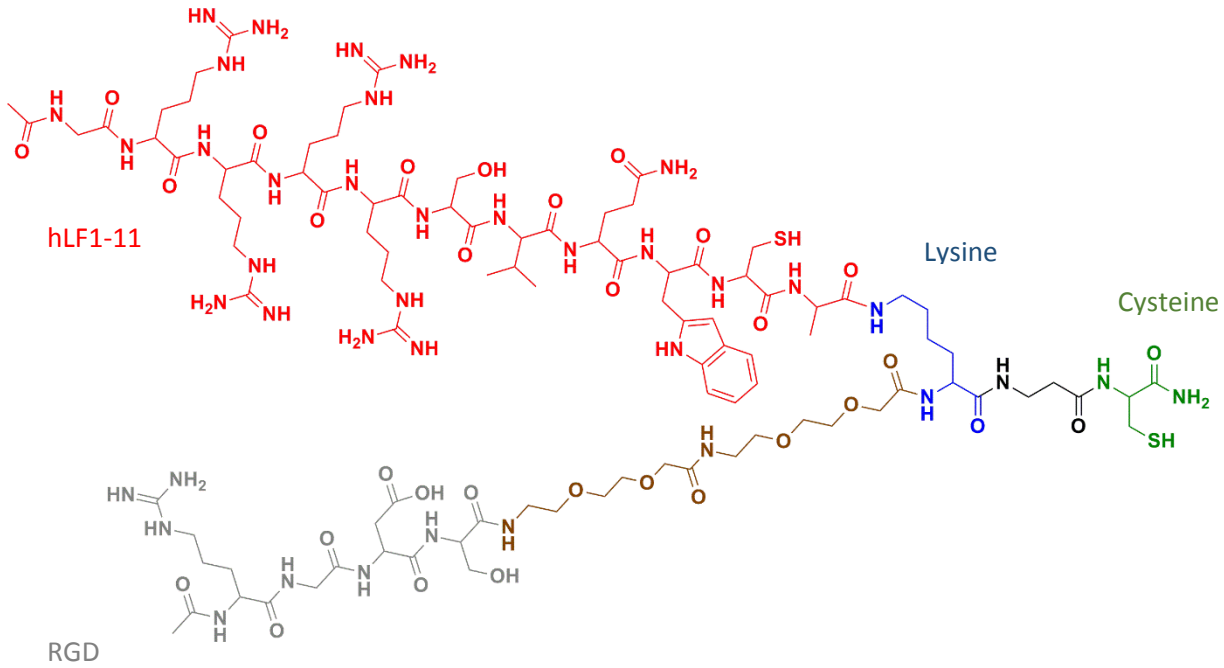

Figure7: Chemical structure of the peptidic platform used for the project [16]

After the functionalization, the surfaces will be physicochemically characterized by SEM, interferometry, contact angle and FTIR.

Its biological efficiency will be studied according to two parameters:

- Adhesion of osteoblast-like cells with LDH assay and by immunofluorescence
- The bactericidal effect on two bacterial strains with agar assays and Alamar Blue.

3. Material and methods

In this part the preparation of the samples, the principle of the equipments used for the experiments, and the protocols of the experiments done during the project will be explained.

It is important to remember that on the graphs, **PEG** means **Ti+PEG**, **CL** means **Ti+PEG+CL,** and **PTF** means **Ti+PEG+CL+PTF.**

3.1 Sample preparation

3.1.1 Hot mounting

The samples are small disks of commercially pure Grade 2 titanium (10 mm diameter, 2 mm thickness). This category of titanium is a good compromise between corrosion resistance and mechanical strength. Moreover, it has a good weldability. It is composed of the following impurities:

% element	N	C	H	Fe	O
Atomic percentage maximum	0.03	0.1	0.015	0.3	0.25

Table 2: Chemical composition of titanium grade 2[10]

Before polishing, the first step is a process of hot mounting, to be able to polish the samples easier. Five samples at a time are put on a piston. This piston is lowered and Bakelite powder is added in the gap (2 spoons of powder approximately). Then the machine is closed and the process can start:

- Strength of 15 kN
- Heating 5 min at 180°C
- Cooling 4 min

The machine used is a Struers Labopress-3.
The configuration of the samples after hot mounting is shown in fig (8):

Figure 8: Hot mounted samples of Ti in Bakelite with Struers Labopress-3

3.1.2 Polishing

For the polishing several SiC grinding papers are used to get a smooth surface (flat and with the less scratches possible) as shown below:

Paper	Speed (rpm)	Force (N)	Time (min)	Direction of sample rotation
P800	150	10	15	Opposite of paper
P1200	150	10	15	Opposite of paper
P2500	150	10	20	Opposite of paper
Solution Al_2O_3 1µm	300	10	60	Opposite of paper
Solution Al_2O_3 0.5µm	300	10	60	Opposite of paper

Table 3: List of polishing steps

For the P800 to P2500, the paper is changed for a new one after 7 min because it gets damaged quickly. Originally the force used was 15 N, and speed was 300 rpm also for P800 to P2500. But the papers were damaged, so the new conditions were used to avoid this defect.

The machine used is a Buehler Phoenix 4000 shown in fig(9)):

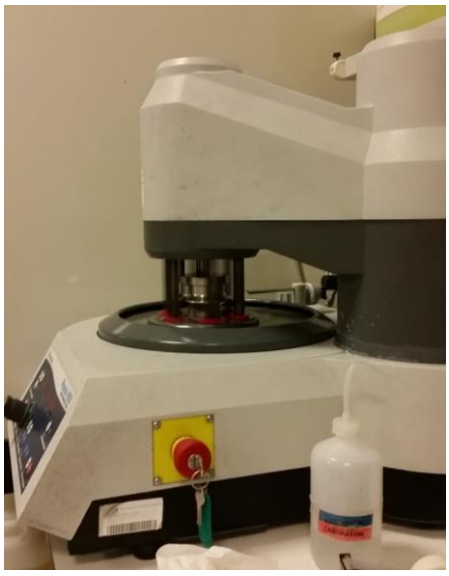

Figure 9: Polishing of hot mounted samples of Ti with Buehler Phoenix 4000

Once polished, a cutting step is necessary to remove the samples off the Bakelite. It consists in locking the Bakelite and samples in a bench vice as in fig (10), cutting the extremities of the Bakelite with a saw and carefully removing the samples of the mold without scratching them.

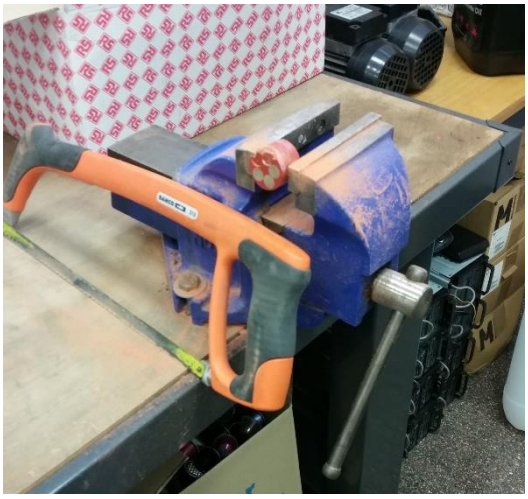

Figure 10: Cutting of hot mounted samples in Bakelite

3.1.3 Cleaning

The aim of the cleaning step is to remove all the unwanted elements on the surface of titanium samples. Since the polarity of the contaminants may not be the same, several solvents with different polarities are used to clean the samples.

For each step of cleaning, the samples are put in a beaker with the solvent, polished face on the top. The beaker is put in an ultrasound bath for 3 min each time.

Here are the different cleaning steps:

- Acetone (organic polar solvent) x1
- Cyclohexane (organic apolar solvent) x3
- Acetone x1
- Distilled water x3 (aqueous polar solvent)
- Ethanol (organic polar solvent, protic) x3
- Acetone x3

3.2 Functionalization

3.2.1 Plasma activation

Before coating titanium samples with polyethylene glycol (PEG), the surfaces need to be activated by an argon plasma treatment. A plasma is a gas to which a surplus of energy was supplied. The peripheral electrons will uncouple with the molecules or atoms, resulting in charged particles. The principle of plasma activation is to supply ionized argon gas on the samples in the vacuum. These particles are charged positively and they have for effect to modify the structure of the passive layer of titanium oxide TiO_2 on the surface of samples, and to charge negatively the samples under the surface. The surface will contain a superior concentration of active groups such as hydroxyls (OH^-).

To achieve stable bonds in the whole coating, the PEG used has amino groups (NH_2) and these groups, which are protonated at physiological pH (NH_3^+) will interact with the negatively charged sample. Thus the samples will be more reactive for the deposition of PEG with the plasma activation.**[17]**

The figure (11) schematically depicts the process of plasma activation:

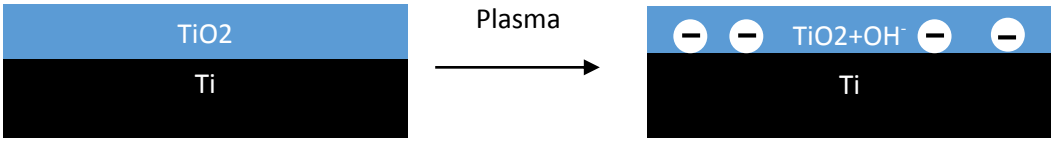

Figure 11: Scheme of influence of plasma activation in vacuum on titanium

The conditions of activation are the following:

- Argon flux
- Pumping down 0.1 mbar
- Gas supply of 0.4 mbar during 2 min
- Plasma power of 33% during 5 min
- Venting during 1 min

3.2.2 PEG electrodeposition

The PEG used for the project is a Poly(ethylene Glycol) bis(3-aminopropyl) terminated with a molar mass of 1500 g/mol. Its structure is the following:

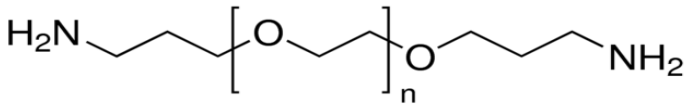

Figure 12: Structure of PEG used for the project[18]

The amino groups of the PEG will be attracted by the negatively charged groups on the titanium, thus they will be bonded with the sample and create a layer of PEG on it. Moreover, some amino groups of the polymer will still be unbounded to the sample after the deposition, which will be useful for the next step of functionalizing. The following figure shows schematically the principle of the PEG coating in this project:

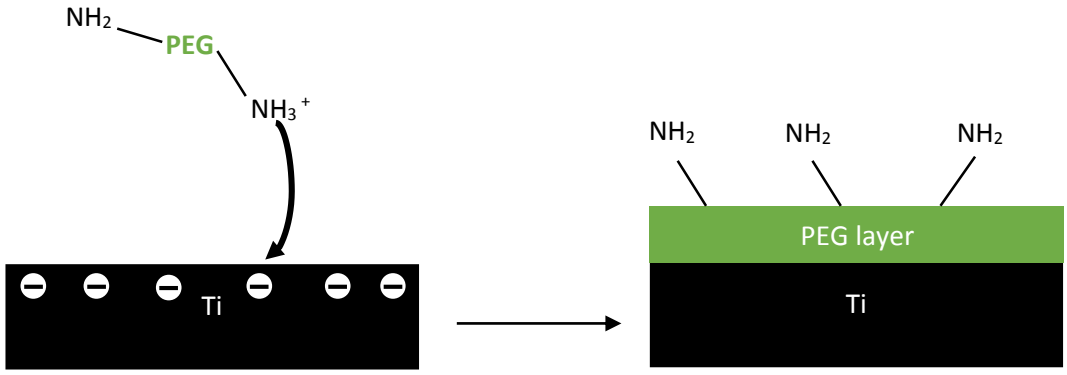

Figure 13: Scheme of PEG coating principle

To deposit a layer of PEG on the titanium samples, we put in a beaker:

- 150 mL of distilled water
- 3 g of PEG in powder
- 2,629 g of NaCl

In this solution is put an electrode of reference of platinum (Pt) and a sample, which is hold by pliers of titanium (those pliers, with the sample, are the second electrode). Then the two electrodes are connected to a generator that send 5V during 5 min, by periods of 8 ms. The system is also connected to an oscilloscope to check if the tension is correct. The figure (14) shows the assembly for the deposition:

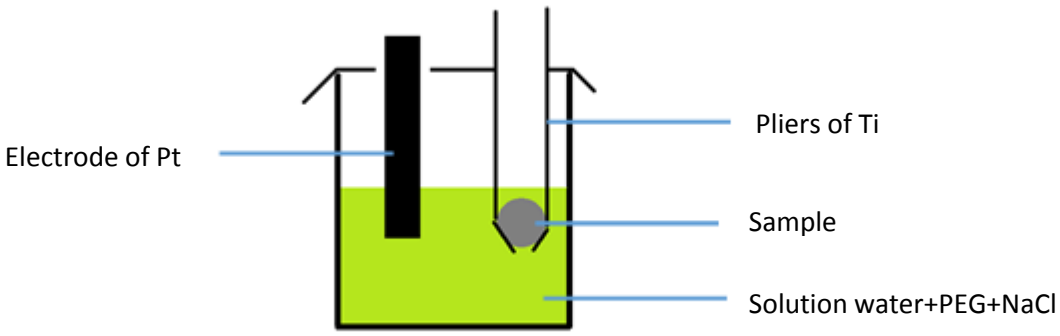

Figure 14: Scheme of electrodeposition of PEG on Ti

After the 5 min, the sample is cleaned with distilled water and kept in a container with the other coated samples.

3.2.3 Crosslinker

The thiol group (SH) of the anchor of the PTF will be used to attach the molecule to the surfaces. However, thiol groups do not react with the NH_2 groups present on the PEG of the samples. Thus, to allow the adhesion of the peptidic platform PTF on the samples with PEG, the use of a crosslinker is needed. This molecule will serve as a link between the PEG and the platform. The crosslinker used is 3-Maleimidopropionic acid N-hydroxysuccinimide ester 99%. Its structure is the following:

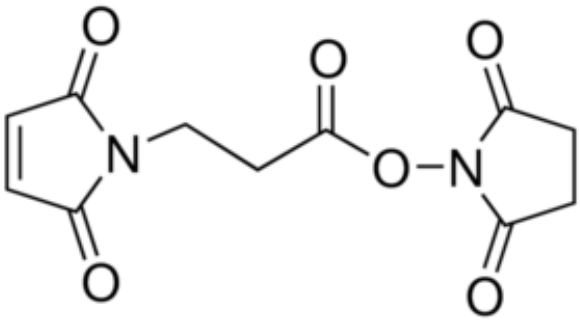

Figure 15: Structure of the crosslinker used for the project [19]

The amino group of the PEG will react with the crosslinker as shown below:

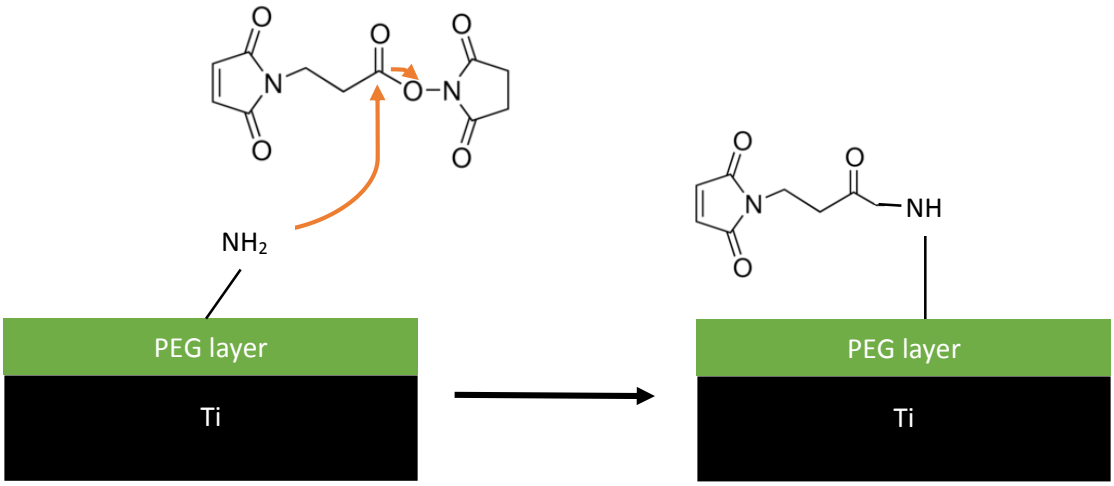

Figure16: Scheme of the reaction between PEG and crosslinker

In a falcon of 15 mL, 0.022g of crosslinker are dissolved in 10 mL of dimethylformamide (DMF). The DMF is an aprotic polar solvent commonly used for chemical reactions. Next, the samples with the side to functionalize on the top are placed in a beaker, and the crosslinker solution is added. The top of the beaker is covered and the samples are left under agitation for 1h with a magnetic stirrer.

Then the samples are put in another beaker and cleaned with:

- DMF x3
- Acetone x1
- Distilled water x3
- Ethanol x3
- Acetone x3 (and let in the beaker)

After that, the samples are dried with nitrogen.

3.2.4 Peptidic platform PTF

As explained previously, the PTF will be linked to the sample via the thiol group of the cysteine (anchor of the PTF). It has a good affinity with maleimide groups, thus it will be able to bond with the crosslinker added in the former step of functionalization. The fig (17) shows the mechanism of the reaction:

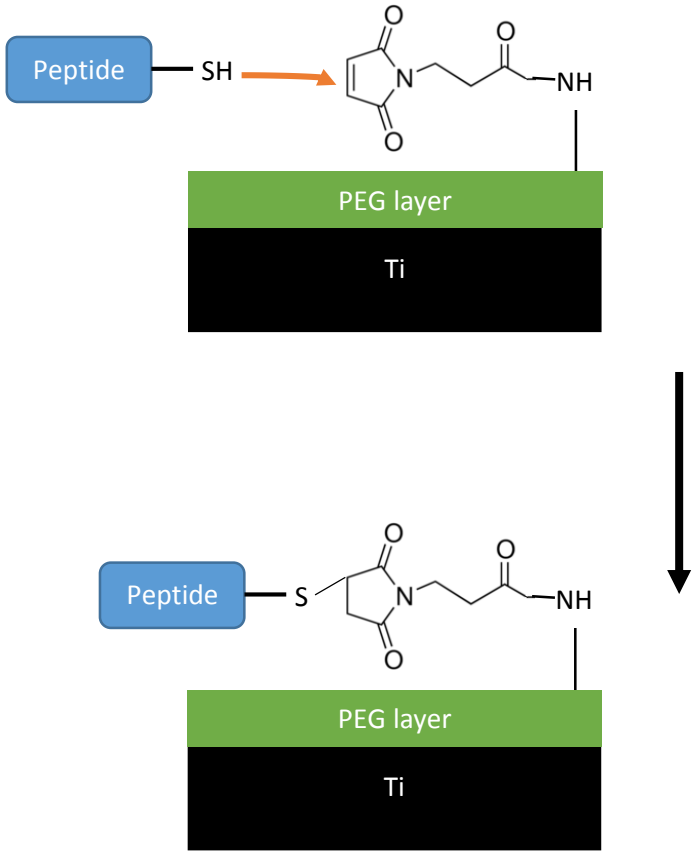

Figure17: Scheme of reaction mechanism between PTF and crosslinker

To functionalize the samples, a solution at 100 µM of PTF in phosphate buffered saline (PBS) at pH 6.5 is used. A drop (100 µL) of this solution is deposited on each sample. The multi-well plate containing the samples is then covered with wet paper (to avoid the drop to dry). The functionalization process is left overnight.

If the functionalization is done the day before a cellular assay, it is important to have the same conditions for the other samples. So if the functionalized samples are treated with a solution of PTF+PBS, the samples of titanium and Ti+PEG should be treated with a solution of PBS at pH 6.5 as well.

After the functionalization, the samples are cleaned with distilled water to perform physicochemical characterization, and with PBS at 7.4 to perform biological assays.

3.3 Physicochemical characterization of functionalized samples

3.3.1 Scanning electron microscopy and focused ion beam

The aim of scanning electron microscopy (SEM) is to study the morphology and topography of the surfaces and also the thickness of the PEG coating. The sample must be electrically conductive (or else, a layer of conductive element must be deposited on it). An electron beam is sent on the surface of the sample. Those electrons then scatter into the sample in a zone called interaction volume (Figure 1).

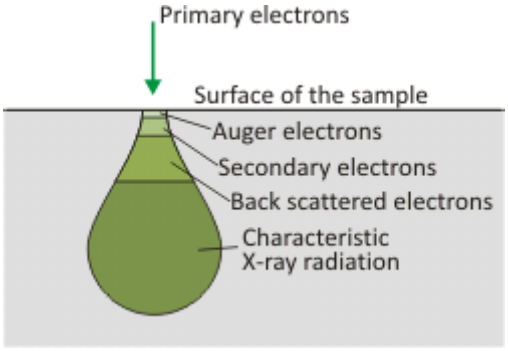

Figure 18: Scheme of the interaction volume[20]

Depending on the acceleration of the electrons, they go more or less deep in the sample. Then electrons are emitted out of the sample. Those electrons are collected in an electrical signal and translated in an image in levels of grey.

The focused ion beam (FIB) is a tool used to cut through the surface of a sample, but at a microscopic scale. An ion beam is sent to the sample and by an effect of sputtering, the atoms on the surface of the sample are ejected away.

In the current study, the SEM-FIB is used to analyze the surface of the titanium samples and the samples with the PEG coating (Ti+PEG), since the peptidic platform is too small to have a visible difference with SEM. But it is also used to check if the layer of PEG has been really deposited on titanium, and to measure its average thickness.

In order to do that, a sputtering of platinum is done on a small rectangular area on the PEG layer. This coating protects the PEG from the cutting step. Moreover, since the PEG is not electrically conductive, a small deposit of carbon must be done on the PEG to be able to see something with the SEM. The FIB does a gradual cut in the sample, and the SEM looks at the sample in an inclined orientation, to have a view of the different layers of the sample.

Two samples of titanium and two samples of Ti+PEG were studied, and 3 measurement of thickness of PEG were fulfilled on each sample of Ti+PEG. The machine used is a Zeiss NEON 40.

Below is schematically drawn the profile of a sample after the FIB cut:

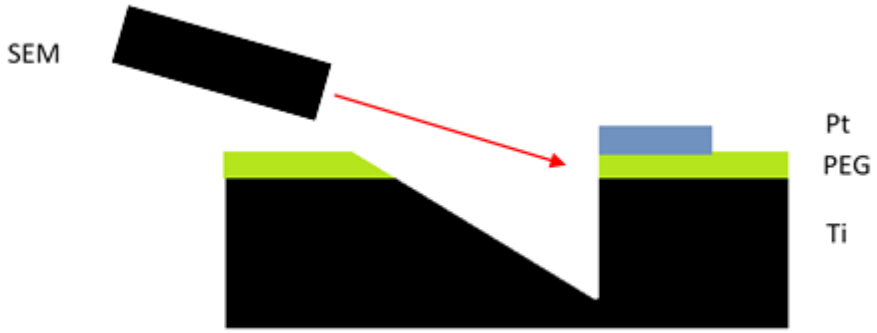

Figure 19: Scheme of sample after SEM-FIB

3.3.2 White light Interferometry

The white light interferometer is used to study the roughness of the samples of titanium before being polished, polished titanium, and Ti+PEG. For the project a Veeco Wyko NT9300 on vertical scanning mode was used.

For the study 3 samples of each condition were used, and the following parameters were measured:

- Ra (average roughness)
- Rsk (surface skewness)

The Ra gives an average idea of the roughness of a surface while the Rsk shows if the surface is porous or not as explained below:

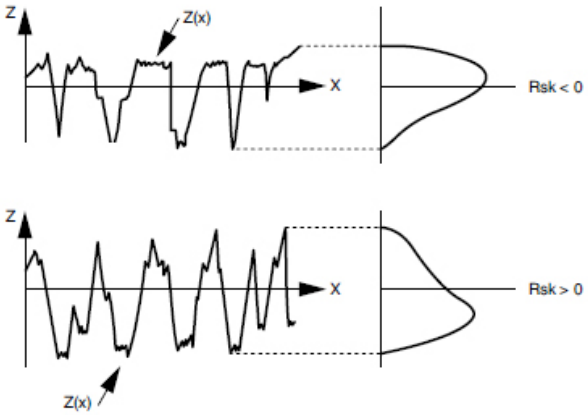

Figure 20: Graph showing difference of Rsk in function of roughness profile [21]

The Rsk gives more details about the roughness profile like the porosity. If Rsk < 0, the top surface is more flat and the cavities are spikier. On the contrary, if Rsk > 0, the top surface is composed of spikes and the bottom of the cavities are flat.

3.3.3 Fourier transform infrared spectroscopy (FTIR)

The FTIR is used for the chemical analysis of the samples. By sending a radiation of different wavelength on a sample, and measuring its absorption, a spectrum is obtained in function of wavenumber (inverse of wavelength) and transmittance. If, for a given wavelength, there is no absorption, then the transmittance is total. But if it is totally absorbed, then the transmittance is equal to zero. On this spectrum, if a peak is noticed for a given wavenumber, it indicates the presence of a specific chemical bond. By knowing the structure of the molecules on the sample, and with the chemical bonds found thanks to the FTIR, the composition of the sample can be analyzed. Moreover, it can be known if the signal corresponds to a stretching or a bending of the chemical bond. The aim of using FTIR in this project is to check if the PEG has been deposited on titanium.

3.3.4 Contact angle

The wettability of a surface can have an influence on the efficiency of biomaterials. But the first objective of measuring the contact angle of the samples in the project is to have another method to check the deposition of PEG on titanium. Since the PEG has a higher hydrophilicity than titanium, a difference in contact angle values should be observed. The presence of the platform will be also analyzed with this method.

Thus, contact angle measurements were performed with water, on 3 samples of Ti, Ti+PEG, Ti+PEG+CL, Ti+PEG+CL+PTF. Three measurements were done on each sample. The volume of drop used is 1 µL and the machine/software used to measure the angle are from Dataphysics. For each drop, the contour of it is drawn on the software, which calculates then the contact angle with spherical approximations.

A contact angle corresponds to a thermodynamically equilibrium between the liquid phase of the drop, the solid phase of the substrate and the gaseous phase of the ambient. Depending on the surface tension of the liquid and the surface energy of the solid, the interaction between the drop and the substrate will be different. The surface energy of the substrate can be modified by a change of roughness or other surface treatments, depending on the application (more or less hydrophilic). The contact angle is the angle ɵ between the interaction liquid/solid ϒLS and the interaction liquid/vapor ϒLV. The lower the angle is, the more hydrophilic the surface is.

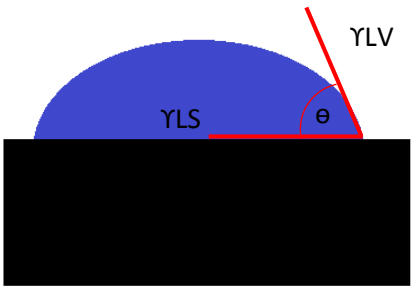

Figure 21: Scheme of contact angle

3.4 Biological characterization of functionalized samples

3.4.1 Cell culture

For this project, human SAOS-2 cells were used. These cells, original from an osteogenic sarcoma, are used because they grow fast and are adhesive (to the flask where they will grow) and can be used as a model of osteoblast cells. Cell culture was done in a hood under sterile conditions.

The first step is to prepare cells fresh medium (50 mL):

-Mccoy SA Medium (42,5mL)

- FBS (foetal bovine serum) (5mL)

- HEPES (1mL)

- Sodium pyruvate (500 µL)

- Penicillin-Streptomycin (500 µL)

- L-Glutamine (500 µL)

10 mL of this solution are added in a flat flask, and then frozen SAOS-2 cells are warmed and added to the flask. The cells are mixed carefully and then incubated at 37°C.

Since the previous cells were frozen in a solution containing dimethylsulfoxide (DMSO) (toxic for the cells if kept too long in contact), it is important to change the medium of the cells the day after of seeding in the flask. In order to do that, the solution in the flask is taken out. Since the cells are attached to the bottom of the flask, they will not be removed while aspiring. First 5 mL of PBS are added to remove non-adherent cells and then 10 mL of new fresh medium without DMSO are added to the flask, which is put back in incubator.

It is important to check regularly on the microscope the state of cells in the flask (their viability). If they are round and float (e.g. move with the liquid), it means that they are no longer attached to the bottom of the flask and thus are dead. Moreover, once the area on the flask is occupied by around 70% of cells, i.e. they reach confluence; they may interfere the growing of each other, and a passage to a new flask is required. To do this, a step of trypsinization is done. It consists of detaching the cells from the flask to then either freeze them or seed them in a new flask.

In the flask with cells, the medium is aspired and 5 mL of a solution of trypsin are added. This solution detaches the cells from the flask; however, it can be deadly for the cells if let too long in contact. The flask is put in an incubator for 2 min, and then the flask is shaken carefully, but enough to unstick the cells from the flask. Next, 5 mL of cell medium are added to the flask to neutralize the effect of trypsin, and then 5 mL more. The content of the flask is then put in a tube of 15 mL and centrifuged during 5 min at 300 rpm. The cells will precipitate at the bottom of the tube. Thus the supernatant can be taken out carefully.

After that step, we can use the cells to perform cell adhesion assays, seed them in a new flask or freeze them for future assays. In these cases: 5 mL of a solution FBS + 10% DMSO are added to the tube and cells are re-suspended. Those 5 mL are then split in cryotubes (1 mL each) or used to seed a new flask. **[22]**

3.4.2 Cells adhesion assay

For both the LDH and immunofluorescence assays, the following protocol was performed:

1) Blocking of the multi-well plate with a 1% w/v BSA solution in PBS, 500 µL in each well, during at least 30 min. The bovine serum albumin (BSA) is a protein from bovine blood commonly used in assays for its blocking properties. This treatment avoids the non-specific adhesion of cells on the multi-well plates, ensuring that cells will only adhere on the samples if cell adhesive motifs (the peptides) are present.

In the plate where the standard curve for LDH will be done, this step must not be applied, because the objective in this case is to have the maximum of cells attached to the plate.

2) Cleaning wells and samples with PBS twice, and put the samples in the blocked plate

3) Preparation of a cell solution:

-Trypsinization of cells in flask

- Counting of cells

- Preparation of the cell solution as explained in the trypsinization step

The solution used for this assay has a cell concentration of 26,875x10^4 cell/mL.

With this solution, two different solutions are done:

-The solution **A** for the assay which is supposed to have a cell concentration of 50 000 cell/mL, and we want 16 mL of this solution.

$$16\ mL * \frac{50000\ cells}{1\ mL} * \frac{1\ mL}{268750\ cells} = 2.98\ mL$$

To obtain the desired cell concentration, the solution **A** is composed of 2.98 mL of the former cell solution + 13.02 mL of cell medium but without FBS.

- The solution **B** for the standard curve composed of 1.86 mL of the cell solution + 3.14 mL of cell medium with FBS (same as in cells culture). We want 5 mL of this solution. It will have an initial cell concentration of 100 000 cell/mL.

$$5\ mL * \frac{100000\ cells}{1\ mL} * \frac{1\ mL}{268750\ cells} = 1.86\ mL$$

This solution is then diluted to have solutions with different concentrations of cells: 50 000, 25 000, 12 500, 7 500, 5 000, 2 500, 0 cell/mL.

4) Put 500 µL of solution **A** on each sample (in the multi-well plate).

5) Put 500 µL of each dilution of **B** in 3 wells each to obtain the standard curve

The incubation last for 4h at 37ºC

The following scheme shows how the plates' configuration should look like after the cell adhesion step:

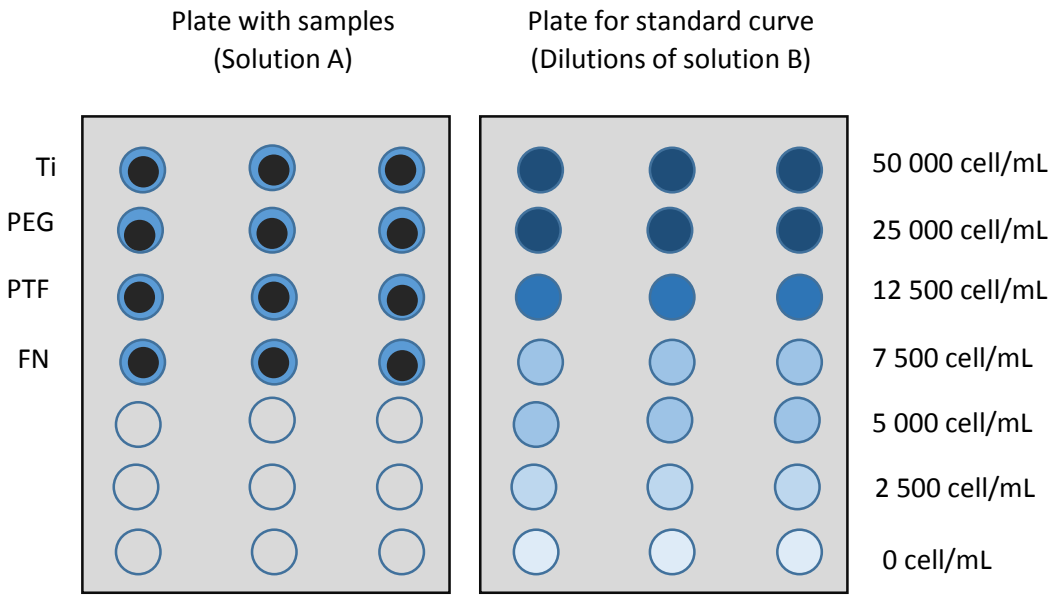

Figure 22: Scheme of plates for LDH (and its standard curve) and immunofluorescence assay, after the cell adhesion step

3.4.3 Assay LDH

This assay is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells. This assay is commonly used for cytotoxicity but in can also be used to determine the number of cells bound to a surface.

In this case, after the adhesion assay, the samples are cleaned with PBS x3 and transferred to a new plate. Then, a lysis buffer, m-PER (mammalian protein extraction reagent), is added to the samples. It lyses the cells membrane thereby releasing the LDH. 350 µL are added in each well. The solutions can be kept at -80°C until the day of assay.

The LDH Cytotoxicity Detection Kit was used for the assay, with the following protocol:

1) Prepare a dye-catalyst solution with the catalyst diluted 46 times (for example 250 µL of catalyst in 11.25 mL of dye for a total solution of 11.5 mL for this assay) and keep it away from light.

2) Add 100 µL of each sample (with M-PER) twice on a 96 well plate.

3) Add 100 µL of the dye-catalyst solution in each well of the 96 well plate. The use of a multichannel pipette can help to do the step faster, and so avoid the time factor in the experiment (since the colorimetric measurements depends on time also).

4) Cover the plate with aluminum foil and let react 10-30 min, until the color starts to develop.

5) Add 50 µL of stop solution in each well to neutralize the reaction.

6) Read the absorbance at 492 nm **[23]**

For this assay the samples used are: Ti, Ti + PEG, Ti + PEG + CL + PTF and Ti + Fibronectin (FN).

Fibronectin is a protein known for its high cellular adhesion properties. In this assay, it will be the positive control (maximum values of cell adhesion; if no cells appear on those samples, it means that the assay did not work) **[24]**

3.4.4 Immunofluorescence

The immunofluorescence permits the visualization of cells by fluorescence microscopy. In order to do that, a specific targeting molecule is added to the sample. This molecule is (or contains) a fluorochrome, whose fluorescence will be detected with the microscope (see figure 23). This step of staining is necessary because the molecules studied in this project are not fluorescent. Thanks to the fluorescence, it will be possible to study the adhesion and spreading of the cells.

For the project are stained: the nuclei of the cells with DAPI to be able to count the number of cells per image, and also actin fibers with phalloidin, which indicate the spreading of cells.

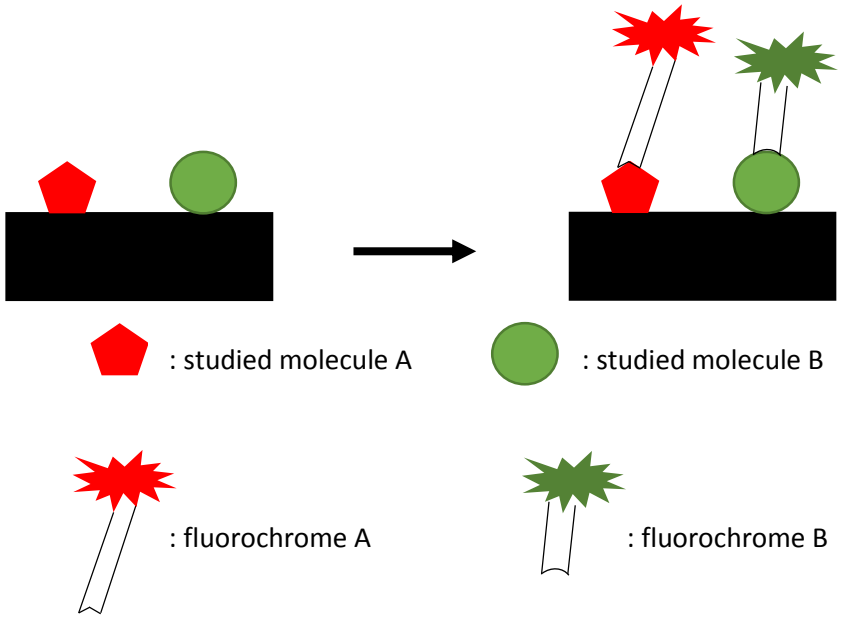

Figure 23: Scheme of principle of immunofluorescence

The protocol used is the following:

1) Cellular fixation with PFA. The paraformaldehyde (PFA) is a fixative polymer used in cellular assays.

- The samples are cleaned after the adhesion assay with PBS x2
- 500 µL of a solution of 4% v/v paraformaldehyde (PFA) in PBS are added in each well and the samples are let during 30 min for fixing.
- The samples are cleaned again with PBS x2 and leave the samples in the fridge (4°C) until the day of the assay.

- 500 µL of PBS-Glycine (for 50 mL of solution, 0.075 g of glycine) are added in each well, 3 times during 5 min to clean the samples. The glycine helps to quench the auto-fluorescence that the PFA could have, so it inactivate its fluorescence.

2) Permeabilization: permits diffusion of the staining molecule into the cells

-500 µL of 0.05% (v/v) Triton in PBS are added in each well, during 20 min. Triton is a detergent that will permeabilize the cells.

- The samples are cleaned with PBS-Gly (500 µL each well) x3

- The samples are transferred to a new plate

3) Blocking: to be sure that the stain will interact only with the desired molecules

- 500 µL of 1% (w/v) BSA in PBS are added in each well during 30-60 min.
The BSA will cover the majority of the cells, and only a molecule with a higher affinity (with actin for example) will replace the BSA for this specific molecule.

- The samples are cleaned with 500 µL of Gly in PBS x3

4) First stain:

This step must be done in the maximum obscurity possible

- On each sample are added 100 µL of a solution triton in PBS (the previous one) with 0.3% v/v Phalloidin-rhodamine, during 1h. Phalloidin is a molecule that has a high affinity with the actin fibers, and the rhodamine is the fluorochrome used to stain them.

- The samples are cleaned with Gly in PBSx3

5) Second stain:

- 500 µL of 0.01% DAPI in PBS-Gly are added to each well and incubated during 2 min (protect with aluminum foil to avoid light). The DAPI is used to stain the nuclei of cells

- The samples are cleaned with Gly in PBS x3

6) Preparation for microscope

- Double face tape is put on a glass support and 3 samples are stuck on it

- A drop of Mowiol is added on each sample. It improves the quality of the results on microscope

-A cover glass is put on each sample, without touching the surface and trying to avoid bubbles of air between the sample and the crystal. The final assembly is represented below:

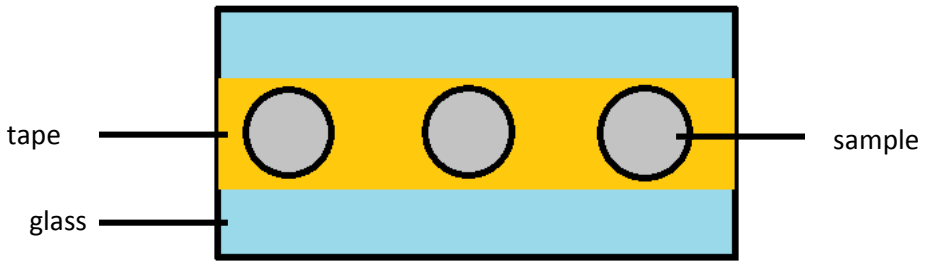

Figure 24: Scheme of the preparation of samples for microscope with fluorescence

For visualizing the samples, the microscope used is a Leica AF7000 in fluorescence intensity mode, with a camera Hamamatsu. The pictures are done with a magnification of 10 times.

With the pictures obtained and the software ImageJ, it is possible to calculate the average area of cells for each condition, and the average number of cells per condition. A picture is composed of two images; on the first one can be seen the nuclei in blue and on the second one the actin fibers in red. The images are made binary (in black and white) and then the software can count the number of nuclei and calculate the cells area. To avoid the counting of artifacts of the picture that are not nuclei or actin fibers, a minimum dimension of element is imposed: 50 µm² for nuclei and 500 µm² for the cell area.

3.4.5 Bacterial assays

The bacteriological assays are performed with *Escherichia Coli* and *Streptococcus Sanguinis*. *E. coli* is an intestinal bacteria and is used because it can be used as a model of gram-negative. *S. Sanguinis* is a bacterial strain present in the human mouth, which can be used as gram-positive model. Both of them might cause infections, thus were selected to study the anti-bacterial efficiency of the coating.

a) Determination of bacterial concentration using Agar

The anti-bacterial efficiency of the samples can be determined by measuring the number of bacteria attached on the samples after a certain time of incubation. The CFU (colony forming unit) is the unit corresponding to a colony of bacteria. The whole protocol is done under a hood and with a flame to avoid contamination. In order to do that, this protocol was followed:

1) Preparation of inoculum: Day before the assay

- 5 mL of bacteria culture medium are added to a flask of 15 mL + 50 µL of a bacteria at 10^8 CFU/mL
The bacteria culture medium is a solution of Todd Hewitt Broth powder TH (36.4 g/L) in distilled water for *S. Sanguinis* and Brain Heart Infusion Broth (37 g/L) in distilled water for *E. coli*. Culture media are always sterilized in an autoclave.

- The flask is not entirely closed and is put in incubator at 37°C until the assay.

For each bacterial type (*S. Sanguinis*, *E. coli*), it is advised to prepare at least 3 inoculums from different Eppendorfs of the same bacteria. In this case, if one of the bacterial strains is not working, other inoculums are available for the assay.

2) Bacteriological assay

Samples of titanium, Ti+PEG and Ti+PEG+CL+PTF are used for this assay. For each assay, 3 samples of each condition are used. The aim is to incubate bacteria on the samples for a period of time, then adherent bacteria are collected in a flask containing PBS. From this solution, 10 times dilutions are prepared (D2, D3, D4). Then 3 drops of 5µL of each dilution D2, D3, D4 are deposited on agar plates. A day after, the CFU of bacteria from each drop are counted.

The following protocol is applied to *E. coli* and *S. Sanguinis*

- In a flask of 15 mL are added 1 mL of inoculum from the previous day in 5 mL of bacteria medium. The aim is that the absorbance of the solution is at least 0.2. If it is lower, more inoculum is added.

The concentration of bacteria desired for the assay is 10^8 CFU/mL, which is characterized by an absorbance of 0.2. Since it is the maximum concentration reachable, it is not important if the absorbance is a bit higher than 0.2.

- The samples and multi well plates are sterilized with ultraviolet during 10 min. The bacteria must not be let under the UV, or they will die.

- The samples are cleaned with sterilized PBS x2

- The samples are transferred into a 48 wells plate

- A drop (10 µL) of bacteria solution is deposited on the top surface of each sample. In order to do that, the solution is mixed and the drops are added randomly.

- Bacteria medium is added in the empty wells around the samples, to avoid the drops to dry.

- Incubation for 4h

- For each sample, 4 eppendorfs with 900 µL of sterilized PBS in each are prepared.

- After incubation, the samples are cleaned with sterilized PBS x3 to keep only the bacteria attached to the samples.

- In a flask of 15 mL, 1 mL of PBS and one of the samples are added (do the same for each sample).

- Agitation by vortex during 5 min to remove the bacteria from the sample and transfer them into the PBS solution. This is the dilution D0.

- After the agitation, 100 µL of solution D0 are transferred fast into one of the previous Eppendorf. This is the dilution D1 (dilution 10 times of D0).

- Then 100 µL of D1 transferred into D2, the same for D3 and D4.

- On the bottom of an agar plate, separations for the drops are drawn

- In each square of the agar box, a drop (5 µL) of the corresponding dilution (D2, D3, D4) is deposited

- When the drops are dried, the agar plates are put in the incubator until next day.

- The day after, check the agar plate and count the number of colonies in each square.

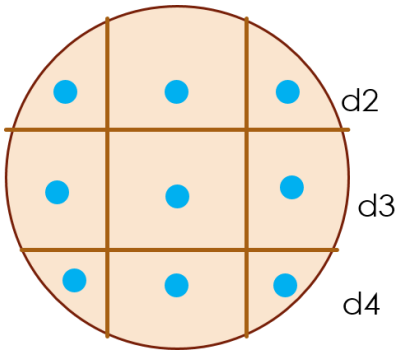

Figure 25: Scheme of agar box with drops for a sample

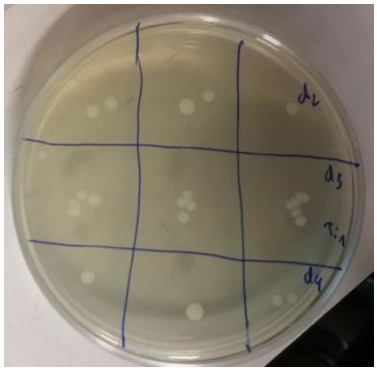

Figure 26: Picture of agar box after an assay on Ti

b) Alamar Blue method

The Alamar Blue (AB) is a colorimetric method commonly used for studying cells. But in this project it has been used to study the antibacterial properties of the surfaces. The principle of the Alamar Blue method is to let react the AB reagent with the samples + bacteria, which will yield a fluorescent signal if bacteria are alive. If the fluorescence is higher, it means that the concentration of bacteria is higher.

The beginning of the protocol is the same as the assay with agar, until the incubation of the samples with bacteria for 4h. The process is done in the maximum obscurity possible:

- After the incubation, the samples are cleaned with PBS sterilized x2

- A solution of 10% v/v Alamar Blue in bacteria medium (BHI or TH + water, depending on the bacteria used) is prepared

- On each sample and in 3 empty wells, 500 µL of the solution with AB are added and let for reaction at least 1h. When the solution (which is blue at beginning) starts to become pink on some samples, 100 µL of each well with solution of AB are transferred 3 times in a 96 wells plate. The 3 wells with only a solution of AB are used as control, to see if the solution has been correctly distributed. No difference of fluorescence should be noticed in those 3 solutions, and if there is, it means that the mixing of the solution has not been done correctly. Thus the results might not be entirely correct. The fluorescence is then measured with an Infinite M200 pro TECAN, at wavelength of excitation of 560 nm and a wavelength of emission of 590 nm.

This method can also be used to study the formation of biofilms on the samples. In this case, the previous samples analyzed by Alamar Blue can be used again:

- The samples are cleaned with PBS x2 after the Alamar Blue step

- 500 µL of bacteria medium are deposited on each sample in the wells

- The plate is placed in incubator until the day after the bacterial assay or 2 days.

- The next day, cleaning of the samples with PBS x2

- Addition of 500 µL of the solution 10% v/v AB in bacteria medium on each samples, plus 3 empty wells for the control. The whole system is let to react as in the previous AB process. Since the concentration of bacteria has increased, the solution will change of color earlier so the reaction will last less than 1h, around 20 min.

- The solution on each sample is transferred in a 96 well plate 3 times.

- The fluorescence is read with the machine.

The percentage of bacteria reduction is calculated with the following formula, given by the fabricant Thermofisher:

$$\%reduction = \frac{FmodifiedTi - FcontrolTi}{FmediumwithAB - FcontrolTi} * 100$$

Where Fx is the fluorescence of the samples (Ti control, Ti+PEG or Ti+PTF) or just the medium with Alamar blue.

4. Results and discussion

4.1 Interferometry: Roughness of the samples

The first roughness parameter studied was the average roughness Ra:

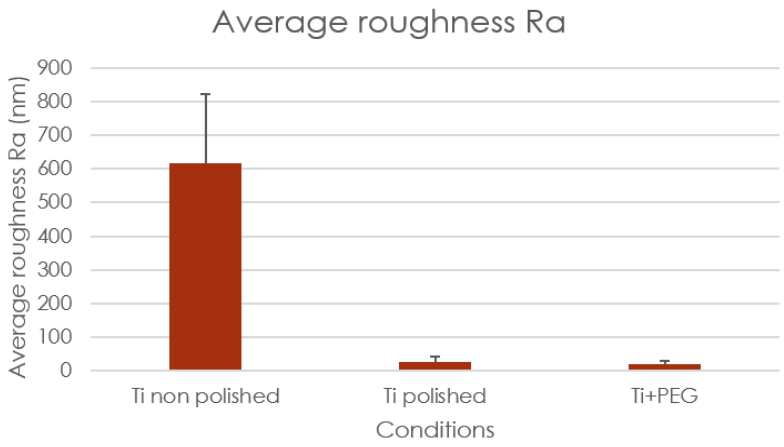

Figure 27: Graph of interferometer results (Ra)

The Ra gives the general trend of roughness of a sample. According to fig (30), the roughness of unpolished titanium is very high (600 nm), and the polishing step reduced it significantly as expected. However, the deposition of PEG does not have an important impact on the roughness of the samples. It reduces it slightly and this can be due to the fact that during the deposition of PEG, it may fill some scratches of the titanium surfaces, involving a smoothing of the roughness profile.

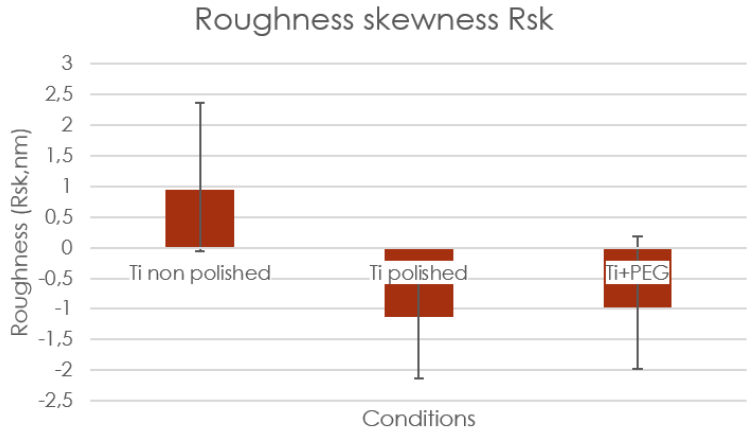

Figure 28: Graph of interferometer results (Rsk)

As shown in fig (28) the Rsk of the unpolished titanium is positive, which means that the surface is spiky. However, the polished samples of titanium and Ti + PEG have a negative Rsk, involving a flat top

surface. This result was expected because the polishing step aims to reduce the roughness of samples. Thus the pikes on the surface of the titanium are chopped into a flat surface as shown below:

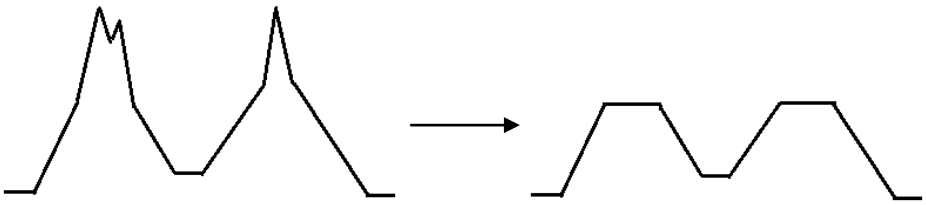

Figure 29: Scheme showing the influence of polishing on Rsk

4.2 SEM: Surface morphology

The SEM is used to study the morphology of the surface of the samples of titanium and Ti+PEG. Two samples of each are studied. The following figures are pictures of the surface of the samples of titanium control or coated with PEG, at different magnifications.

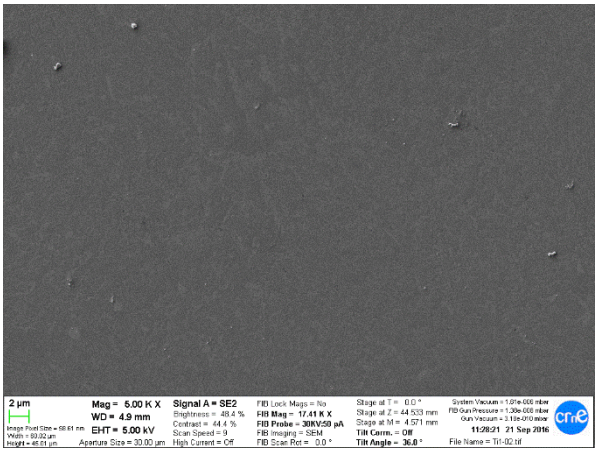

Figure 30: Sample 1 Ti with SEM, mag 5k

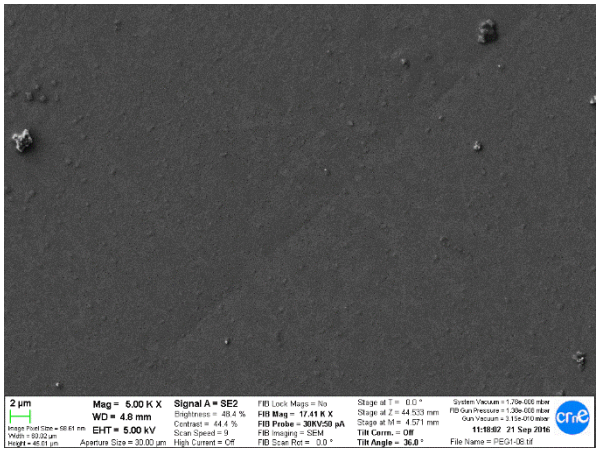

Figure 31: Sample 1 Ti+PEG with SEM, mag 5k

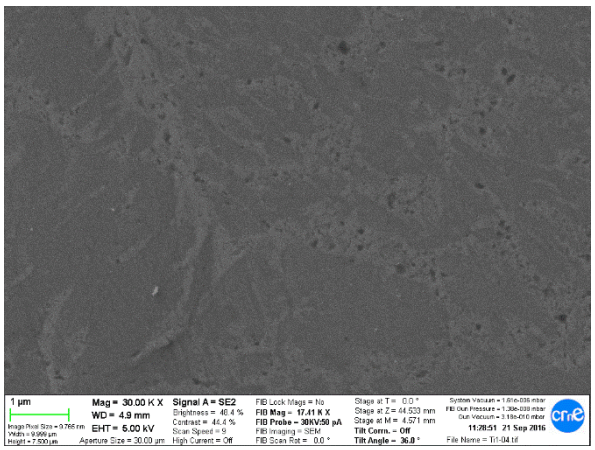

Figure 32: Sample 1 Ti with SEM, mag 30k

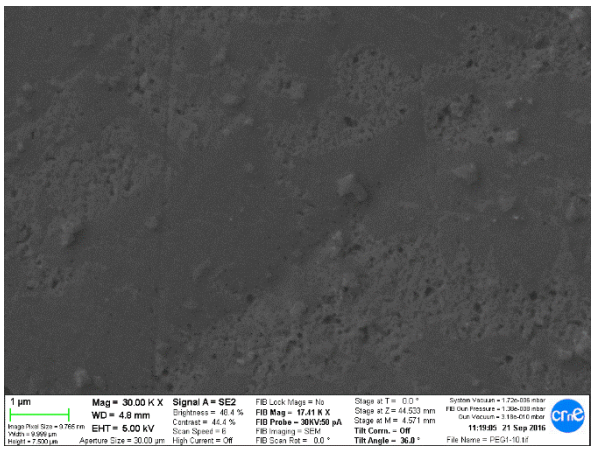

Figure 33: Sample 1 Ti +PEG with SEM, mag 30k

According to the pictures obtained by SEM, the samples of Ti + PEG show some granules or particles, which are not present on control titanium samples and could be attributed to the PEG coating. However, further studies should be performed to analyze the identity of these particles.

4.3 SEM-FIB: Measurement of PEG layer thickness

SEM-FIB is used to confirm the presence of PEG and measure its thickness. To measure the thickness of the PEG layer, a coating of platinum is done on the samples to protect the PEG. Then the FIB cuts the sample and with the SEM it is possible to see all the layers of the sample:

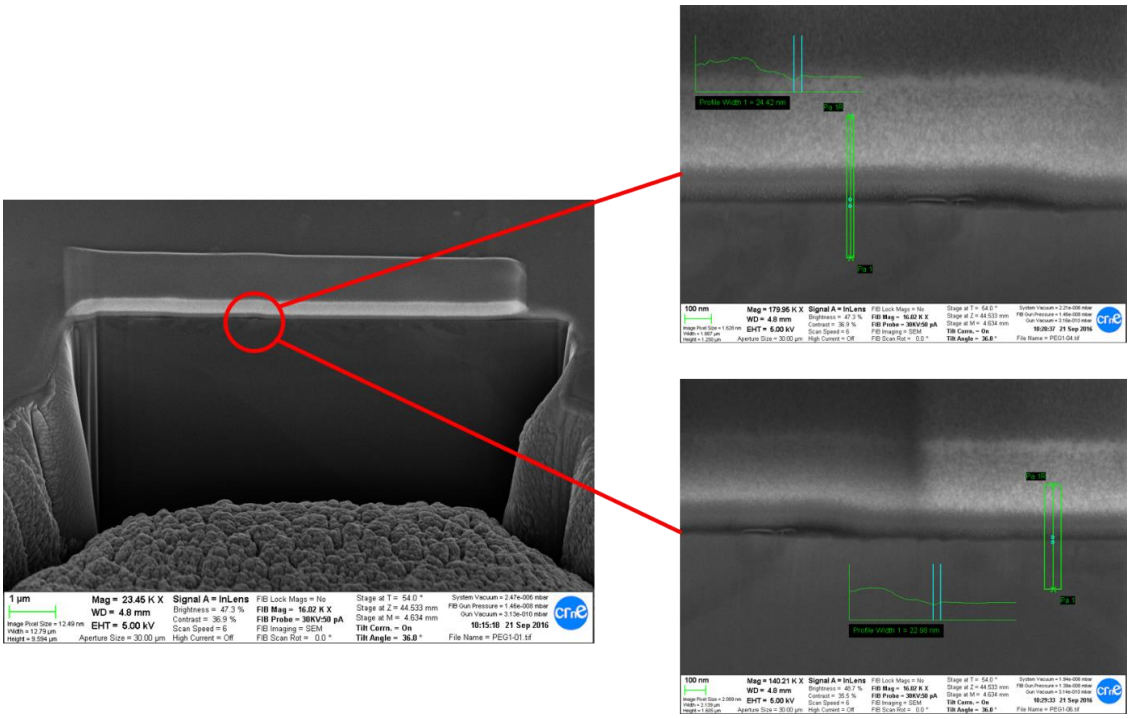

Figure 34: Measurement of PEG layer thickness with SEM

A zoom is realized on the cut as in fig [34] and the thickness is measured in several points of the picture. The PEG is the darkest layer of the picture so the thickness is measured by looking at the darkest area of the picture on a light graph.

On the following picture can be seen the different elements of the structure studied:

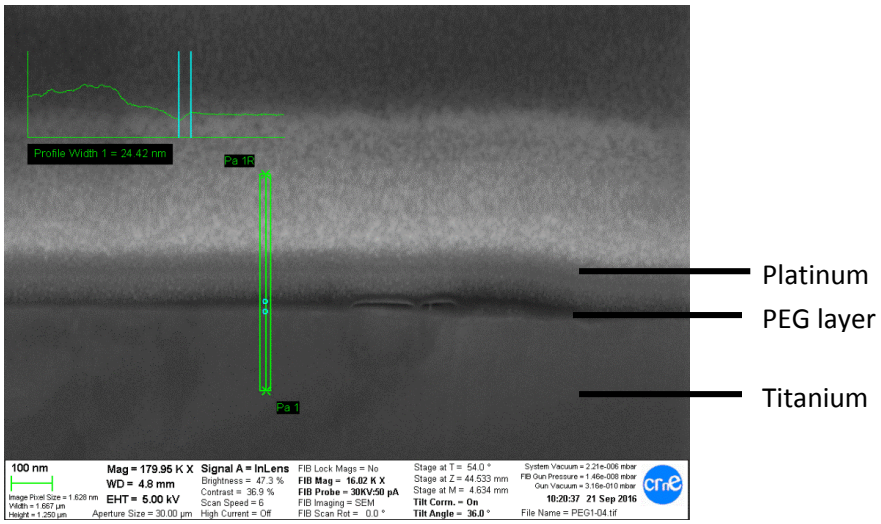

Figure 35: SEM of sample 1 of Ti+PEG, mag 180 K

The SEM shows that the PEG layer has been correctly deposited on the Ti. Moreover, its average thickness is **23 nm.** From previous studies, the thickness obtained by electrodeposition is between 5 and 30 nm thus the results obtained seems legitimate **[26]**. The pictures shown below are examples of images used to calculate the average thickness of PEG layer:

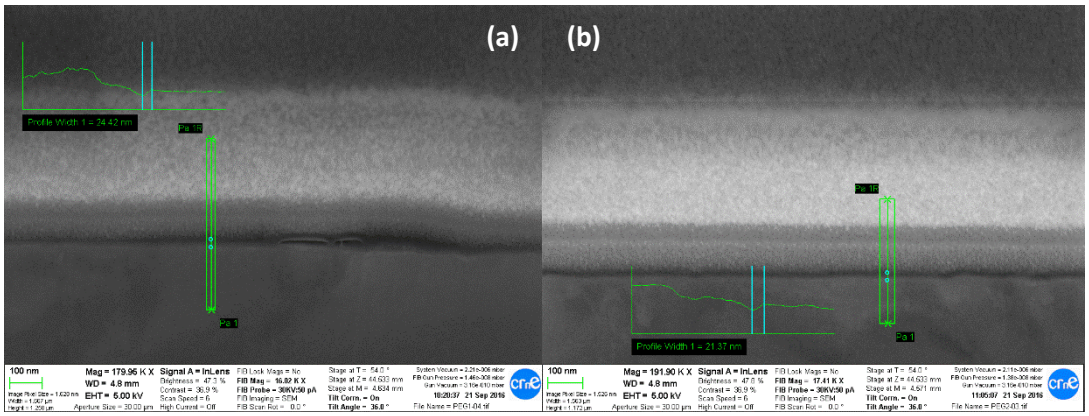

Figure 36: Representative SEM pictures used to measure the thickness of PEG layer with (a) from sample 1 and (b) from sample 2 of Ti+PEG

4.4 FTIR: Chemical composition of the surfaces

The FTIR is used to chemically characterize the presence of the PEG on titanium. The following spectrum is obtained from a titanium control and a Ti+PEG samples.

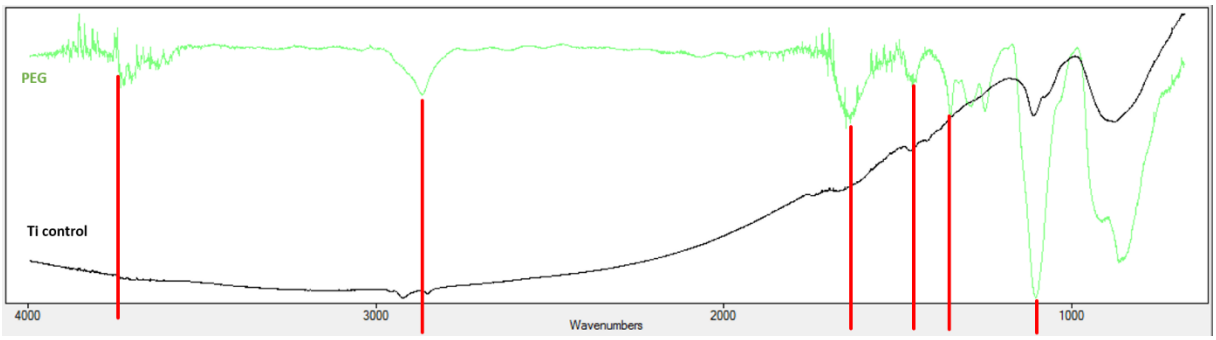

Figure 37: FTIR spectrum of Ti and Ti + PEG

According to the tables, several specific bonds can be determined in function of the wavenumber: **[25]**

Wavenumber (cm^{-1})	**1100**	**1340**	**1470**	**1645**	**2870**	**3600**
Bond	**C-O stretching**	**O-H bending**	**C-H bending**	**C-C stretching**	**C-H stretching**	**O-H bending**

Table 4: Table of bond found in the Ti+PEG sample

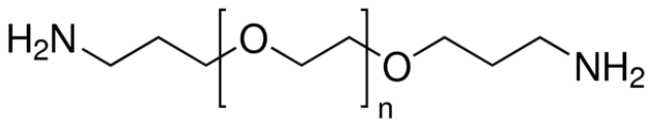

Figure 38: Sketch of PEG used for the project [18]

Now when we look at the molecule of PEG, the bonds **C-O, C-C** and **C-H** found in the FTIR spectrum are present in the molecule. Therefore the presence of the PEG on the surfaces is confirmed. It should be mentioned that the bonds N-H or N-C are not visible on the spectrum; however, the presence of these chemical groups is much lower that for example the C-O or C-C groups.

4.5 Contact angle

To study the effect of the surface treatment (PEG deposition and functionalization with PTF) on the wettability, a contact angle study has been done, with the following results:

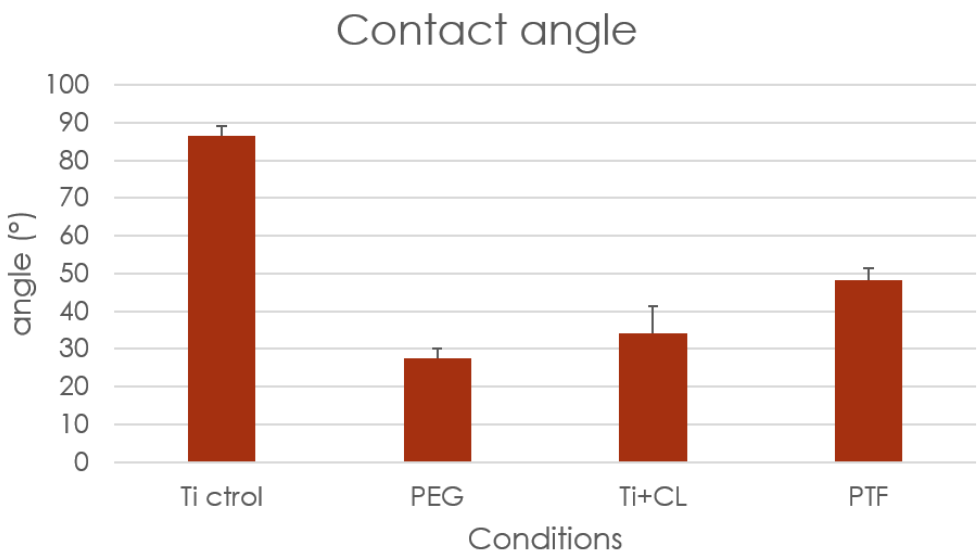

Figure 39: Graph of the average contact angle in function of the conditions

It can be seen on the graph that titanium is the more hydrophobic sample, with an average contact angle of 86°. The decrease of hydrophilicity due to the PEG coating is important since the angle is less than 30°. This difference of wettability is due to the fact that the PEG is more hydrophilic than titanium. On the contrary, addition of the crosslinker increases the contact angle, and the immobilization of the peptidic platform further increases the angle. However the angle is still lower than control titanium (non-functionalized).

The possible explanation is that the PEG is very hydrophilic and increased the wettability. However, although the crosslinker and the platform are hydrophilic, are less hydrophilic than the PEG. Since they cover the PEG, this is why the wettability has been reduced during the silanization step. But the main objective of the contact angle study has been reached because the fact that the wettability changes for the Ti+PEG and Ti+PTF shows that the functionalization process has been correctly done.

4.6 LDH: Cell adhesion efficiency

An LDH assay is performed to know the efficiency of cell adhesion on the samples. With this process, the concentration of cells on a sample can be determined.

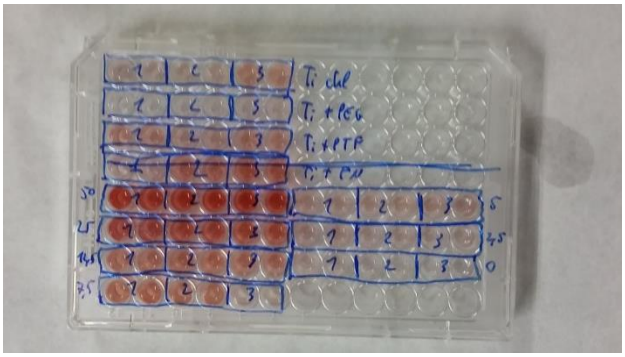

Figure 40: Plate for LDH assay

With the absorption measurement obtained with the solutions with specific concentrations of cells, a standard curve is established:

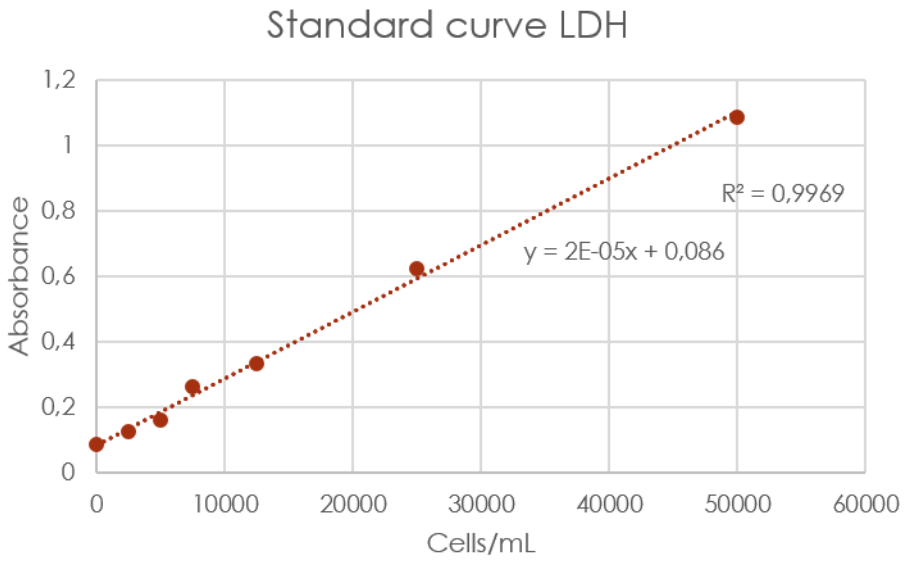

Figure 41: Standard curve of LDH showing the absorbance in function of the concentration of cells

From the standard curve, a trend is obtained with its equation **y = 2E-05x + 0,086**

With this equation, it is possible to translate the absorbance of the solutions into cell numbers on the samples: **Cells concentration = (absorbance – 0,086)/2E-05**

Thus the following graph is obtained from the results translated with this equation:

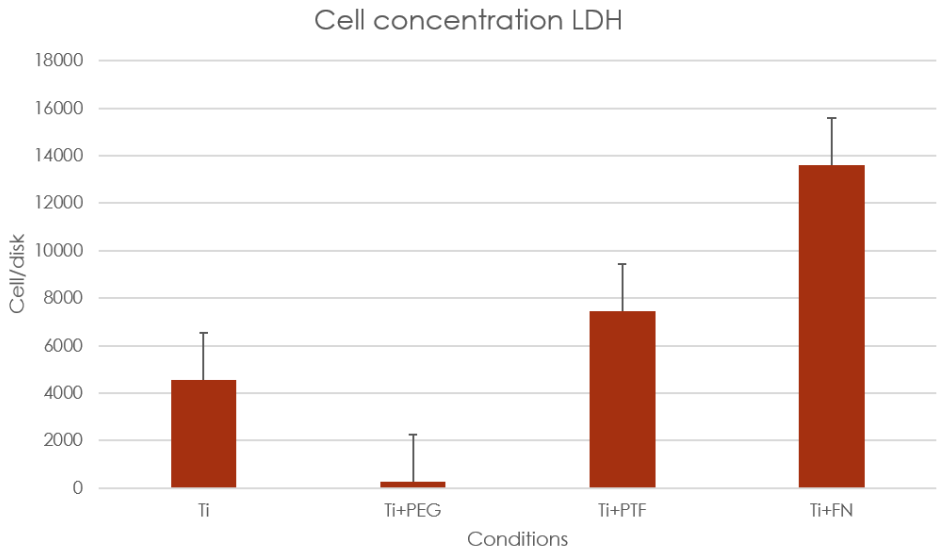

Figure 42: Cell concentration for LDH assay depending on the conditions

On fig(42) can be seen that cells adhere on titanium samples (4500 cells/disk). This is normal because the titanium is a biocompatible material, commonly used for prosthesis, and thus osteoblasts are expected to adhere.

The samples Ti + Fibronectin (FN) are the positive control, they are supposed to have the best cell adhesion, because FN is a natural cell adhesive protein from the ECM. These samples have the higher number of cells (13500/disk), which means that the assay worked.

As expected, cell binding on the titanium + PEG samples is strongly reduced. This is due to the anti-fouling properties of the PEG coating.

In contrast, the samples functionalized with the PTF (Ti + PEG + PTF) show a really good cell adhesion (7000/disk), much higher than PEG and significantly higher than titanium control. It means that the cell adhesion properties of the peptidic platform works because the molecule not only overcomes the anti-adhesive effect of the PEG, but also improves the cell adhesion compared to non-biofunctionalized titanium.

4.7 Immunofluorescence: Cell adhesion and spreading

With the immunofluorescence it is possible to count the cells on samples, and to measure their spreading, which is a good indicator of the quality of cell adhesion. If the adhesion on the samples is good, the cells will spread and show a higher contact area with the surface. However, if the adhesion is poor, cells will be small and round because they have no possibility to stick to the sample (i.e. there is no integrin binding), so they will have a circular morphology.

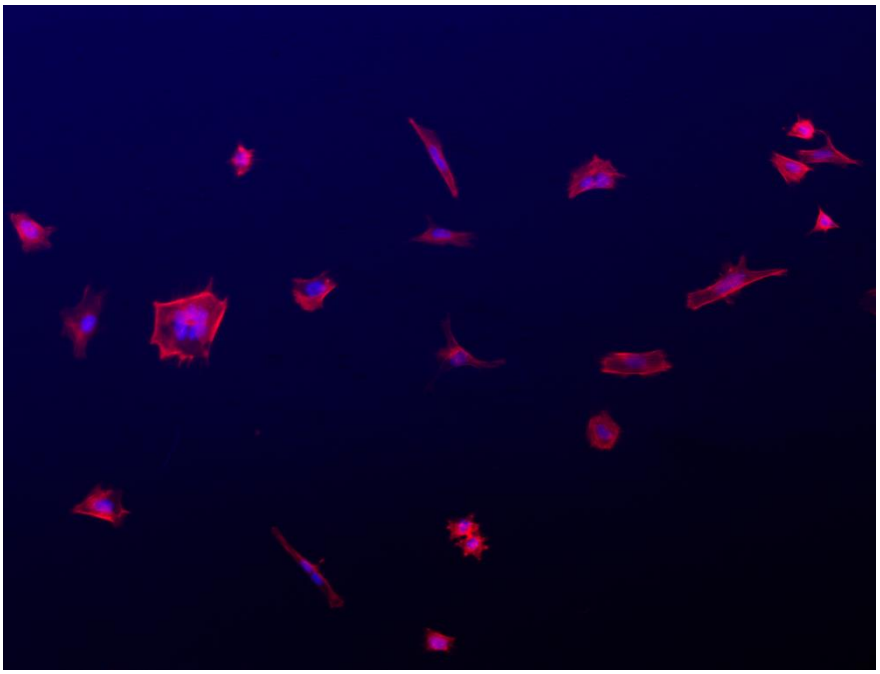

Figure 43: Picture of cells on Ti by fluorescence, mag 10x

The red filaments are the fibers of actin of the cells, while the blue color correspond to the nuclei of the cells. The fig(43) shows the cells on the samples of titanium. The number of cells is relatively low but their adhesion is relatively good because they are not small and round.

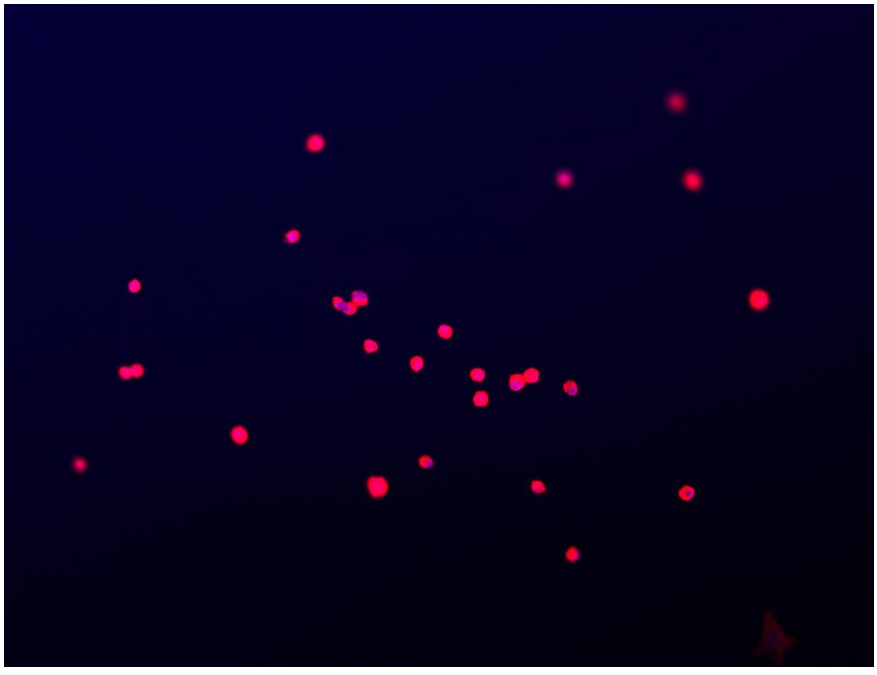

Figure 44: Picture of cells on Ti+PEG by fluorescence, mag 10x

The cells on the samples of Ti + PEG (fig(44)) are not numerous and are round which means they have no adhesion on the samples. It is even possible that some of these cells are dead. The anti-adhesion property of the PEG here is thus verified at a cellular scale.

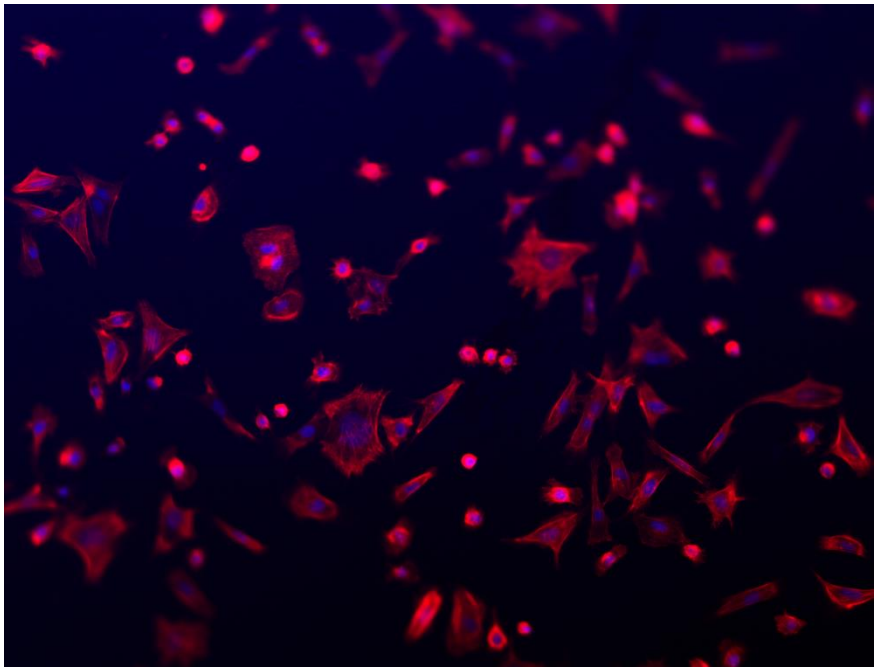

Figure 45: Picture of cells on Ti+PEG+CL+PTF by fluorescence, mag 10x

On the fig(45), which shows a titanium sample functionalized with the PTF, numerous cells can be seen, and most of them are very well spread. However, some of the cells are round like in the Ti + PEG samples. A possible explanation to this heterogeneity is that during the functionalization of the Ti + PEG, the platform's immobilization was not homogeneous. Thus the areas where the cells are round correspond to surfaces where there is no platform, and thus the cells are directly in contact with the PEG, which blocks the adhesion.

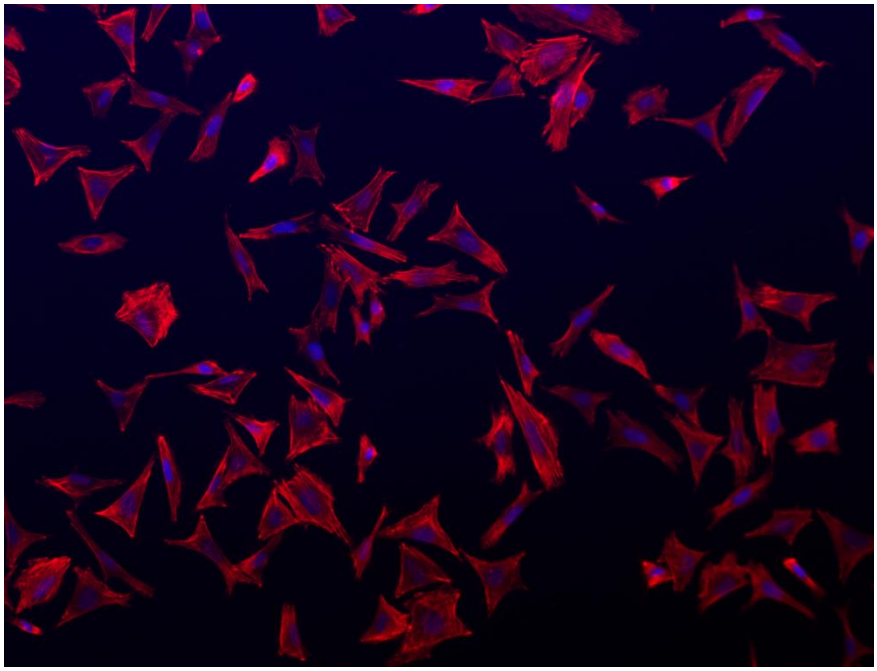

Figure 46: Picture of cells on Ti+FN by fluorescence, mag 10x

The fig(46) shows the cells on the samples of Ti + FN. It can be seen that those cells are well spread and numerous. It is normal because as explained previously, the fibronectin is the positive control so it is supposed to show the best results.

The figure (47) presents the number of nuclei on each sample obtained from the previous pictures:

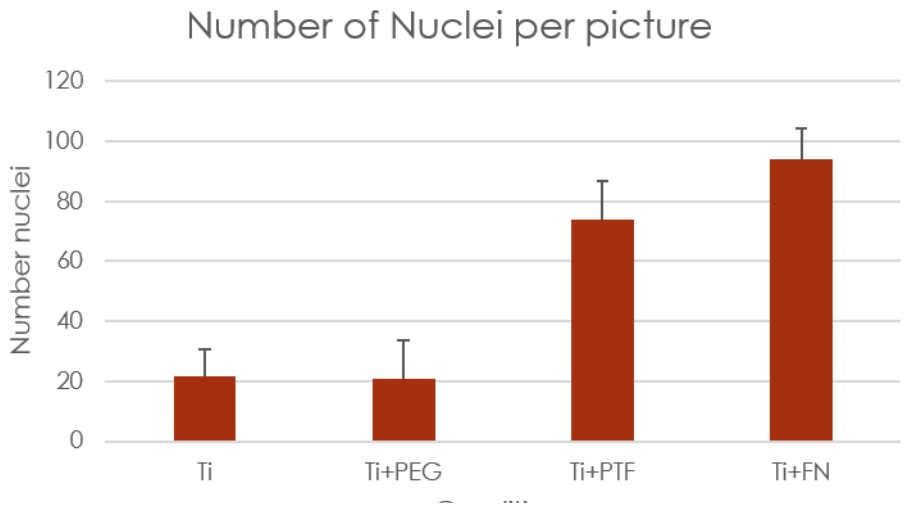

Figure 47: Graph of average number of nuclei per image in function of the condition

According to the graph above, the titanium and the Ti+PEG have a very low number of nuclei so a low number of cells. However, there are more than the double of nuclei for the Ti+PTF, and still more for the Ti+FN. Thus the adhesion efficiency of the peptidic platform is proved by immunofluorescence, with a result close to the fibronectin.

The figure (48) presents the area of cells on each sample obtained from the previous pictures:

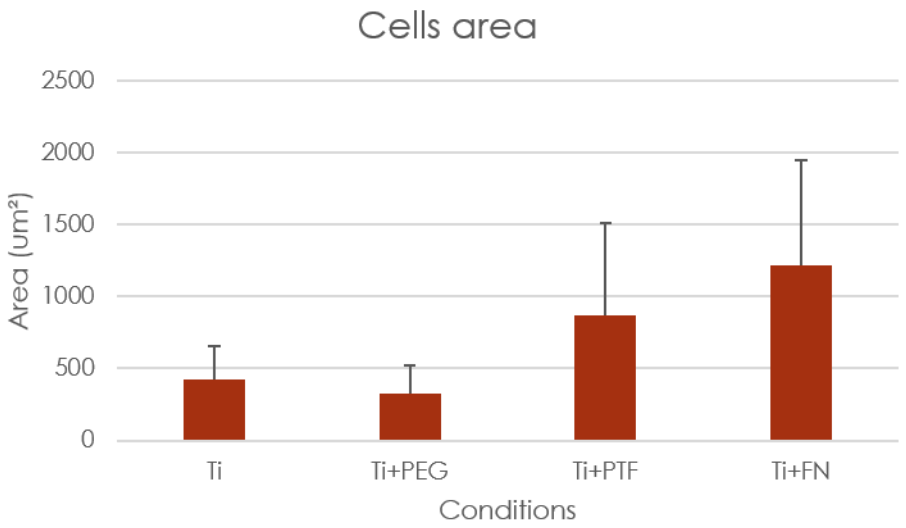

Figure 48: Graph of average cell area in function of the condition

The cell area in the case of the Ti+PEG is the lowest. This is due to the fact that there is no adhesion, so the cells are round and do not spread, involving a small area. On the contrary, the cells on Ti+FN have the highest area because they are the most spread. For control titanium samples, the cell area is relatively low, but still higher than the Ti+PEG. So it seems that the cellular adhesion on the titanium is not really good because the cells area is small so they do not spread well. However the previous pictures show that the cells are not as round as in the samples of Ti+PEG. Finally, the cells on Ti+PTF have a high area, almost the double of the cell area on titanium. It is correlated with the previous picture obtained by fluorescence and it confirms the efficiency of the PTF for the cellular adhesion. Moreover, the average cell area on those samples could be even higher if the platform was more homogeneously deposited on the samples. Indeed, the round cells on the samples of Ti+PTF reduce the average cell area for this condition. But those cells are in areas of the samples where there is no PTF and are directly on PEG as explained previously; therefore they do not adhere well on the samples.

4.8 Anti-bacterial efficiency

4.8.1 Agar plate

The first bacteriological assays were done counting CFU grown on agar plates.

A first assay was realized with *S. Sanguinis* on titanium, Ti+PEG and Ti+PTF. However, the bacteria did not grow. In order to check the viability of the protocol and optimize it, and given the low quantity of PTF available for the project, the next assays were done only with titanium and Ti+PEG. Unfortunately, due to a lack of time, it has not been possible to repeat the bacteriological assays with Ti+PTF.

An assay for each bacterial strain was first realized on titanium and Ti+PEG, with dilutions D2, D3, D4. For this assay bacteria were left to adhere on the disks for 4 h.

However, in the case of *S. sanguinis*, the bacteria did not grow on the agar plates. On the contrary, for *E. coli* the following results were found:

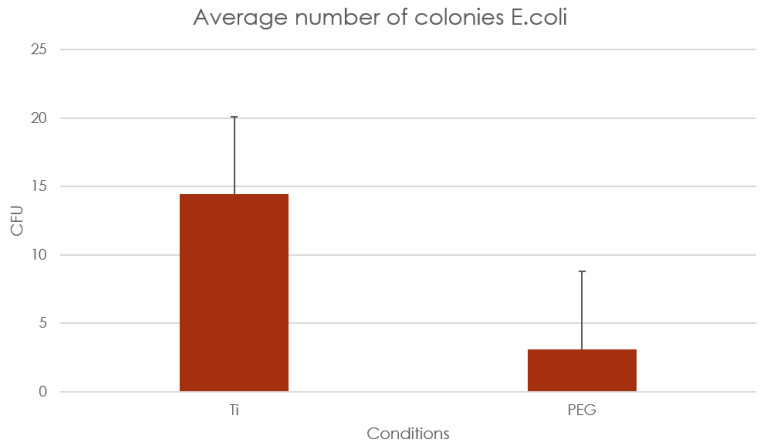

Figure 49: Graph of number of colonies for a dilution d3 with E. Coli

In the dilution D2, the colonies were too numerous thus it was impossible to count them. According to fig(49), there are more colonies on control titanium, which is the expected result. Now, in order to

know if there is a reduction of the concentration of bacteria with the PEG, we have to translate the number of colonies into concentration (CFU/mL).

Since a drop deposited on agar contains 5 µL of solution if we have **x** CFU for a D2, then the concentration **y** is:

$$y = \frac{x}{5 * 10\text{^} - 3} * 10\text{^}2$$

The factor 10^2 is due to the fact that a D2 is the initial solution diluted two times by 10. For a D3, the factor will be 10^3. Then the average number of CFU/mL is calculated for each condition, and with it, the reduction due to the PEG.

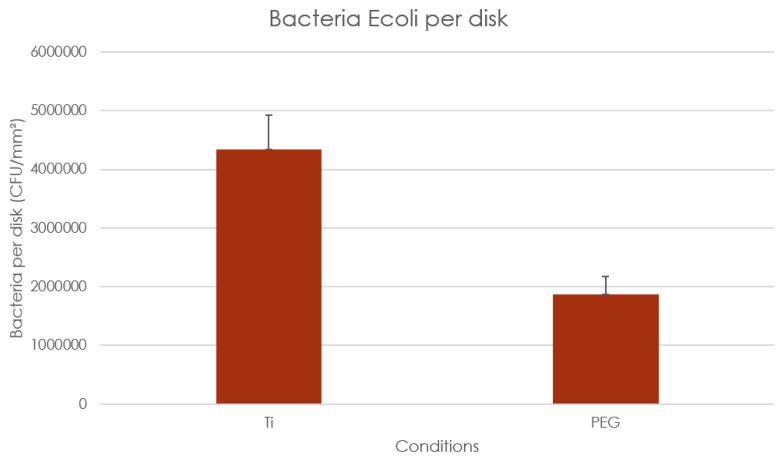

Figure 50: Graph of bacteria E. coli per disk depending on the conditions (calculated from dilution D3)

The fig (50) shows a reduction of 50% in bacteria concentration for the Ti+PEG condition Therefore, the PEG coating seems to reduce the number of bacteria attached to the surface. However, since the assay is not very precise, it would be necessary to repeat it to draw more conclusions. Moreover, since the deposition of PEG was done more than a month before the assay was performed, it could be hypothesized that the coating has lost effectiveness.

4.8.2 Alamar blue method

Since the results obtained with the agar assay were not conclusive, the Alamar blue method was tested as alternative.

a) E coli and S. Sanguinis:

The first assay was done during the agar assay, which gave the previous results with the same strains. The fluorescence varies with the bacteria concentration. The higher the concentration is, the faster will react the AB solution, so the fluorescence will increase faster. The following values were obtained for *E. Coli* (fig 51) and *S. Sanguinis* (fig 52):

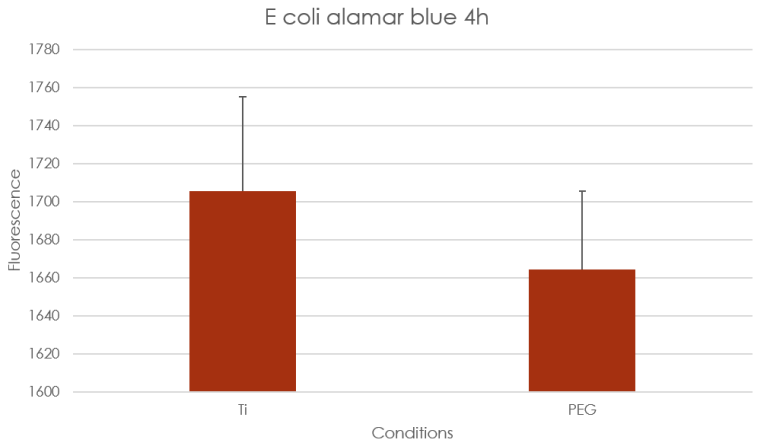

Figure 51: Graph of fluorescence of Alamar blue solution in function of the condition (E. coli)

For *E. Coli* strain, a significant reduction of the fluorescence can be noticed on PEG samples, thus indicating the number of bacteria attached to these surfaces is decreased. These results correlate with the previous observations using the agar method.

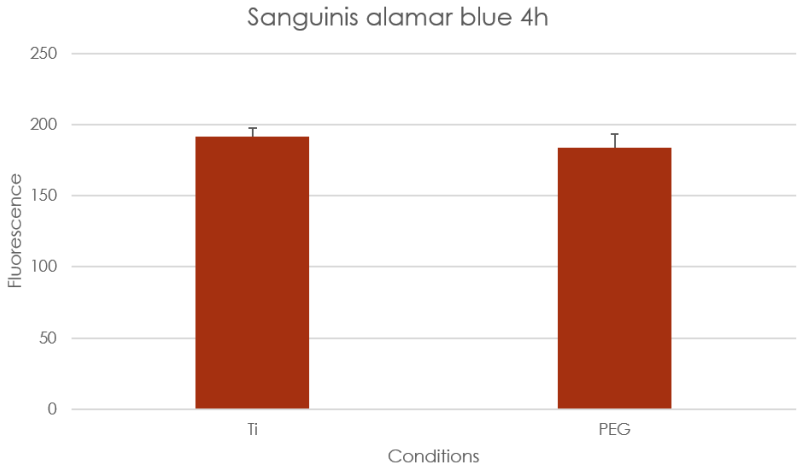

Figure 52: Graph of fluorescence of Alamar blue solution in function of the condition (S.Sanguinis)

However, the effect on *S. Sanguinis* is much less pronounced: the samples with PEG seem to have a lower concentration of bacteria, but the reduction is lower compared to the results observed for E. coli.

b) Biofilm E. coli and S. Sanguinis 48h

The study of biofilm was done with the samples used for the previous assay. Biofilm formation was analyzed after 48h of incubation.

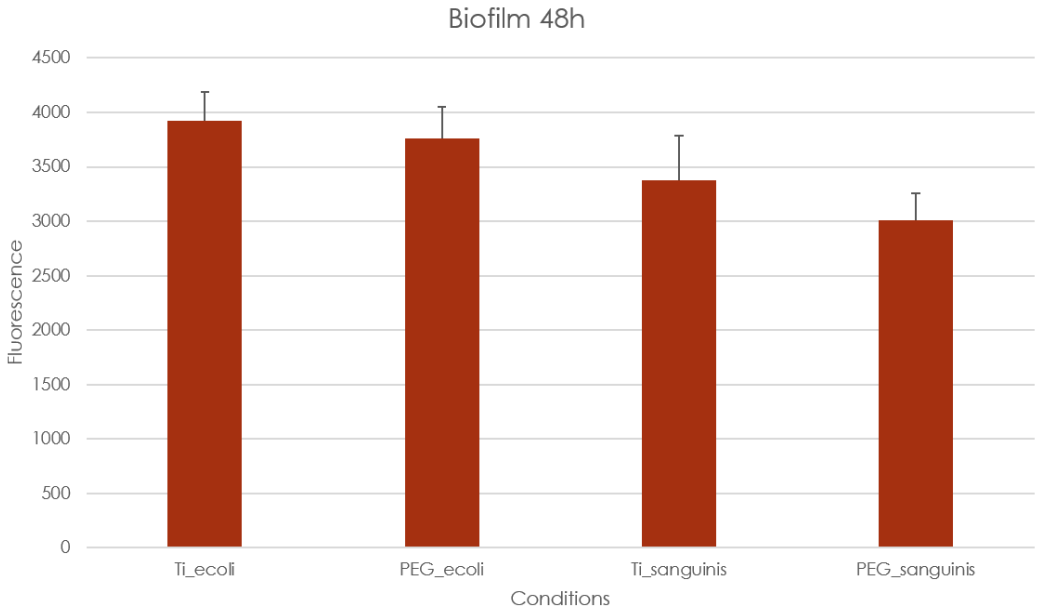

Figure 53: Graph of fluorescence of AB solution from biofilm in function of the condition (E.coli & S.Sanguinis)

Reduction	48h
E. coli	4,74%
S .Sanguinis	12,3%

Table 5: Reduction of bacteria concentration between Ti and Ti+PEG after 4h according to the fluorescence difference for S. Sanguinis and E. coli

It can be seen on the graph that the fluorescence is higher for the biofilm (48h) than for the bacterial adhesion assay after 4h. This is due to the fact that the bacteria grow during this time so the concentration is higher. But as previously mentioned, the same trend can be noticed; the concentration of bacteria on samples coated with PEG is lower. Thus, not only the adhesion of bacteria seems to be reduced with the PEG, but it also seems to be inhibited the development of a biofilm. Nevertheless, this is just a conclusion based on a trend. The next step for further investigations would be to realize the Alamar Blue and agar assays with PTF functionalized samples to know its efficiency against bacteria.

5. Conclusion

The first main objective of this project was the characterization of the functionalization treatment done on the titanium samples, with PEG and PTF. A study of roughness demonstrated that the PEG deposition did not impact significantly the topography of titanium. A small reduction of roughness was, however, noticed. This smoothing is probably due to the PEG deposition filling the gaps on the surface of the titanium. The small variation of roughness may be due to the relatively small thickness of the PEG layer measured with SEM-FIB. The SEM is used in order to see the influence of PEG on the aspect of the surface. It has been seen that the surface of the samples coated with PEG presented more granules or spherical particles. The PTF layer is thinner than the PEG, therefore it will not impact the roughness of the surfaces.

The PEG did not impact significantly the roughness of the samples, but it increased drastically the wettability of the samples. This is due to the fact that the hydrophilic behavior of PEG is higher than for titanium. With the same reasoning, the PTF deposited on PEG decreased the wettability of the samples because it is less hydrophilic than PEG. Finally, the analysis by FTIR proved chemically the presence of PEG on the samples by showing similar chemical bonds on the spectra.

As expected, the LDH assay gave good results toward the cell adhesion efficiency of the PTF. The cell adhesion was similar to that of fibronectin, which is a natural cell adhesive protein known for its efficiency. Thus the synthetic PTF succeeded to almost reach the cell adhesion ability of a natural protein, in spite of the antifouling behavior of the PEG coating. Therefore it could be interesting to test the efficiency of the PTF on a sample without PEG to determine the influence of the PEG coating on the cell adhesion ability of the PTF functionalized samples.

A hypothesis emitted is that the deposition of the PTF on the samples could not be homogeneous, involving a reduction of adhesion in areas where the PTF is not correctly deposited. This hypothesis is supported by the results of immunofluorescence. Indeed, The PEG proved its antifouling effect with circular cells on the pictures, which means they did not adhere on the surface. However, for the PTF the majority of cells were well spread, but some were circular. This inhomogeneity in cells shape is an additional argument to the hypothesis of uneven repartition of PTF. An improvement concerning the deposition of PTF on the samples could, in the future, improve the distribution and coverage of the PTF and thus increase the cell adhesion efficiency on the functionalized titanium.

As explained previously, due to a lack of time, it has not been possible to prove the bactericidal effect of the PTF. However, encouraging results were found for the PEG. According to agar assays, a reduction of bacteria concentration on PEG coated samples has been noticed. In order to have more accurate results, the Alamar Blue assay has been initiated. A trend of bacteria reduction has been noticed for both short term (4h) bacterial adhesion and biofilm formation (48h) on PEG-coated samples, thus confirming the antifouling ability of PEG.

Future perspectives

To improve the characterization of the PTF, an XPS analysis could be done to characterize the chemical presence of the PTF and PEG on the samples. The antifouling effect of the PEG could be tested with a protein adsorption assay, to study its behavior toward the proteins too. The cell adhesion efficiency of the PTF could be also analyzed with a proliferation assay. The antibacterial effect of the PEG and PTF needs to be tested again by more agar assays or Alamar Blue.

And as explained previously, the homogenization of the deposition of the PTF on the samples can be a way to improve the properties of the functionalized titanium in the future.

6. Economic impact

In this part will be presented the economic impact of this project in the tables below. It is important to know that most of the following prices are estimations and only help to give an overview of the approximate total cost of the project.

Sample preparation			
name	**quantity**	**Price**	**Cost (€)**
Titanium disk of 10 mm diameter and 2mm thickness	90,00	0,7369€/disk	73,69
Bakelite embedment LaboPress	6h	10€/h	60
Automatic polishing machine Buehler	15h	30€/h	450
Grind of 305 mm diameter P800	10	150€/100	15
Grind of 305 mm diameter P1200	10	100€/100	10
Grind of 305 mm diameter P2500	10	450€/100	45
Velvet grind of 305 mm diameter	2	500€/25	40
Colloïdal alumina 1 µm-0,5 µm	4L	60€/L	240
24-well plates	2	65€/5	26
48-well plates	20	381€/100	76,2
PEG	5g	90€/5g	90
Crosslinker	132mg	80€/25mg	422
Total cost			1547,5

Figure 54: Estimated cost of the sample preparation

Reagent used for cleaning and other treatments			
name	**quantity**	**Price**	**Cost (€)**
Acetone	1L	16,70€/L	16 ,7
Ethanol	1,5L	25,50€/L	38,25
Cyclohexane	0,7L	37,20€/L	26
Distilled water	5L	0,1€/L	0,5
Dimethylformamide	0,5L	60€/L	30
Total cost			111,45

Figure 55: Estimated cost of reagents used

Cell and bacteria assays			
name	**quantity**	**Price**	**Cost (€)**
Mccoy 5A Medium	500mL	25,6€/500mL	25,6
FBS	50mL	100€/500mL	10
HEPES	10mL	91,75€/100mL	9,2
Sodium pyruvate	5mL	12,28€/100mL	0,6
Penicillin-Streptomycin	5mL	14€/100mL	0,7
L-Glutamine	5mL	15,26€/100mL	0,76
Todd Hewitt Broth powder	73g	72,6€/100g	53
Brain Heart Infusion Broth powder	74g	36,4€/100g	27
Bacteriological Agar	40g	986€/kg	40
PBS tablets	4	178€/100 tablets	7,12
Total cost			174

Figure 56: Estimated cost of the cell and bacteria assays

Equipment used			
name	**quantity**	**Price**	**Cost (€)**
White light interferometry	3h	50€/h	150
Contact angle device	3h	40€/h	120
Fluorescence microscopy	6h	60€/h	360
FTIR	6h	35€/h	210
Plasma activation	2h	20€/h	40
SEM	3h	40€/h	120
Test tubes, flasks, pipettes, etc	-	-	400
Total cost			1400

Figure 57: Estimated cost of the equipment used

Work involved			
name	**quantity**	**Price**	**Cost (€)**
Junior engineer	800h	-	0
PhD Student	700h	30€/h	21000
Senior engineer	100h	50€/h	5000
Total cost			26000

Figure 58: Estimated cost of the work involved

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