Abstract

Titanium implants are nowadays often used in orthopedics and dentistry to repair or replace bone injuries or defects caused by aging, accident or diseases. Even if titanium is biocompatible, a low osseointegration between the implant and the surrounding tissue may result in graft rejection and a series of unwanted reactions.

To overcome these issues, two main strategies have been investigated: a physicochemical modification of the implant surface (e.g. roughness, energy or chemistry of the surface) or a functionalization of the surface with cell adhesive molecules.

This research project will mainly focus on the second approach which consists on the biofunctionalization of the surface. There are many types of cell adhesive molecules that can be used for such purpose. One of the most common family of cell adhesive molecules used are the proteins found in the extra cellular matrix (ECM). The cell bonding activity of these proteins is usually contained within a few amino acids. This is the case of the sequence Arg-Gly-Asp (RGD) found in many proteins of the ECM. Thus, the use of these short peptide sequences has been explored to biofunctionalize materials and to improve cell adhesion.

To create a covalent and stable binding between the peptides (organic molecules) and the titanium surface (inorganic surface), the use of linker molecules is required. This can be achieved by using organosilanes.

The aim of this project is to improve the adhesion of the osteoblasts onto the titanium graft. In order to do so, different activation methods of the titanium surface will be explored followed by a silanization step that ensure the attachment of the peptides to the surface. Then, RGD peptides will be immobilized to improve the binding of the cells on the surface.

The central theme of this research will be to compare two methods that increase and accelerate the adhesion of the cells in order to decrease the risk of complications. The first method is a simple physisorption of the peptide onto the implant surface and the second method is a creation of a strong covalent bond between the surface of the implant (made of titanium) and the peptides. The effect of the distinct activation treatments on the physicochemical properties of the surfaces will be investigated by
means of contact angle measurements, interferometry, scanning electron microscopy and X-ray photoelectron spectroscopy. The biological properties of the functionalized materials will be explored by means of cell adhesion assays with osteosarcoma cells.
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**Abbreviations**

Ti: Titanium

RGD: sequence of amino acids Arg-Gly-Asp

ECM: extra cellular matrix

SEM: scanning electron spectroscopy

XPS: x-ray photoelectron spectroscopy

SAOS-2: sarcoma osteogenic cells

PBS: phosphate buffered saline

FBS: fetal bovine serum

LDH: lactate dehydrogenase

BSA: bovine serum albumin

M-PER: mammalian protein extraction reagent

PFA: paraformaldehyde

Gly: glycine

DAPI: 4',6-diamidino-2-phenylindole

CA: contact angle

SE: surface energy
1. Introduction

1.1. State of the art

Every year, millions of metal devices are implanted in the human body in order to repair injuries or defects caused by aging, accident or diseases. Even if those implants are biocompatible, it can happen that the body rejects this graft leading to another operation in order to save the life of the patient. In fact, rejection but also a loosening of the implant can lead to hypersensibility, internal bleedings, embolisms and other complications that force the surgeons to re-operate the patient. A poor biointegration will also result in a low mechanical fixation of the implant with the bone leading to other surgeries. All those revision surgeries cost hundreds of millions to the society and this is why this problem needs to be fixed.  

There are two ways to decrease the rate of implant rejections:

- The use of immunosuppressant that will for a moment neutralize the immune system so that the leucocytes and the macrophages will not recognize the implant as a threat.

- The use of biocompatible materials that will facilitate the osseointegration of the implants.

Bone formation is the result of a succession of events that begin with recruitment and proliferation of osteoprogenitor cells from surrounding tissues, followed by osteoblastic differentiation, matrix formation, and mineralization. In bone grafting there are three basic biological mechanisms to favor bone regeneration:  

- Osteogenesis: In osteogenesis, viable osteoblasts or osteogenic cells (bone forming cells) are transplanted from one part of the body to the site where new bone is needed.

- Osteoconduction refers to the ability of some materials to serve as a scaffold on which bone cells can attach, migrate, grow, and divide. As only living cells can form new bone, the success of any bone-grafting procedure is dependent upon having sufficient osteogenic cells in the area.

- Osteoinduction: The induction of bone formation refers to the capacity of some natural substances in the body to stimulate primitive stem cells or immature bone cells to grow and mature, forming healthy bone tissue.
Therefore, in order to facilitate osseointegration, implants must be modified to increase the adhesion of osteoblasts onto the implant surface. A successful osseointegration of the implant with bone tissue will decrease to a large extent the complications previously explained.

Over the last centuries, few materials have been selected to create implants based on their biocompatibility and their physical and chemical properties. The relatively corrosive environment combined with the poor tolerance of the body to even microscopic concentrations of most metallic corrosion products eliminates from discussion most metallic materials. \(^3\)

Attempts to use titanium for implant fabrication go back to the late 1930’s. Titanium's lightness and good mechanical and chemical properties are salient features for implant applications. Titanium, nevertheless, has poor shear strength, making it less desirable for bone screws, plates and similar applications. Titanium derives its corrosion resistance to the formation of a surface oxide film. Under in vivo conditions, the oxide layer is the only stable reaction product.\(^3\)

Titanium is thus, one of few materials that naturally match the requirements for implantation in the human body because it is light, strong and totally biocompatible.

Titanium can be used to make implants for:

- Bone and Joint Replacement (Fig. 1). Millions of patients worldwide are treated annually for total replacement of arthritic hips and knee joints.

![Figure 1: Titanium hip implant.](image-url)
- Dental Implants (Fig. 2). A titanium 'root' is introduced into the jaw bone and with time the implant will become part of the bone helped by the osseointegration (bone cells will migrate to the implant and bind it to the jaw). The superstructure of the tooth is then built onto the implant to give an effective replacement.

- Cardiovascular devices. Titanium is regularly used for pacemaker cases and defibrillators, as the carrier structure for replacement heart valves, and for intra-vascular stents.

Even if titanium is biocompatible and a perfect candidate for osseointegration, this phenomenon does not occur fast enough. Titanium is biocompatible but not bioactive. A lot of researches have been made in order to improve osseointegration.

- There are several methods to improve the osseointegration. One of the main methods is a modification of the titanium surface.

  1) By a modification of the surface morphology as for example a modification of the roughness of the implant. Modifying the roughness of the titanium surface will have a significant influence on the cell behavior. This influence can be positive or negative depending on the effect chosen. In fact, many
studies show that roughness can have a great effect onto the cell adhesion but also on the proliferation and the differentiation of the cells.  

2) By a modification of the surface chemistry using inorganic chemistry as for example an anodic plasma oxidation to produce a structured titanium oxide layer. Depending on the parameters of the plasma oxidation, amorphous or nanocrystalline structures can be obtained.

- Another strategy would be to functionalize the surface implant with cell adhesive molecules to increase the adhesion of cells.

1) In the bones, the extra cellular matrix (ECM) consist of about 20% of an organic phase (osteoid) and 70% of an inorganic phase (hydroxyapatite). The organic phase is composed of more than 90% of type I collagen. One way to functionalize the implant would be to use hydroxyapatite, a cell adhesive molecule mainly used in many modern implants to promote osseointegration. 

Another type of cell adhesive molecules are ECM proteins like laminin, collagen or fibronectin. Those molecules can be deposited onto the implant surface by physisorption or covalently attached.

2) Instead of using complete ECM proteins, smaller proteins called peptides are synthesized. In fact, while whole proteins can be used, the peptides will be chosen to simplify the coupling and subsequent characterization. They also act as regulatory receptors that can initiate intracellular signal pathways. Certain short amino acids sequence bond to cell surface receptors, and mediate at least some of an entire protein’s biological activity. Because cell adhesion receptors recognize only certain amino acid sequence, the use of an appropriate peptide can lead to a cell-selective surface. One of the most effective peptide is the RGD peptide (Fig 3), a peptide with the sequence Arg-Gly-Asp. It is found in fibronectin or vitronectin, a component of the ECM.

![RGD sequence](image-url)
Those RGD peptides can interact with integrins. Integrins (Fig 4) are a major family of cell surface receptors that are responsible for anchoring cells to ECM, and that function as cell-cell adhesion molecules. They are transmembrane proteins located at the surface of the cells that will interact with the RGD peptides sending a signal to cells to bind with the implant surface.\textsuperscript{15}

Of all those methods previously described, the functionalization of surfaces with RGD peptides represents a very promising strategy and so far has shown an improvement of osseointegration \textit{in vitro} \textsuperscript{9,16} but also \textit{in vivo} \textsuperscript{17,18} by creation of a continuous bone layer.

Various studies have been made in order to functionalize the titanium surface with RGD peptides. To realize a simple physical adsorption, the RGD peptides just need to be deposited onto the titanium implant. However, physisorption is useful for proteins but not for peptides because the bond is unstable since it is based on weak interactions such as Van der Waals interactions or hydrogen bonds\textsuperscript{19}. Alternatively, peptides can be covalently attached. There are many approaches to bind them to the surface of the titanium implant. One of them is silanization. A silanization is first made to create a binding between an inorganic material (metals) and an organic material (peptides). A silanization is the covering of a surface with organofunctional alkoxysilane molecules.
To be able to silanize the surface, this one must be activated with the creation of hydroxyl groups. The more hydroxyl groups are created on the surface, the better the silanization will take place. This activation can be done by various methods like an acidic or basic etching\textsuperscript{20,21} or plasma treatment\textsuperscript{22}. The hydroxyl groups at the surface of the titanium will then attack and react with the alkoxy groups on the silane and form a covalent binding Si-O-Si.

The aim of this project will be to determine which method (between physisorption and covalent binding) would the best one to functionalize titanium surfaces with RGD peptides in order to improve the osseointegration. To this end, the functionalization of the surface will be studied in terms of physicochemical and biological properties.

1.2. Objectives of the project

This project is composed of two main objectives:

1) Characterize the process of titanium functionalization (activation, silanization and peptide immobilization) in terms of physic-chemical properties of the surface. Compare distinct activation methods.

2) Study and compare the adhesion of sarcoma osteogenic cells on surfaces functionalized with RGD peptides via two strategies: covalent binding and physical adsorption.

The first objective will be achieved by doing several chemical treatments to activate the surface before the silanization.

Chemical treatments:
- Plasma cleaning
- Sodium hydroxide etching
- Nitric acid etching

The different activation/functionalization protocols will be analyzed in terms of:

- Surface physic-chemical properties (contact angle, surface energy, roughness, SEM images, XPS )
- Cell adhesion with sarcoma osteogenic cells
2. Experimental methods

2.1. Preparation of the samples

- **Cutting process**

  The original piece was a 1 meter bar of commercially pure Grade 2 titanium (initial diameter: 10 mm).

  The titanium samples were put with an automatic machine (Fig. 5) (Brand: STRUERS, model: Accutom-50).

  **Parameters**: speed of cut of 0.025 mm/s at the beginning of the cutting process and from 0.040 to 0.050 mm/s during the rest of the time.

  After the cutting process, all the samples had a 10 mm diameter and a 2.5 mm width.

- **Polishing process**

  The samples were polished using two automatic machines.

  1) The samples were glued to a disk and then polished automatically with a polishing machine (Fig. 6) (Brand: BUEHLER, Model: Ecomet 4)
Figure 6: samples unpolished glued on the disk (on the left) and the automatic polishing machine (on the right)

2) The samples were put into Bakelite (Force: 20 N, 5 minutes of heating, 3-4 minutes of cooling) and then polished with an automatic machine (Fig. 7) (Brand: STRUERS, model: RotoPol-31 y RtoForce-4)

Figure 7: automatic polishing machine

Several grinds were used to polish the samples:

- P 400 (quick step to remove the big impurities, abrasive)
- P600 (10 to 20 minutes)
- P 800 (10 minutes)
- P1200 (10 minutes)
- P2400 (10 minutes)
- P4000 (10-15 minutes)
- Silica solution with 1 micrometer diameter particles. (5-20 minutes)

The force used on each machine was 10 N for the polishing grind and 5 N for the silica solution. The rotation of the disks was at 300 rpm.

After the polishing process, the samples are like small mirror without scratches.

2.2. Activation treatments

2.2.1. Plasma activation

Goal: the aim of this treatment is to clean and activate the surface of the sample by creating the formation of hydroxyl groups that will allow its further functionalization. This treatment is also a cleaning treatment so no washes will be needed afterwards.

The samples were put in a glass cup and put into the plasma cleaner chamber. After having made the vacuum with the pump during 2 minutes, the main chamber was filled with O₂. The treatment lasted 5 minutes after the apparition of the O₂ plasma with a high level of RF (radio frequency).

Plasma cleaner characteristics:
- Brand : HARRICK
- Model : PDC-002
2.2.2. Nitric acid activation

**Goal:** The nitric acid is a very corrosive and oxidizing acid. The aim of this treatment is to clean and activate the surface of the sample by oxidizing Ti and introducing hydroxyl groups to facilitate the silanization\(^\text{20}\).

After having put the samples into a beaker, the samples were covered with nitric acid at 65 %. The reaction lasted 1 hour at room temperature. After the treatment, several washings were made to clean the surface from any impurities left or made during the treatment. The samples were washed 3 times with water, 3 times with ethanol and 3 times with acetone. They were finally dried with N\(_2\).

2.2.3. Sodium hydroxide activation

2.2.3.1. 2 hours treatment

**Goal:** The aim of this treatment is to clean and activate the surface of the sample by creating a sodium titanate layer and subsequent hydroxyl groups in order to facilitate the silanization\(^\text{21}\).

Samples were treated with a 5M NaOH solution for 2h under sonication in an ultrasonic bath. After this treatment, the samples were put in distilled water in an ultrasonic bath for 30 minutes to be washed. This operation was repeated once. The last step has been a series of washes with ethanol (3 times) and acetone (3 times). To finish, the samples were dried with N\(_2\).
2.2.3.2. Overnight treatment

**Goal:** the aim of this treatment is to clean and activate the surface of the sample by creating a sodium titanate layer and subsequent hydroxyl groups in order to facilitate the silanization\(^\text{21}\).

Samples were treated overnight in a **5M solution of sodium hydroxide at 60°C** (about 12 hours). After the treatment, the samples were washed following the same protocol as for the 2h treatment.

2.3. Silanization

2.3.1. Reagents

- **Toluene**
  
  ![Toluene](image)

  **Formula:** C\(_7\)H\(_8\)
  
  **Density:** 0.8670 g/cm\(^3\)
  
  **Molar weight:** 92.1384 g/mol

- **3-chloropropyltriethoxysilane (CPTES)**
  
  ![3-chloropropyltriethoxysilane](image)

  **Formula:** C\(_9\)H\(_{21}\)ClO\(_3\)Si
  
  **Density:** 1.025 g/cm\(^3\)
  
  **Molar weight:** 240.8 g/mol

- **Diisopropylethylamine (DIEA)**
  
  ![Diisopropylethylamine](image)

  **Formula:** C\(_8\)H\(_{19}\)N
  
  **Density:** 0.742g/mL
  
  **Molar weight:** 129.24g/mol
2.3.2. Protocol

**Goal:** Create a covalent binding between the hydroxyl groups at the surface of the sample and the silane in order to ‘bind’ a non-organic material (the metallic samples) and an organic material (peptide).

After putting the samples in an erlenmeyer and adding a magnetic stirrer, the erlenmeyer was closed with a plastic cup and the vacuum was made in order to remove all entities present in the air that could lead to undesirable reactions. Then, to be sure that no oxygen can alter the reaction, a purge with N\textsubscript{2} has been made. Once the erlenmeyer is ready to be used, **10 mL of toluene** were added with a syringe as solvent. Then, **0.2 mL of CPTES** were added and to finish **0.1 mL of DIEA** were added.

The silanization reaction was done on a heating magnetic agitator at **70 °C during 1 hour**. After the reaction, the erlenmeyer was put 5 minutes in an ultrasonic bath to remove all silanes not covalently bound to the surface.

The last step of this treatment is a series of washes with toluene (3 times), ethanol (3 times), isopropanol (3 times), water (3 times) and acetone (3 times). Finally, all the samples were dried with N\textsubscript{2}.

2.4. Immobilization of the RGD peptide

2.4.1. Reagents

- Sodium carbonate buffer

  Sodium carbonate: Na\textsubscript{2}CO\textsubscript{3}

  » Dissolution in water/PBS to give a basic buffer.

  \[
  \text{Na}_2\text{CO}_3 + 2 \text{H}_2\text{O} \rightarrow 2 \text{Na}^+ + \text{H}_2\text{CO}_3 + \text{HO}^-
  \]
2.4.2. Protocol

- Peptides covalently bound

100μL of a 100μg/mL solution of RGD peptide in a sodium carbonate buffer were added to each sample. The functionalization was left overnight at room temperature.

- Physisorption

100μL of a 100μg/mL solution of RGD peptide in a PBS buffer were added to each sample. The functionalization was left overnight at room temperature.

2.5. Contact angle and surface energy

The contact angle, also called wettability, is the angle between the interface solid/liquid and liquid/vapor (Fig. 10).

The equipment used for was a contact angle system OCA (Fig. 9). The software used is SCA20.
The solvents used were distilled water and diiodomethane.

The drop deposited was of 1μL and the dosing rate was 1μL/s.

The method used for the contact angle measurements was the sessile drop method and the approximation used to determine wettability were the ones of Laplace and Circle fittings.

The energy at the surface of a biomaterial is defined by its general charge density and the net polarity of the charge.$^{23}$

The equation used to calculate the surface energy was the one of Ström et al using water and diiodomethane as solvents.
2.6. Scanning Electron Microscopy (SEM)

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. The SEM is also capable of performing analyses of selected point locations on the sample; this approach is especially useful in qualitatively or semi-quantitatively determining chemical compositions (using Energy dispersive X-ray Spectroscopy), crystalline structure, and crystal orientations.
Figure 11: Schematic drawing of the electron and X-ray optics of a SEM combined with an electron probe microanalyzer. 

2.7. White light interferometry

This technique will allow us to measure the roughness of the samples after distinct activation treatments to determine variations on the surface topography.

An interferometer is an optical device that splits a beam of broadband light in the infrared range (1300 nm) into two separate beams. One beam is directed at the object surface, and the other at a mirror in the interferometer reference arm. The probe receives and recombines the reflected light from the sample and reference arms and records the resulting interference from which a wide range of information about the object can be retrieved: its surface shape, roughness or waviness (Fig. 12).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra</td>
<td>arithmetic roughness average</td>
</tr>
<tr>
<td>Rq</td>
<td>root mean square roughness</td>
</tr>
<tr>
<td>Rt</td>
<td>maximum height of the surface (distance from the highest peak to the lowest)</td>
</tr>
<tr>
<td>Rz</td>
<td>average of the extreme heights (ten lowest and ten highest)</td>
</tr>
</tbody>
</table>

Figure 12: Roughness parameters
The values were taken at Mag 50 in mode VSI. In VSI Mode, the white-light source is filtered with a neutral density filter, which preserves the short coherence length of the white light. The magnification objective moves through focus in a controlled manner. The detector measures the modulation corresponding to every focus point on the surface as the objective moves vertically providing the degree of fringe modulation, or coherence (while PSI mode measures the phase of the interference fringes). The intensity signal leads to the modulation data which allows calculating the surface heights.

The software used to analyze the data was Vision 4.1. To ameliorate the data and reduce the texture component wave, a filter was used (Windows filtering: Fourier filtering, Fourier filtering: high pass, Fourier filtering window: Gaussian, High cut off: 55 1/mm)

2.8. X-ray Photoelectron Spectroscopy chemistry

This technique is used to characterize the surface of a material. It gives information about the composition and the chemical state of the elements that are found at the surface of the sample.

Principle: there is an irradiation of the solid surface with monochromatic X-ray photons (E~1000 eV, λ~1 nm) and then an energy analysis of emitted photoelectrons is done.

![XPS principle](image)

According to the level of energy that the photoelectrons have, an analysis indicates which kind of elements was at the surface. Graphics will be obtained and after a
deconvolution of the different graphic, it is possible to characterize the composition of the surface and the type of elements.

A typical XPS spectrum is a plot of the number of electrons detected (Y-axis) versus the binding energy of the electrons detected (X-axis). Each element produces a characteristic set of XPS peaks at characteristic binding energy values that directly identify each element that exist in or on the surface of the material being analyzed. These characteristic peaks correspond to the electron configuration of the electrons within the atoms, e.g., 1s, 2s, 2p, 3s, etc.\textsuperscript{28, 29}.

The analysis was done with the software CasaXPS.

<table>
<thead>
<tr>
<th>Type of elements detected</th>
<th>Range of binding energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titanium oxides</td>
<td>528-531</td>
</tr>
<tr>
<td>Hydroxyl groups</td>
<td>531-532</td>
</tr>
<tr>
<td>Water</td>
<td>533-534</td>
</tr>
</tbody>
</table>

Table 1: Range of binding energy for the analyzed elements

2.9. Cellular studies

2.9.1. Type of cells

The cells used in this project are sarcoma osteogenic cells known as SAOS-2 cells. Those cells are of human origin from an osteogenic sarcoma. They were established from the primary osteogenic sarcoma of an 11-year-old Caucasian girl in 1973. This type of cells was chosen because those cells secrete particulate matters containing bone inducing agents thought to comprise bone morphogenic proteins. They are also adherent to a surface (the flask), fast growing (doubling time of 43 hours) and can easily differentiate into osteoblastic cells.\textsuperscript{30}

Culturing conditions: the cells were cultivated in culture flasks Nunc with plastic filtered cups and let in an incubator to grow and proliferate. The medium used was McCoy’s completed with FBS. It was changed every 2-3 days. Each flask was filled with a total of 12 mL of medium with cells.
2.9.2. Preparation of the medium

The medium used all cellular studies was made of:

- Medium McCoy’s 5M (41 mL): this medium was without L-glutamine, ref M8403, conservation 4°C.

- Fetal bovine serum (7.5 mL): it is used to help the cells to grow, multiply and adhere to the flask (10%). Ref 10270-106, conservation -20°C and +4°C in use.

- Sodium pyruvate (0.5 mL): it is used as an additional source of energy, but also as a protective defense against hydrogen peroxide that can cause apoptosis of the cells (process of death). Ref 11360-039, conservation +4°C.

- L-glutamine (0.5 mL): it is an amino acid that helps the cells to grow (1%). Ref 25030-024, conservation -20°C and +4°C in use.

- Penicillin (0.5 mL): it is used to avoid any bacterial contamination (1%). Ref 15140-122, conservation -20°C and +4°C in use.

2.9.3. Incubator

The incubator is used to facilitate the growth of the cells and to reproduce physiological conditions.

The following conditions are used:

- 5% of CO₂

- 95 % of relative humidity

- Usual temperature : 37°C

- Maximum temperature : 40°C

2.9.4. Trypsinization

The trypsin used is called TrypLE™ Express Stable. It is a trypsin-Like Enzyme with phenol. Ref 12605-028.
Trypsinization is done to detach cells from the culture flask.

This process is done according to the following steps:

- First, 2 washes of 10mL of PBS are done to remove dead cells.
- Then, 2mL of trypsin pre-heated at 37°C are added in the flask.
- 3mL of medium with FBS added after 1-2 minutes are added to stop the effect of trypsin. The 5mL of cells in solution are then transferred into a 15mL tube.
- 5mL of medium with FBS are added in the flask to remove any cells that still might be in the flask. Those 5mL are transferred into the tube.
- The tube with the 10 mL is centrifuged (5 minutes, 300 RCF).
- A pellet of cells is obtained after the removal of the solution. This liquid is then re-diluted into medium according to the needs for the assay. (usually 4mL of medium with or without FBS)

2.9.5. Cell adhesion assay

Each assay was done following the same protocol.

1) Preparation of the polystyrene multi-well plate and the samples
   - Pre blocking of 1 hour with BSA 1% in PBS (1mL/well) followed by 2 washes with PBS
   - Immobilization of the peptides (if necessary) followed by 3 washes with PBS
   - Blocking of 1h with BSA 1% in PBS (1mL/well)

2) Preparation of the cell solutions
   - 2 washes of the flask with PBS
   - Trypsinization following the protocol
   - Counting of the cells using a Neubauer chamber
   - Preparation of the concentrated solution of cells for the samples and for the standard curve if LDH assay is required

3) Sterilization of the plate and the samples
   - 2 washes of PBS (to remove the BSA)
- 10 minutes sterilization with ethanol (1mL/well) followed by 3 washes with PBS
- Add of the cells : 500 μL of solution/well depending on the concentration needed

2.9.6. **LDH cytotoxicity test**

After the incubation time, a reagent called M-PER (mammalian protein extraction reagent) is added into the wells. This reagent is a non-denaturing detergent formulation that dissolves cell membranes and extracts total soluble protein in 5 minutes.

The LDH Cytotoxicity Detection Kit (Fig. 14), (REF: 04 744 934 001, Version 05) offers a simple way to measure plasma membrane damage, based on the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in most cells.

The first step of this test is to transfer 100μL of each sample solution into a 96 well plate. The second step is to prepare the test solution with the LDH kit using a catalyst and a dye (1:46).

Next, 100 μL of the catalyst-dye solution is added in each well of the 96 well-plate. The reaction is left for 15 to 30 minutes protected from the light. The mixture will developed over time leading to a dark pink color.

50 μL of stop solution in each well will finally stop the reaction.

The results are analyzed with a multi-plate reader (Fig 15) at the wavelength of 492 nm and the reference wavelength is 690nm.

**Multi-plate reader:**
- Brand : TECAN
- Model : Infinite M200 Pro
2.9.7. **Immunohistochemistry analysis**

Immunohistochemistry refers to the process of detecting antigens (e.g. proteins) in cells by exploiting the principle of antibodies binding specifically to antigens.\textsuperscript{31}
This protocol describes the staining of actin, focal adhesion and nuclei of cells attached on metallic samples.

The reagents and steps used for this analysis are described below:

- Paraformaldehyde (PFA) 4% in PBS for 20 minutes to fix the cells on the samples and prevent deterioration

- 3*5 minutes washes with PBS-Gly. The presence of Gly helps to quench some autofluorescence observed for PFA

- Triton 0.05% in PBS for 15 minutes to permeabilize the samples and allow the diffusion into the cells of the antibodies that will be used. (followed by 3*5 minutes washing with PBS-Gly)

- BSA 1% in PBS for 30 minutes to ensure that only specific interactions take place in the following steps.
- Primary antibody: mouse anti-vinculin (1:200) in BSA 1% for 1h to stain the focal adhesions. (followed by 3*5 minutes washing with PBS-Gly)

- Secondary antibody: anti-mouse Alexa 488 (1:2000) in triton 0.05% for 1h
- Phalloidin-rodhamine (1:300) in triton 0.05% (add with the secondary antibody) for 1h to stain actin filaments. (followed by 3*5minutes washing with PBS-Gly)

- DAPI (1:1000) in PBS-Gly for 2minutes to stain the nuclei. (followed by 3*5minutes washing with PBS-Gly)

- Finally, samples are located in microscope slides. To protect the integrity of the samples and to reduce bleaching of fluorescent tags, a mounting, anti-fade solution called mowiol is added to each sample (1-2 drops).

12 samples have been studied (2 samples of each activation treatment + 2 physisorpted samples+ 2 control samples)

The microscope used was a Nikon E-600 upright microscope for transmitted and fluorescence illumination. The software to take pictures of the staining of the cells was cell^F and the one to analyze and mount the cells pictures was ImageJ. Three types of filters (Fig 53) were used according to the type of staining desired.

<table>
<thead>
<tr>
<th>Filter</th>
<th>Excitation Filter</th>
<th>Dichroic</th>
<th>Emission filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-2A</td>
<td>450-490</td>
<td>500</td>
<td>515</td>
</tr>
<tr>
<td>G-2A</td>
<td>510-560</td>
<td>565</td>
<td>590</td>
</tr>
<tr>
<td>A</td>
<td>330-380</td>
<td>400</td>
<td>420</td>
</tr>
</tbody>
</table>

Table 2: Description of the various filters used during the analysis
3. Results and discussion

3.1. Wettability

To study the effect of activation treatments on the hydrophilicity of the Ti samples, the wettability of the samples will be measured.

Each sample will be analyzed in 3 different spots following the same axis. To have a good statistic, each kind of treatment will be analyzed using triplicate of samples (9 measures / treatment). The wettability will give us information about the hydrophilicity of the surface. The more hydrophilic the surface get, the more effective the activation treatment is.

3.1.1. Polished/Unpolished

- Results

![Contact angle: Unpolished/Polished](image)

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>Diiodomethane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpolished</td>
<td>67.6</td>
<td>37.0</td>
</tr>
<tr>
<td>Polished</td>
<td>59.4</td>
<td>37.4</td>
</tr>
</tbody>
</table>

Figure 17: CA values obtained with unpolished and polished samples

- Discussion

As shown in Fig. 17, it seems that the polishing process decreases the contact angle of the samples. In fact, because after the polishing, the surface of the sample is smoother and without any kind of irregularities (scratches, dust …), the drop of water or diiodomethane will just spread more easily and
widely onto the surface and so the contact angle will decrease. We can correlate that there is a link between the surface state and the hydrophilicity. The smoother the surface is, the higher the hydrophilicity will be because the surface is removed from any hydrophobic impurities.

3.1.2. Activation treatments

- Results

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water Contact Angle</th>
<th>Diiodomethane Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54.3°</td>
<td>33.4°</td>
</tr>
<tr>
<td>Plasma</td>
<td>32.5°</td>
<td>36°</td>
</tr>
<tr>
<td>Nitric Acid</td>
<td>47.4°</td>
<td>35.5°</td>
</tr>
<tr>
<td>NaOH-2h</td>
<td>13.2°</td>
<td>9.7°</td>
</tr>
<tr>
<td>NaOH-o/n</td>
<td>9.7°</td>
<td>9.4°</td>
</tr>
</tbody>
</table>

Figure 18: CA values obtained on samples after different activation treatments

- Discussion

The first constatation that we can make after this contact angle measurements (Fig. 18) is that in general, the activation treatments tend to decrease the contact angle. This means that the affinity between the liquid deposited (water or diiodomethane) and the surface is increased. This could be explained by the formation of hydroxyl groups at the surface of the samples during the activation. Indeed, the main goal of the activation treatments is to form hydroxyl group at the surface of the samples.

Regarding all the treatments, it seems that sodium hydroxide etchings (during 2 hours or overnight) might be the more effective ones to increase the hydrophilicity of the surface because the contact angle with water goes from 54.4° (33.4° with diiodomethane) to 13.15° (9.7° with diiodomethane) for the 2
hour treatment and to 9.7° (9.35° with diiodomethane) for the overnight treatment.

If we have to make a ranking of the activation treatment in terms of hydrophilicity based on those results it would be:

(+ hydrophilic) NaOH o/n ≈ NaOH 2h > Plasma > HNO₃ (- hydrophilic)

### 3.1.3. Silanization

- **Results**

![Contact angle: Silanization](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water Contact Angle</th>
<th>Diiodomethane Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59,4</td>
<td>37,4</td>
</tr>
<tr>
<td>Plasma</td>
<td>53,4</td>
<td>36,3</td>
</tr>
<tr>
<td>Nitric Acid</td>
<td>53,2</td>
<td>38,4</td>
</tr>
<tr>
<td>NaOH-2h</td>
<td>12,0</td>
<td>11,1</td>
</tr>
<tr>
<td>NaOH-o/n</td>
<td>18,0</td>
<td>9,8</td>
</tr>
</tbody>
</table>

*Figure 19: CA value obtained on samples silanized after different activation treatments*

- **Discussion**

As shown of Fig. 19, the silanization seems to increase the contact angle. Indeed, the silane are hydrophobic molecules so it seems normal that the contact angle increase a bit compared to the activation but in any case, the wettability is still higher on the samples silanized than on the control samples.

Since for the activation treatments, there was a significant difference between the sodium hydroxide etchings and the two other treatments (plasma cleaning and nitric acid etching), this difference is even more obvious after the silanization. Indeed, now, the samples silanized after a plasma cleaning or a nitric acid treatment give almost
the same results whereas for the sodium hydroxide treatment the difference is of 40-45 ° from the control samples.

If we have to make a ranking of hydrophilicity the activation treatment based on those results it would be:

(+ hydrophilic) NaOH o/n ≈ NaOH 2h > Plasma > HNO₃ ( - hydrophilic)

3.1.4. Immobilization of peptides

As shown in Fig. 20, after the immobilization of peptides, the contact angle increased. Normally, the theory would say that the contact angle should decrease because the peptides used (RGD peptides) are hydrophilic but we observe the contrary. This can probably be explained by the spacer used in the peptides. Indeed, the peptides in itself is hydrophilic but the spacer is a molecule that is hydrophobic. That is why we obtain results showing that the surface is more hydrophobic.

For the case of the sodium hydroxide etching overnight, we observe a slight decrease of the wettability but this can be explained by the difficulty to obtain good results during the experiments. In fact, for the two sodium hydroxide etching samples,
the drops of liquid spreaded so fast that it was almost impossible to take a good measurement.

Regarding the samples where peptides were immobilized by physical adsorption, we observe a decrease of the contact angle meaning that the surface became more hydrophilic. The main difference here is there are no silanes and the surface is not activated.

Again, if we have to make a ranking of the activation treatment based on the hydrophilicity of the surface after treatment, the trend previously obtained is maintained.

(+ hydrophilic) NaOH o/n ≈ NaOH 2h > Physisorption > Plasma > HNO₃ (- hydrophilic)

3.1.5. Summary

Figure 21: summary of all the CA results
In each case of activation, silanization and then immobilization of peptides, the same trend is followed. We observe an important decrease of the contact angle after the activation treatments and a small increase after the silanization. The value obtain are still lower than the value obtained for the control samples. After adding the peptide, we observe an increase of the contact angle even if normally we should have a decrease of the contact angle.

The physisorpted samples seem to have a surface more hydrophilic than the plasma and nitric acid treated surface whereas this surface is less hydrophilic than the surface of the sodium hydroxide treated samples.

The results clearly show that NaOH is the activation treatment that has a higher influence on the hydrophilicity of the surface. This could be correlated with an increase of the type of titanium oxide layers or a number of hydroxyl groups. It might also be that this treatment is the one removing more efficiently the hydrophobic impurities of the surface.

3.2. Surface Energy

Since the surface energy directly influences two important phenomena for an efficient cell/biomaterial interaction: proteins adsorption and cell attachment\(^{34,35}\), it could be interesting to know how the surface energy is evolving through all these treatments and so know which activation treatment should be the optimal for cell adhesion.

3.2.1. Polished/Unpolished

- Results
• Discussion

As shown in Fig. 22, after the polishing process, the surface energy is higher on the polished samples.

The surface energy can easily be linked with the wettability and the hydrophilicity. In fact, the surface energy is given by 3 components: the surface energy itself, the dispersive component and the polar component. The polar component correspond to the polarity of the samples. The more hydrophilic the surface is, the higher the polar component will be and the higher the hydrophilicity will be. The polar component is proportional to the hydrophilicity.

Moreover, since the surface energy is closely related to the contact angle and because we observed in the last experiment that the contact angle decrease quite a lot after polishing the samples and making the surface more smooth, it is logical to observe an increase of the surface energy after the polishing process.

3.2.2. Activation treatments

• Results
**Discussion**

All activation treatments increased the surface energy. The surface energy is the energy that is available to bind with other molecules. After the activation, hydroxyl groups are deposited onto the surface and those groups can easily react with other molecules. This is why the activation treatments increased the surface energy.

If we had to rank the activation treatment in terms of surface energy:

(+ energetic) NaOH o/n ≈ NaOH 2h > Plasma > HNO₃ ( - energetic)

Indeed, it seems that the plasma cleaning and the sodium hydroxyde etching modify the polarity of the sample by creating more hydroxyl groups than for the nitric acid activation.

### 3.2.3. Silanization

- Results
As shown on Fig. 24, when the activation was done with a plasma cleaner or a nitric acid etching, it seems that there is no significant change of surface energy regarding the value obtained for the control samples but the surface energy decreased regarding the values acquired after the activation treatments. The samples treated with sodium hydroxide (2 hours or overnight) seems to keep the same surface energy level as after the activation treatment. The difference of surface energy between plasma/nitric acid etching and sodium hydroxide etching seems to be in the polar component of the energy. Indeed, the polar component of the sodium hydroxide etching is about 2 times higher than for the two other treatments.

The silanization is consuming a big amount of surface energy and this is probably why after it, the surface energy of the plasma and the nitric acid activation is close to the initial level.

If we had to rank the activation treatment, it would give something like :

(+ energetic) NaOH o/n ≈ NaOH 2h > Plasma > HNO₃ (- energetic)
3.2.4. Immobilization of peptides

- Results

The results of the surface energy after the immobilization of peptides is similar to the results obtained for the silanization. The sodium hydroxide etching tend to produce more surface energy than the plasma cleaning or the nitric acid cleaning. The difference in the polar component is even higher than for the silanization. We can also observe that the physisorpted samples have a higher surface energy than the plasma and nitric acid treated samples.

The major difference is that in this scenario, after the add of peptides, for the plasma cleaning and the nitric acid etching activation, we observe an surface energy lower than the surface energy of the control samples.

If we had to rank the activation treatments in terms of surface energy :

(+ energetic) NaOH o/n ≈ NaOH 2h > Physisorption > Plasma > HNO₃ ( - energetic)
3.2.5. Summary

Figure 26: summary of the SE results for each activation treatments.

- If we summarize the whole activation, silanization and immobilization of peptides, the results are the same for the plasma and nitric acid activation treatment: there is an large increase of the surface energy after the activation, a return to the initial surface energy after the silanization and a light decrease of surface energy after the adding step of peptides.

- It seems that after the activation, the surface energy of the samples treated with sodium hydroxide reach a stable maximum level that does not change even after the adding step of peptides (step that decreases the surface energy under the control level). The SE remains unaltered after the activation with sodium hydroxide. This could be explained by a silanization that was not efficient. If the activation treatment do not create enough hydroxyl groups, then the silanization is not efficient and no peptides (or few) will be attached.
The physisorpted samples are (as for the contact angle results) giving a higher surface energy than the plasma and nitric acid treated samples but a lower surface energy than the sodium hydroxid treated samples.

The method that yields a higher SE is NaOH treatment. This is correlated with CA (wettability) values. Cell adhesion will be influenced by this parameter.

The hydrophilicity repends not only on the number of hydroxil groups. It can be influence by the amount of titanium oxyde created or the concentration of peptide used.

3.3. SEM

After having characterized how hydrophilic and energetic the surface is after the activation treatments, the silanization and the immobilization of the peptides, another important parameter in cell adhesion needs to be studied: the surface morphology after the activation treatments. The SEM images will give us a preview of the apparent roughness of the samples and an idea how affected the surface can be after these treatments.

3.3.1. Unpolished

As we can observe on Fig. 27, those samples are really dirty. There are a lot of scratches due to the cutting and the various uses.
3.3.2. Polished

Figure 28: Polished sample, Mag = 1000 (on the left), 10 000 (on the right)

Those samples seem smoother. We can still see few scratches but not really deep (Fig. 28). At a magnitude of 50 000, we can see contaminations with various polishing product like silica, zirconia or zinc.

3.3.3. Plasma activation

Figure 29: Plasma activated sample, Mag = 1000 (on the left), 10 000 (on the right)

As we can observe on Fig. 29, the surface of the plasma treated samples is really smooth. There are no more scratches (like if the plasma removes the smallest scratches. After the magnitude 10 000, we can observe small strains.
3.3.4. Nitric acid activation

As we can observe on Fig. 30, the samples treated with nitric acid seem similar to the plasma treated samples: clean, smooth and without scratches.

3.3.5. Sodium hydroxide 2h and overnight

As we can observe on Fig. 31, the samples treated with sodium hydroxide seem similar to the plasma treated samples: clean, smooth and without scratches.
As we can observe on Fig. 31 and 32, at magnitude of 1000, nothing particular strikes in the images but at a magnitude of 50 000, it seems obvious that this treatment radically affected the surface because there is like a network of holes that was created during this treatments. The surface became porous.

3.3.6. Discussion about the SEM images

It seems that only one kind of treatments affected the surface of the samples. After treatments, the samples treated with plasma or nitric acid tend to be the most cleaned but the significant changes might be the ones observed in the samples treated with sodium hydroxide. Indeed, there is a real modification of the surface because of the creation of this network that looks like a foam with a new composition.

The sensibility of this technique does not indicate if a change of roughness was done after the activation treatments or if a layer of sodium titanate was created. Unfortunately, this layer was too small for the SEM to see and that is why we used the interferometer.

3.4. Roughness

For these measurements, 3 samples of each category were analyzed. Roughness measurements for each sample were done in 3 different spots.
• Samples analyzed:
  - Unpolished samples
  - Polished samples
  - Samples activated with a plasma treatment
  - Samples activated with a nitric acid solution
  - Samples activated with a sodium hydroxide solution during 2h
  - Samples activated with a sodium hydroxide solution overnight
  • Results and discussion

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Ra (nm)</th>
<th>Rq (nm)</th>
<th>Rz (µm)</th>
<th>Rt (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpolished Average</td>
<td>273,07</td>
<td>359,07</td>
<td>6,62</td>
<td>10,00</td>
</tr>
<tr>
<td>SD</td>
<td>13,20</td>
<td>24,25</td>
<td>1,39</td>
<td>3,51</td>
</tr>
<tr>
<td>Polished Average</td>
<td>49,79</td>
<td>62,52</td>
<td>0,69</td>
<td>1,03</td>
</tr>
<tr>
<td>SD</td>
<td>7,00</td>
<td>8,34</td>
<td>0,29</td>
<td>0,49</td>
</tr>
<tr>
<td>Plasma Average</td>
<td>62,00</td>
<td>105,37</td>
<td>2,27</td>
<td>2,97</td>
</tr>
<tr>
<td>SD</td>
<td>19,20</td>
<td>64,73</td>
<td>2,52</td>
<td>3,18</td>
</tr>
<tr>
<td>HNO3 Average</td>
<td>64,85</td>
<td>86,07</td>
<td>1,03</td>
<td>1,59</td>
</tr>
<tr>
<td>SD</td>
<td>24,24</td>
<td>35,38</td>
<td>0,76</td>
<td>1,29</td>
</tr>
<tr>
<td>2h Average</td>
<td>93,85</td>
<td>124,22</td>
<td>1,78</td>
<td>2,38</td>
</tr>
<tr>
<td>SD</td>
<td>15,61</td>
<td>21,16</td>
<td>0,79</td>
<td>1,34</td>
</tr>
<tr>
<td>o/n Average</td>
<td>104,02</td>
<td>136,25</td>
<td>1,83</td>
<td>2,52</td>
</tr>
<tr>
<td>SD</td>
<td>11,54</td>
<td>14,47</td>
<td>0,28</td>
<td>0,55</td>
</tr>
</tbody>
</table>

Table 3: Summary of the roughness data

The results obtained with the interferometer clearly show that the polishing of the samples reduced their average roughness (Ra in Table 3).

All activation methods seemed to slightly increase the roughness of the polished samples, although the standard deviation is high.

Nonetheless, the treatment with NaOH overnight seems to be the one that caused the highest increase of roughness.
Those results can be correlated with the SEM pictures previously discussed. Indeed, it seemed that the plasma and nitric acid treatments did not change a lot the appearance of the surface and this is probably why we have a lower increase of the roughness compared to the sodium hydroxide treated samples. Those NaOH treated samples showed on the SEM pictures that some kind of porous oxide layer had been created. This porous layer is certainly why the average roughness goes from 70 nm for the polished samples to 136 nm for the NaOH overnight treated samples.

We also explained in the state of the art that roughness has a significant influence on cell adhesion. The rougher the surface is, the more cells will tend to attach. However, what we want here is the smoothest surface to be sure that what helped the bonding of the cells is the peptides and not the roughness of the cells. Anyway, since we will covalently bind silanes to the surface and then, after the immobilization of peptides, block it with BSA, this change of roughness should not affect the results.

3.5. XPS analysis

To characterize the formation of hydroxyl groups and the formation of titanium oxide layers, an XPS analysis was made. This analysis will give us the amount of hydroxyl groups and titanium oxide layer and the ration –OH/O² will be determined.

Figure 33: XPS graphics for samples untreated a) and treated with plasma b)
Figure 34: XPS graphics for samples treated with a) nitric acid, b) sodium hydroxide for 2hours and c) sodium hydroxide overnight

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Type of element analyzed</th>
<th>Position of the binding energy peak</th>
<th>% of this element</th>
<th>Ratio OH/O$_2^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>O$^2-$</td>
<td>529.611</td>
<td>47.10</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>530.963</td>
<td>15.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>533.245</td>
<td>37.45</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>O$^2-$</td>
<td>529.984</td>
<td>51.14</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>530.698</td>
<td>44.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>533.034</td>
<td>4.68</td>
<td></td>
</tr>
<tr>
<td>Nitric acid</td>
<td>O$^2-$</td>
<td>530.036</td>
<td>56.25</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>531.042</td>
<td>43.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>NO</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>NaOH-2h</td>
<td>O$^2-$</td>
<td>529.675</td>
<td>56.65</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>530.34</td>
<td>43.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>NO</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>NaOH-o/n</td>
<td>O$^2-$</td>
<td>529.925</td>
<td>82.79</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td>531.37</td>
<td>17.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>NO</td>
<td>NO</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: XPS results obtained with the software
As we can see on Figure 33-34, the activation treatment reduced the amount of water in the samples. Water only remains with the less aggressive treatment which is the plasma cleaning.

In table 4, we can observe that all activation treatments increased the amount of hydroxyl groups by a factor of 3. It also confirms that titanium oxide has been created (47.10% of titanium oxides for the control samples and more than 50% for the rest of the samples). The increasing of oxide amount is following the same trend that for the roughness of the samples. The less rough activated samples (plasma) is the one with the lower amount of oxides (47.10%) and the rougher (NaOH-o/n) has the highest level of oxide (82.79%). The exception is the NaOH overnight activation treatment which increases slightly the amount of hydroxyl groups but doubles the presence of titanium oxides.

The activation treatments reduce the amount of water in the samples and increase the amount of hydroxyl groups and titanium oxides. Except for the NaOH-o/n treatment, the ratio amount hydroxyl groups on amount of oxide increased. It also confirms that hydrophilicity not only depends on the concentration of hydroxyl groups but also in other proprieties like the amount of oxides at the surface of the samples (e.g. the amount of hydroxyl groups is the lowest for the sodium hydroxide overnight treated samples but those samples also give the best results in terms of contact angle and surface energy).

All the activation methods used were successful.
- Plasma treated samples and nitric acid treated samples: we can observe an increase of the amount of hydroxyl groups and an increase of the OH/O$^{2-}$ ratio. There is also a rise of the surface energy and of the roughness.

- Sodium hydroxide 2 hours treated samples: we can observe an increase of the amount of hydroxyl groups and an increase of the OH/O$^{2-}$ ratio. There is also a rise of the roughness but the surface energy values could be a sign that the silanization is not efficient.

- Sodium hydroxide overnight treated samples: we can observe an important increase of the amount of titanium oxide and a decrease of the amount of hydroxyl groups leading to a lower ration OH/O$^{2-}$. As for the sodium hydroxide 2 hours treated samples, there is a rise of the roughness and the surface energy values could be a sign that the silanization is not efficient and that no peptides were attached.

### 3.6. Optimization of the cell adhesion assays.

In order to establish the optimal conditions to perform the cell adhesion assay, we will optimize all the condition of the assay. We will determine the optimal concentration of cells for the assay, the effect of FBS on the cell adhesion, the optimal incubation time, the efficiency of the blocking step done with BSA 1% in PBS, the optimal concentration of peptide solution and also the impact of the pre-coating with PBS overnight on the polished control samples.

All the assays were done with SAOS-2 cells.
3.6.1. Cell number and influence of FBS

3.6.1.1. Cell adhesion assay

The aim of this first assay was to learn the effect of FBS onto the cells but also to determine how many cells we will use for the cell adhesion assay.

In order to do so, we prepared 3 plates:

- One with Ti samples and 4 different concentration of cells in a medium with FBS
- One with Ti samples and 4 different concentration of cells in a medium without FBS
- One standard curve that will help determine how many cells attached onto the samples. Indeed, in the standard curve well plate, no Ti samples are deposited and we make the hypothesis that all the cells that are seeded will attach to the plate well surface. These types of multi-well plates are made of polystyrene and are pre-treated to allow the binding of proteins and cells. The medium used for the standard curve is with FBS.

The samples used in this assay were just polished. No activation treatments were done.
The incubation time was 4h.

The cells were at passage P21.

### 3.6.1.2. LDH results

**Cell adhesion after 4h with FBS**

![Graph showing cell adhesion with FBS](image1)

**Figure 36**: LDH results of the assay done in a medium with FBS

**Cell adhesion after 4h without FBS**

![Graph showing cell adhesion without FBS](image2)

**Figure 37**: LDH results of the assay done in a medium without FBS
• Discussion

This assay has been done to quantify the effect of FBS on the cell adhesion and the proper cell concentration to use for the future assay. Whereas in medium with FBS the adhesion rate varies from 57 to 76 %, the adhesion rate is only varying from 20 to 52 % in medium without FBS, which is below the lower adhesion rate obtained with the medium containing FBS. Those results are showing that FBS is helping the cells to bind to the surface. This assay also shows that the number of cells attached increases with the number of cell seeded. Since the cells we use (SAOS-2) are really fast growing, it is better to seed 25 000.

⇒ FBS helps the cells to bind to the surface and the best option for the next assays is to seed 25000 cells per well to be sure that the amount of cells attached is high enough to be measured.
3.6.2. Incubation time

3.6.2.1. Cell adhesion assay

Figure 38: Plate 1 = medium with FBS for 4h, Plate 2 = standard curve for 4h, Plate 3 = medium with FBS for 6h, Plate 4: standard curve for 6h

The aim of this assay was to determine the optimal incubation time for a cell adhesion assay.
In order to do so, we prepared 4 plates:
- One with 3 titanium samples with a concentration of 50,000 cells/mL of cells in a medium with FBS that will be incubated for 4 hours
- One standard curve that will help determine how many cells attached onto the samples that will be incubated 4 hours.
- One with 3 titanium samples with a concentration of 50,000 cells/mL of cells in a medium with FBS that will be incubated for 6 hours
- One standard curve that will help determine how many cells attached onto the samples that will be incubated 6 hours.

The samples used in this assay were just polished. No activation treatments were done.

The cells were at passage P23.

3.6.2.2. LDH results

![Cell adhesion after 4h and 6h](image)

Figure 39: LDH results of the assays done during an incubation time of 4 and 6 hours.

- Discussion
This assay was done to determine the optimal time of incubation during an assay. The results are showing an adhesion rate which is superior to 100%. This can be explain by calculation or experimental errors done when the cells were added. It is possible that the concentration was slightly superior to 25,000 cells. However, we can conclude that there is a 100% adhesion rate after the assay or at least a adhesion rate close to 100%. So according to the graphic, there is no real difference between 4 hours of incubation or 6 hours. After this assay, we can conclude that eitherway, we obtain a full adhesion. To reduce experimental time, 4h was chosen as timepoint for the following studies.

3.6.3. Efficiency of the blocking step

3.6.3.1. Cell adhesion assay

![Image of cell adhesion assay]

Plate 1

Plate 2

Figure 40: Plate 1 = medium with or without FBS with 25,000 cells/well and BSA only in the wells or in the wells and on the samples for 4h, Plate 2 = Standard curve for 4h

The aim of this assay was to determine whether or not the blocking step with BSA (bovine serum albumin) is efficient. In order to do so, we will completely block the wells and the samples with BSA and see how effective is this step with or without FBS.

In order to do so, we prepared 2 plates:
One with 12 titanium samples with a concentration of cells in a medium with or without FBS of 50 000 cells/mL that will incubate for 4 hours. Those samples were either blocked with BSA or not as described in Figure 40.

One standard curve that will help determine how many cells attached onto the samples that will incubate 4 hours.

The samples used in this assay were just polished. No activation treatments were done. The cells were at passage P25.

3.6.3.2. LDH results

![Assay with samples blocked with BSA](image)

Figure 41: LDH results of the assays done with BSA for 4h.

- Discussion

During this assay, we noticed two things:

- First, the FBS definitely helps the cells to bind as we can see on the graphic (full adhesion with FBS and only 22% without).

- Second, the blocking step is really efficient because when we add BSA on the samples and if we do not put FBS to help the cells to bind then there is only
7% of adhesion. The FBS is probably removing the BSA or neutralizing it. Further experiments could be done to determine what exactly happens.

The idea would be that the effect of BSA is lower if there is FBS in the medium.

3.6.4. Summary of the first step of the optimization

The 3 first steps of the optimization process were:
- To determine the cell concentration for the future assay and the effect of the FBS
- To determine the incubation time
- To check how effective was the blocking step

After the analysis of all the results, we will now continue with the following parameters for the assays:
- A concentration of 25 000 cells/well
- A medium without FBS
- 4 hours of incubation
- A blocking step of 1 hour with BSA 1% in PBS will be done in the wells and on the samples before adding the cells.

3.6.5. Peptide concentration

After having optimized the required conditions for the cellular assays, the effect of an RGD peptide physisorpted on the surface in terms of osteoblast adhesion was evaluated. To this end, the RGD peptide was dissolved in PBS at different concentrations and Ti samples were coated with these solutions overnight.
3.6.5.1. Cell adhesion assay

As described in Fig. 42, Ti samples were coated with 100 μL/well of RGD peptides at a concentration of 100, 500 and 1000 μg/mL. Ti samples coated with only PBS solutions were used as control samples.

The aim of this assay is to determine the optimal concentration of peptides in order to improve the cell adhesion rate. We will determine the percentage of adhesion in each case. Normally, the more peptides we immobilize on the sample surface, the higher the adhesion rate should be.

In order to do so, we prepared 2 plates:

- One with the 12 titanium samples prepared as explained in the plate 1 (Figure 44) with a concentration of cells in a medium without FBS of 50 000 cells/mL that will be incubated for 4 hours. Those samples were blocked with BSA 1% in PBS.

- One standard curve that will help determine how many cells attached onto the samples that will incubate 4 hours.
The samples used in this essay were just polished. No activation treatments were done.

The cells were at passage P37.

3.6.5.2. LDH results

This assay worked correctly. The physisorpted samples gave a higher adhesion rate than the control samples. As we can see on the Figure 43, there is a 12.6% of adhesion in the wells that contained blocked control samples. It corresponds approximately to the adhesion rate obtained in the previous assays (Fig. 41: 7% of adhesion in a well blocked with medium without FBS). As we can see in the figure 43, the adhesion rate is increasing as the concentration of peptide in the coating solutions increases. The increase is significant (by a factor of 3). However the standard deviation is high. For the samples coated with the 500 µg/mL solution, a higher adhesion rate should be expected but because of experimental errors, we obtained a lower adhesion rate.

We will chose for the future assays a concentration of peptides of 100 µg/mL because it gives a clear enhancement in adhesion rate. Moreover, the peptides
are expansive to produce, therefore, a low concentration will save money for the project.

3.7. Cell adhesion assay

3.7.1.1. 1st Cell adhesion assay

![Cell adhesion assay diagram]

Figure 44: Plate 1 = medium without FBS with 25,000 cells/well and BSA in the wells and on the samples for 4h, Plate 2 = Standard curve, 4h

The aim of this assay was to determine the effect of our functionalization strategy (activation, silanization, immobilization of peptides) on the cell adhesion rate of osteoblasts. The covalent immobilization of the peptides was compared with a simple physical adsorption.

In order to do so, we prepared 2 plates:

- One with 18 titanium samples with a concentration of cells in a medium without FBS of 50,000 cells/mL that will incubate for 4 hours. Those samples were blocked with BSA 1% in PBS.
- One standard curve that will help determine how many cells attached onto the samples that will incubate 4 hours.

The samples used in this essay were polished, activated, silanized and covered with 100 µL of 100 µg/mL concentration of peptides following the protocols previously described and as explained on the Figure 44.

Parameters:
- Coating of 100 µL of a 100 µg/mL
- Cells at P27

- LDH results

![Cell adhesion assay, 4h](image)

Figure 45: LDH results of the assays done without FBS, with BSA for 4h

As shown on Fig. 45, the physical adsorption of the peptides yielded the higher adhesion rate. No significant differences were observed within the distinct activation treatments.

In this assay, control samples are missing and therefore it is not possible to determine whether there is an improvement in cell adhesion compared to non-functionalized samples or not.
3.7.1.2. Last cells adhesion assays

The previous assay was repeated, including control samples. For this new series of assays, a new lot of SAOS-2 cells was used.

To avoid any unspecific signal in the LDH measurements, before adding the lysis buffer (M-PER), samples were transferred to new plates.

The samples were activated, silanized and peptides were immobilized on the samples following the same scheme as in figure 4.

The new cells were at passage P21.

![Cell adhesion assay, 4h](image)

Figure 46: LDH results of the assays done without FBS, with BSA for 4h

Only physisorption and plasma treated samples give increased values of cell adhesion compared to control samples. Although adhesion rates are low (below 20%), there is a 30% increase of the adhesion rate on physisorpted samples and a 36% increase for the plasma treated samples. Sodium hydroxide and nitric acid treatments do not seem to increase cell adhesion.

The assay is repeated with an increase of the concentration of peptides up to 500 µg/mL. Cells were at passage P22.
Again, physisorption increases the adhesion rate (rise of 25%) but this time, the plasma treatment does not seem to affect the cell adhesion rate. The nitric acid treatment also increases the adhesion rate by 25%. The samples treated with sodium hydroxide give a lower adhesion rate than the control samples.

The assay is repeated with 6 hours of incubation. The cells were at passage P23.

The physisorbed and plasma treated samples yield the highest adhesion rate, slightly higher than control sample adhesion rate. Again, sodium hydroxide and nitric
acid treatments do not seem to have an effect on the adhesion rate. Adhesion rate for this assay were really low. A closer study of the cells showed that they were not behaving properly (slow growth and proliferation).

- The physical adsorption of RGD peptides enhances the cell adhesion.
- Although standard deviation values are too high and the performance of the cells in the last 3 assays was not optimal, it seems that plasma treated samples yields the best values of cell adhesion and the sodium hydroxide treated samples the worse. This last statement can be correlated by the contact angle and surface energy values. Indeed, even if sodium hydroxide treated samples give the highest surface energy, the silanization and the immobilization of peptides did not change the level of energy meaning that maybe the peptides are not coated correctly.

3.8. Immunofluorescence tests

These analyses are done to analyze the spreading of the cells and the quality of the adhesion by checking actin filament and focal adhesion.

3.8.1. Control samples

Figure 49: staining of the nuclei and the actin filament at a) Mag 4, b) Mag 20

There are not a lot of cells attached to the samples but the dispersion seems homogenous. The stains of the nuclei and the actin filaments are considerably similar. The cells are rounds.
3.8.2. Physisorption

Figure 50: staining of the nuclei and the actin filament at a) Mag 10, b) Mag 20

It seems that more cells are attaching to the border of the samples than in the middle. Globally, more cells are attaching. The actin filament stains start to be more wide.
3.8.3. Plasma

Figure 51: staining of the nuclei and the actin filament at a) Mag 4, b) Mag 20, c) Mag 40

The dispersion of the cells is more homogenous than in the control or physisorpted samples. The concentration of cells is also increased compared to control samples and also the physisorpted samples. Cells are more spread and we start to really see actin filaments.
3.8.4. Nitric acid

![Images showing staining of nuclei and actin filament at different magnifications: Mag 4, Mag 10, Mag 20.]

Figure 52: Staining of the nuclei and the actin filament at a) Mag 4, b) Mag 10, c) Mag 20

The number of cells compared to the plasma treated samples is less significant. The repartition of the cells over both samples was not homogeneous: cells were concentrated in groups on the borders of the samples. Still, the stains seem wider than in the control samples.
3.8.5. Sodium hydroxide 2h

The cell number is significantly more important than in the other samples with a perfect homogenous repartition. However, some unknown molecules (probably from the staining assay) appear on the pictures. The staining of the actin filaments and the nuclei is similar. Cells are more spread than in the control samples but less than the physisorpted or plasma treated samples.

3.8.6. Sodium hydroxide overnight

As for the sodium hydroxide 2hours treated samples, there are a lot of cells. However, it seems that the stains of the actin filaments are wider.
3.8.7. Discussion

The blue stains correspond to the nuclei fluorescence, the red stains correspond to the actin filament fluorescence and the purple/magenta stains correspond to the overlap of both nuclei and actin filaments.

In all the samples, whether peptides or not had been immobilized, no focal adhesion could be noticed.

➔ By observing the staining of Figure 49, it seems that the cells in the control samples are really round. There are few cells with real actin filament meaning that the adhesion is not strong.

➔ There is a significant difference between the control samples and the physisorpted samples: the number of cells attached increased and the size of the actin filament too. It seems that the cells in the physisorpted samples are more spread and that the quality of the adhesion is improved.

➔ All the samples that had been activated show an improvement in the cell adhesion spreading but only the plasma treated samples and the nitric acid treated samples show a real improvement in the adhesion quality.

➔ The increase of cell number in the sodium hydroxide treated samples could be due to the increase of the roughness if we correlated this with the absence of peptides observed with the other experiments (SE, LDH results).
4. Conclusion

The first main objective was to characterize the process of titanium functionalization (activation, silanization and peptide immobilization) in terms of physico-chemical properties of the surface and to compare distinct activation methods.

The CA and SE results clearly showed that the hydroxide sodium activation treatments (2 hours or overnight) are the ones that give the higher hydrophilicity as we can see on Fig 21 and 26. The other activation treatments also increased the hydrophilicity but the raise was less significant. The physisorpted samples seem to have a higher hydrophilicity and surface energy than the plasma and nitric acid activated samples. However, even if the sodium hydroxide treated samples give the higher values of surface energies, there are no changes of this energy through the various treatments that followed the activation (silanization and immobilization of peptides). This is probably due to a low efficiency of the silanization leading to less covalent bindings of the peptides.

In terms of roughness, the activation treatments increased the average roughness of the samples as we can see on Table 3. All the activation treatments increased the average roughness. The sodium hydroxide overnight etching showed a rise of a factor 2 of the average roughness. The plasma activation and the nitric acid etching barely change the roughness of the samples.

SEM pictures of the plasma and nitric acid treatment showed a surface quasi intact and cleaned. The SEM pictures of the sodium hydroxide etchings showed a new layer of sodium titanate that seems to be porous. However, the composition of this layer (sodium titanate) has not been confirmed.

The XPS data also showed that the activation treatments were efficient to create titanium oxide layer and hydroxyl groups. However, it seems that plasma and nitric acid treatments create more hydroxyl groups.

*In terms of physico-chemical properties, the optimal activation treatment should be the plasma treatment. However, it seems that the physical adsorption give better results although stability issues need to be taken in account.*
The second objective was to study and compare the adhesion of sarcoma osteogenic cells on surfaces functionalized with RGD peptides via 2 strategies: covalent binding and physical adsorption.

The LDH results that we gathered during this project showed that physisorption improved more the adhesion rate than the other covalent binding. It also showed that plasma treated samples seem to give higher values of cell adhesion rate than the rest of the activation treatments.

The immunohistochemical test clearly showed that the physisorption and the silanized samples were improving the adhesion quality of the cells. The cells are starting to spread and are more numerous when peptides were immobilized whereas on the control samples the cells are round and less numerous. The adhesion quality was increased no matter how the peptides were immobilized. Nevertheless, the cells were less spread when the peptides were covalently bound. The plasma activation treatment seems to be the optimal treatment whereas the sodium hydroxide treatment seems to be the worst. This is probably due to the absence of peptides at the surface of the samples or to a hydrophilicity to important.

In term of cell adhesion quality the optimal activation treatment would be the plasma activation treatment or the nitric acid activation treatment. However, the physical adsorption is still the optimal option to enhance to adhesion rate and the adhesion quality.
5. Economic and environmental impact

5.1. Economic impact

In this part, I will consider the divers reagents, the utilization of equipment and the cost of the technicians.

- Preparation of the samples

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- Activation treatments, silanization, immobilization of peptides

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- Cell related material

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- Equipment

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- Personal

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- Other costs

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This includes the gloves, plastic cup, boxes for the samples, etc.

**Total cost of the project: 27376,23€**

5.2. Environmental impact

The environmental impact of this project was taken into account during the whole project. This is why all the reagents, even used in small amounts had been treated in a friendly environmental manner.
5.3. Residue management

Whether in the biomaterials laboratory or in the IBEC laboratory, the management of the residues is very strict and follows various protocols.

In the biomaterials laboratory, only chemical residues are treated. After the use of a chemical product, according to the type of the product, this one will be stored in the accurate recipient. Those recipients are placed strategically in the laboratory according the type of products used in the different laboratory-rooms.

Once the polypropylene recipients are full, there are placed in a safety closet. ECOCAT, a company that deals with the management of the residues, comes every 6 months to collect the residues.

In the IBEC laboratory, various protocols had been created to take into account the chemical residues but also the biological, domestic and general residues.

The domestic biological and general residues are collected in separate trash cans whereas the chemical products are collected in polypropylene recipient constantly in a working hood.

Those residues are taking care of by the Scientific Park which deals with the management.
Acknowledgment

First of all, I would like to thank Doctor Manero Planella, the director of this project, who gave me the opportunity to realize this project and to increase my knowledge in the biomaterial field.

Then, I would like to thank Carles Mas Moruno for his help during this entire project. Thank you for your patience, your comprehension, your guidance and your ability to teach me how to properly work in a laboratory.

I also would like to thank the laboratory BIBITE and all its coworkers who welcomed me as a new member. Thank you for always being available for guidance or technical help during the project.

I would also like to thank all the technicians and lab managers who helped me through this amazing project in the nanotechnology center but also in the IBEC facilities.
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