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Bachelor Thesis

"Chemical surface modification of bio-functionalized thin films using cross-linking agents"

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ABSTRACT

The biomaterials group at CTB has developed a new technique for the biofunctionalization of a wide range of materials with primary amines. Numerous proteins and other biomolecules were covalently bound to the amine groups using the EDC/NHS chemistry as cross-linking agents. The aim of this work is to study material bio functionalization with other crosslinking agents; Sulfhydryl group will be studied as a reversible way of crosslinking proteins to a surface through disulfide bond formation. We hypothesize that this molecules will yield better material-cell interactions and improve the bioactivity of biocompatible materials such as silicon, titanium and glass. Complementarily the protein adsorption process onto a solid surface will be investigated to characterize it in both static and dynamic conditions.

Keywords: protein adsorption, biofunctionalization, crosslinking agents, biocompatibility

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GLOSSARY

LDH – Lactate Dehydrogenase

AFM – Atomic force microscopy

Abs – Absorbance (a.u.)

DTT - Dithiothreitol

AVS – Activated vapor silanization

NADH – Nicotinamide adenine dinucleotide (reduced form)

NAD+ - Nicotinamide adenine dinucleotide (oxidized form)

U – Enzymatic activity (μmol /min * mg)

Δn_{NADH} – NADH variation

Δt – Time variation

m_{LDH} – Mass of LDH (mg)

OD_{340} – Absorbancec at 340nm (a.u.)

$V_{cuvette}$ – Volume in the cuvette (970 + 30μl)

ε - Molar extinction coefficient

l – Length of the spectrophotometer cuvette (cm)

n – Number of previous measurements

V_{essay} – Remaining volume of NADH after the sample extraction at time t_n (600 – 30n μl)

V_{sample} - 30 μl sample volume extracted each time

ΔG – Gibbs free energy

q – Probability of a step towards the sample

Φ – Dimensionless reaction rate

v_a – Average velocity of random walk (cm/s)

C_m – Concentration of the monolayer (mg/cm^2)

D – Diffusion coefficient (cm^2/s)

C_b – Initial bulk protein concentration (mg/cm^3)

k – Boltzmann constant (erg/molec*K)

T – Temperature (K)

M_w – Molecular weight (g/molecule)

A_{LDH} – Area of an adsorbed LDH molecule (cm^2)

r – Radius of an adsorbed LDH molecule (cm)

η – Solvent viscosity (kg/m*s)

r_{sol} – radius of LDH in solution (cm)

Q – Dimensionless observed slope

L – Distance to the boundary layer (cm)

z – Distance from the inlet to the flow cell

γ_w – Shear strain rate at the surface (1/s)

X – Volumetric flow rate (mL/s)

R – Radius of the pipe (cm)

P – Sticking coefficient

Γ – Adsorbed amount of protein at time t

1. INTRODUCTION

1.1. Motivation of work

Protein adsorption on biomaterials is of great interest in the biomedical industry both for diagnostics (bioimaging and affinity-detection techniques) and therapeutics (implants, surgical and laboratory material…) as it enhances biological performance, affinity and biocompatibility.

This protein adsorption process is not well-characterized nor understood, and as critical as it is in biomaterial outcome and success of the application, it should be thoroughly studied.

Thiol groups arouses great interest in bioconjugation research as it opens promising possibilities for creating reversible interactions with proteins and other biomolecules. Some techniques have been previously reported in the literature for attaching thiol groups on solid surfaces such as mercapto-silane self-assembly on silaceous and metal surfaces, self-assembly on gold using thiol groups on both ends of the conjugating molecule, immobilization of thiol containing conjugators on otherwise functionalized surfaces or continuous phase glow discharge polymerization using thiol containing monomers. Each of these techniques have their own drawbacks, being some of them complicated processing, substrate specificity or the loss of thiol group functionality [21].

1.2. Goals

In this work a new reversible three-step biofunctionalization method for silicon thin films is developed based on the exposure of thiol groups on the solid surface that will later on interact with a protein via disulfide bond formation. Since it relies on disulfide bond formation, the protein adsorption or immobilization (depending on the application) can be reverted with commonly used disulfide reducing agents such as DTT.

The efficiency of this crosslinking scheme is intended to be proved by using the enzyme lactate dehydrogenase as model. As a first step in this experimental program, the protein adsorption process is analyzed so that its interaction with the substrate may be understood. Both static and dynamic adsorption conditions are characterized through enzymatic assays. Also, a mathematical model based on the experimental results is used for further unraveling of protein adsorption kinetics. LDH is used as the protein model since the reaction it catalyzes can be easily followed by spectrophotometry measurements.

2. STATE OF THE ART

2.1. Current situation

2.1.1. Biomaterials, its role in the biomedical industry and the importance of biofunctionalization

"A biomaterial can be defined as a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body". *(Consensus definition, 2nd Biomaterials Consensus Conference, 1992, Chester, UK). According with this definition, the range of materials and applications that could be considered is very broad: the several types of implants that serve to replace a diseased or non-working part of the body or to enhance or correct its function, materials used to aid in the process of healing as in the case of sutures, to act as drug delivery systems (hydrogels, NPs…), as scaffolds for tissue engineering and of course the many applications in diagnosis acting as biosensors, or for laboratory material [3][13][15].

Depending on the application, one type of material or another will be selected (metals, polymers, ceramics or composite materials), as they have different properties that make them suitable for certain applications. To this concern one can differentiate between the bulk properties of the material (that govern the durability and its mechanical behavior) and the surface properties, which are the ones who really determine the biological response of the biomaterial. Here it must be introduced the term of biocompatibility: "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1987) [19].

According to the affirmation that surface properties are the ones responsible for the biological response (that is indeed the biocompatibility of a material) it becomes apparent that surface modification of a certain material can provide several advantages in the use of that given material. In particular, when this surface modification is performed for the material to have an enhanced biological property or function (that can be either permanent or temporary) this process is defined as biofunctionalization. Surface modifications fall mainly in two categories: Physicochemical surface treatment and biological methods.

The material selected in this work as a model of biomaterial is silicon, whose main biomedical applications are in the field of diagnosis and therapeutics (being used mainly for biosensors and bioimaging and in some cases for drug delivery, cancer therapy and

tissue engineering applications) [4][6][9]. However, it is expected that the biofunctionalization techniques explored in this work can be also applied also for other biomaterials with similar properties (like Titanium) that may have very different applications in the biomedical field.

In many cases, biofunctionalization implies the binding of biomolecules to the surface (biological surface modification). The immobilization of the biomolecule can be performed with several techniques: physical adsorption, physical entrapment and covalent attachment, being the latter the most stable one. In the case of biosensors, proteins offer a wide range of possibilities thanks to its broad variety and flexibility, and several types of receptors can be built based on these proteins. It is worth-mentioning the application of AFM probe functionalization for detection of antibodies, receptors or other types of markers on the cell surface [20]. Some other relevant applications require biological surface modification motifs that consist of binding fibronectin RGD containing peptides to promote cell attachment, heparin and heparin sulfate binding peptides to enhance cell adhesion and growth factors to induce specific cell responses.

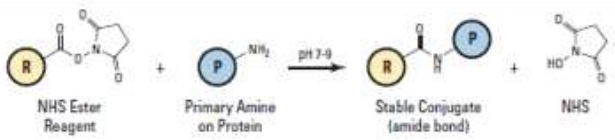
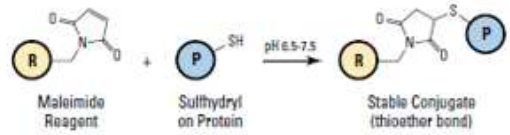
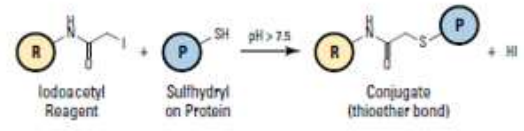
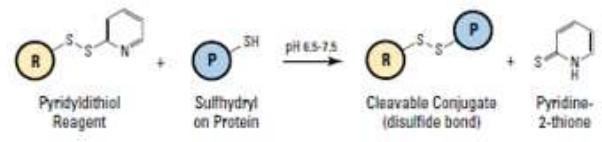
"Crosslinking reagents (or crosslinkers) are molecules that contain two or more reactive ends capable of chemically attaching to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules." (Thermo Fisher Scientific,"Chemistry of Cross-linking") [17]. Therefore they emerge as promising tools for bioconjugation of proteins. Depending on whether the two functional groups of the cross-linker are identical or different they can be classified in homobifunctional or heterobifuntional crosslinkers. On the other hand, if we classify them by the type of functional group they interact with, we find Primary Amine Reactive Cross-Linkers, Sulfhydryl Reactive Cross-Linkers, Carbohydrate Reactive Cross-Linkers, Carboxyl Reactive Cross-Linkers and Photoreactive Cross-Linking Reagents. The most widely used types of crosslinkers for protein immobilization are heterobifunctional crosslinkers that interact with proteins via amino or sulfhydryl groups, as these are numerous and readily available in peptide groups and cysteines. Within these sets of cross-linkers we can find different chemistries:

- Primary Amine Reactive Cross-Linkers: Imidoesters and N-Hydroxysuccinimide-Esters (NHS-Esters). Imidoesters leave amidine protonated bonds whereas NHS esters leave amide bonds. Since NHS esters are more stable and efficient they are more widely used.

- Sulfhydryl Reactive Cross-Linkers: Maleimides, Haloacetyls and Pyridyl disulfides. It must be noted that even though all of them attach to thiol groups, the only ones that leave a disulfide bond are pyridyl disulfides; Maleimides and haloacetyls leave tioether bonds.

One main difference between each type of crosslinker is the pH at which the reaction takes place (and therefore the buffer that must be selected for each of them). Table 1 presents a summary of the reaction mechanism of each type of crosslinker and the pH at which the reaction takes place.

TABLE 1: REACTION MECHANISMS AND PH CONDITIONS FOR SOME TYPES OF CROSSLINKERS

CROSSLINKER CLASS	REACTION MECHANISM	pH
AMINE-REACTIVE CROSSLINKERS		
NHS esters		**Slightly alkaline conditions. pH=7.2-8.5**
Imidoesters		**Alkaline conditions. pH=8-10**
SULFYDRYL REACTIVE CROSSLINKERS		
Maleimides		**Neutral conditions. pH=6.5-7.5**
Haloacetyls		**Physiologic pH**
Pyrydyl disulfide		**Broad pH range.** **Optimum pH=4-5**

*Adapted from [17]

For the use of these type of molecules on a solid surface, the surface needs to be previously functionalized with one of the aforementioned functional groups in order to be able to react with the protein. Therefore, the full biofunctionalization process would involve 3 steps: (1) existence of available functional groups on the surface of the material, (2) reaction of the crosslinker with the functional group on the surface and, finally, (3) reaction between the crosslinker and the protein. (Figure 1)

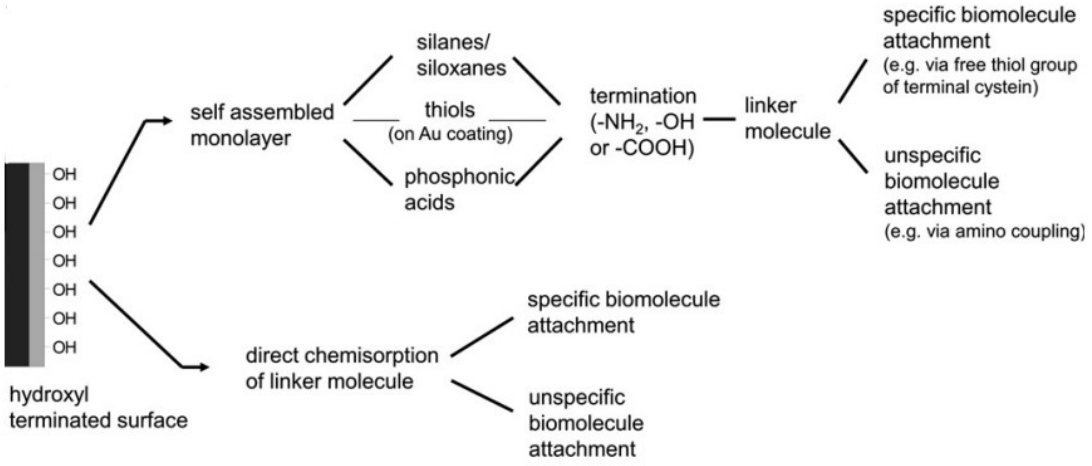


Figure 1: General scheme of a full 3-step biofunctionalization procedure. (Bauer et al, 2013) [18]

More specifically, in this work the silicon thin film was biofunctionalized with amine groups using activated vapor silanization (AVS), which is a type of physicochemical surface treatment. Other methods for generating amine groups on metallic surfaces include immersion silanization and different types of plasma treatments.

AVS is based on the chemical vapor deposition (CVD) technique in which vaporized APTS is deposited on the sample surface. Activation of the APTS molecules occurs at high temperatures (700ºC) and both activation and deposition of APTS on the surface takes place at low vacuum. This silanization technique has proven to produce thin films with a homogeneous distribution and high density of amine groups and to have more efficiency than other biofunctionalization techniques. [8]

2.1.2. The importance of protein adsorption and its characterization

Protein adsorption is the process whereby a protein is adhered onto a solid surface. It is the first event in blood-material interaction, affecting greatly blood coagulation, complement activation and bacterial and cell adhesion. These protein surface interactions will condition to a great extent the fate of the blood-biomaterial interface. From a

thermodynamic point of view, protein adsorption is favored if it reduces ΔG: ΔGadsorption = ΔGprotein + ΔGsolvent + ΔGsurface

Before a protein can adhere to a substrate, it must be transported to its vicinity by different mechanisms (diffusion, convection and/or coupled transport). Afterwards, it undergoes conformational changes (known as molecular spreading) exposing functional groups ready to create new contacts with the surface and making desorption less likely.

The protein adsorption process is highly dependent on the protein properties (size, hydrophobicity, charge and protein unfolding). Nevertheless surface properties are equally important (topological features, surface chemical composition and of course the presence of biofunctional motifs). [5][18]

Being this process as vital as it is to determine the biocompatibility of the material, it was aimed to gain a better understanding of the process and conditions under which it takes place. In addition, the protein employed, lactate dehydrogenase (LDH) is intended to be used as model molecules to evaluate the efficiency of the whole biofunctionalization program.

Currently there are several mathematical models that try to parametrize the protein adsorption phenomenon, each of which making different assumptions on transport processes and protein and surface properties.

Figure 2 shows an overview of the main mathematical models currently used in research in this field. Some other approaches to model the kinetics of protein adsorption are with computational tools such as Monte-Carlo simulations, molecular dynamics simulations and energy optimization methods. [11]

However theoretical models must be compared with the experimental reality to fit it to the conditions under study with label-free or fluorescence techniques. Several parameters used in this type of models are protein and/or surface dependent, as for example the diffusion coefficient, sticking coefficient, or on-rate and off-rate constants. Consequently there is a need of detailed characterization of the protein and surface and its interaction by experimental methods to fully understand protein adsorption in a given condition.

2.2. Design of solutions

2.2.1. Background of cross linker chemistry

The biomaterials group at CTB had already worked on protein immobilization on solid surfaces [2] [8][16]. However the techniques used were permanent. In an attempt of creating reversible protein functionalization, the disulfide bond between thiol groups emerged as a good possibility for exploring new biofunctionalized schemes, as this type of bond is strong (with a typical dissociation energy of 60kcal/mol), are naturally found between thiol groups in cysteines of proteins and is reversible by treating it with reducing agents as DTT.

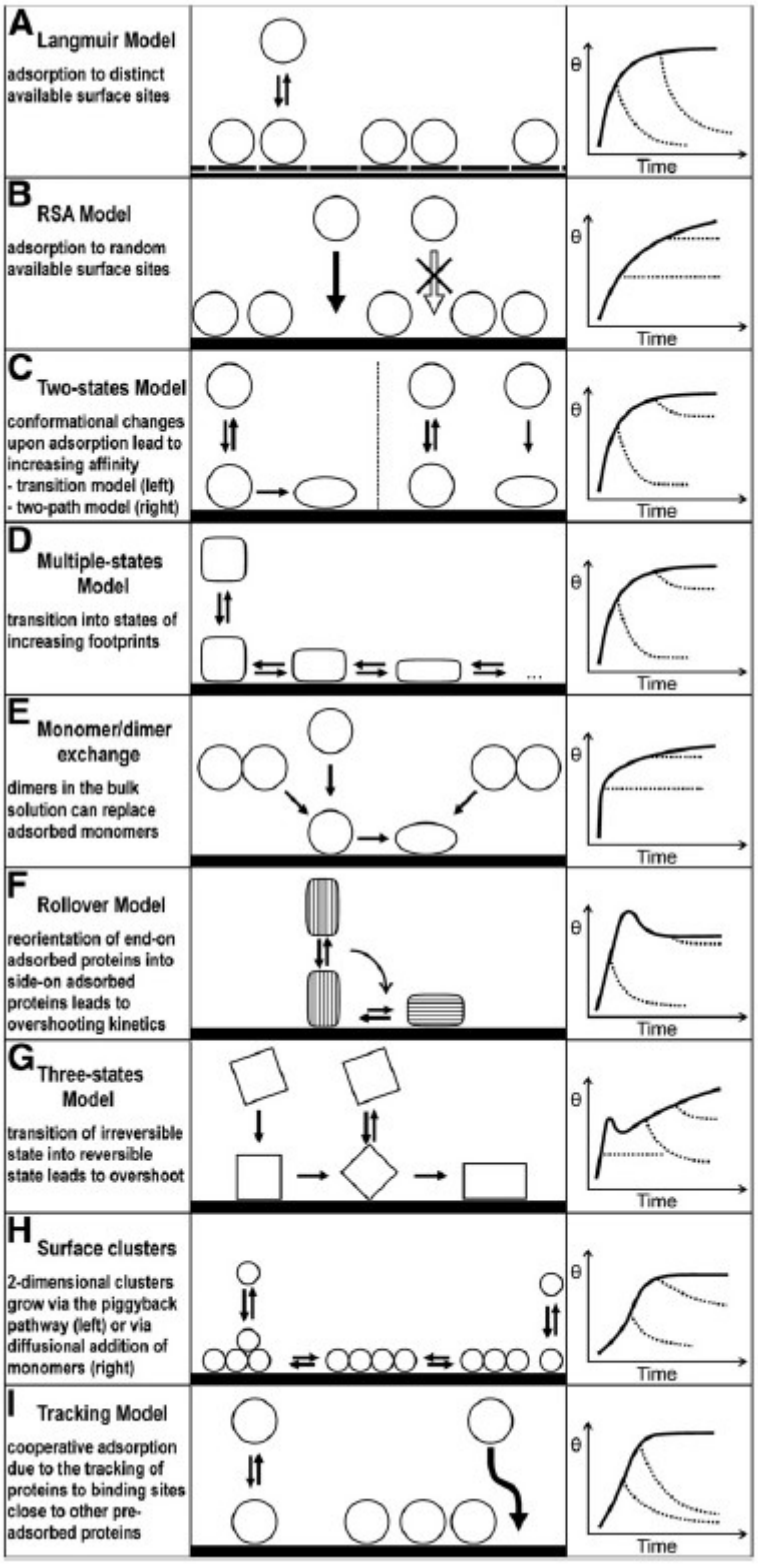
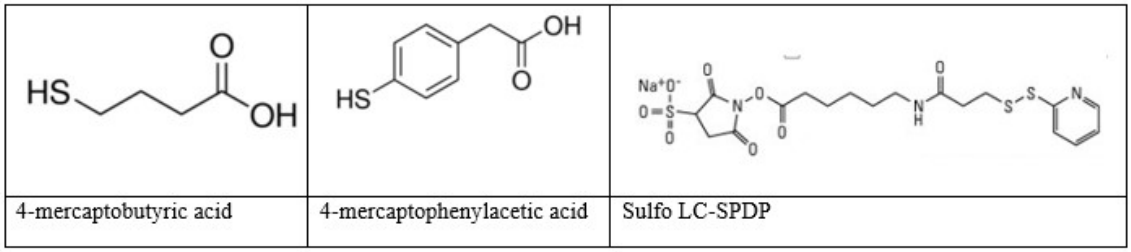


Figure 2: Overview of the main models of protein adsorption. (M. Rabe et al. (2011) [11]

Keeping in mind the objective of forming disulfide bonds between the cross-linker and the protein of interest and having amine groups exposed on the surface of the biomaterial, the choices of crosslinker were narrowed as the two functional groups had been already set. Three different possibilities were found viable for the applications: 4-mercaptobutyric acid, 4-mercaptophenylacetic acid and sulfosuccinimidyl 6-(3'-(2- pyridyldithio)propionamido)hexanoate (Sulfo LC-SPDP) (Figure 3).



4-mercaptobutyric acid	4-mercaptophenylacetic acid	Sulfo LC-SPDP

Figure 3: Chemical structure of crosslinkers to be studied

For the attachment of both 4-mercaptobutyric and 4-mercaptophenylacetic acids the EDC/NHS reaction chemistry was selected as a way of adhering the molecule to the aminated surface. EDC is a carbodiimide (a type of carboxylic reactive crosslinker) that causes direct conjugation of carboxylic groups to amines. In the EDC reaction chemistry, an unstable intermediate (O-acylsourea) is formed. Adding NHS (or its water soluble analog (sulfo-NHS)) increases the efficiency of the reaction as the intermediate formed in this case (an NHS ester) is considerably more stable. In addition, EDC reaction occurs at acidic pH whereas when coupled to NHS, it allows the reaction to happen efficiently at physiological pH. Figure 4 shows the mechanism of EDC/NHS reaction chemistry.

For the Sulfo LC-SPDP the reaction mechanism is shown in Figure 5.

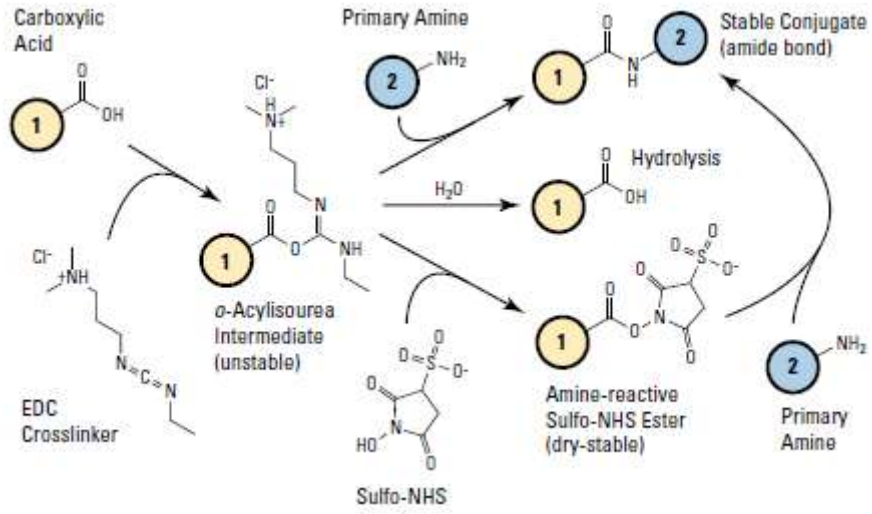


Figure 4: EDC/NHS reaction chemistry. Carboxyl-to-amine crosslinking using the carbodiimide EDC and Sulfo-NHS. Addition of NHS or Sulfo-NHS to EDC reactions (bottom-most pathway) increases efficiency and enables molecule (1) to be activated for storage and later use. [17]

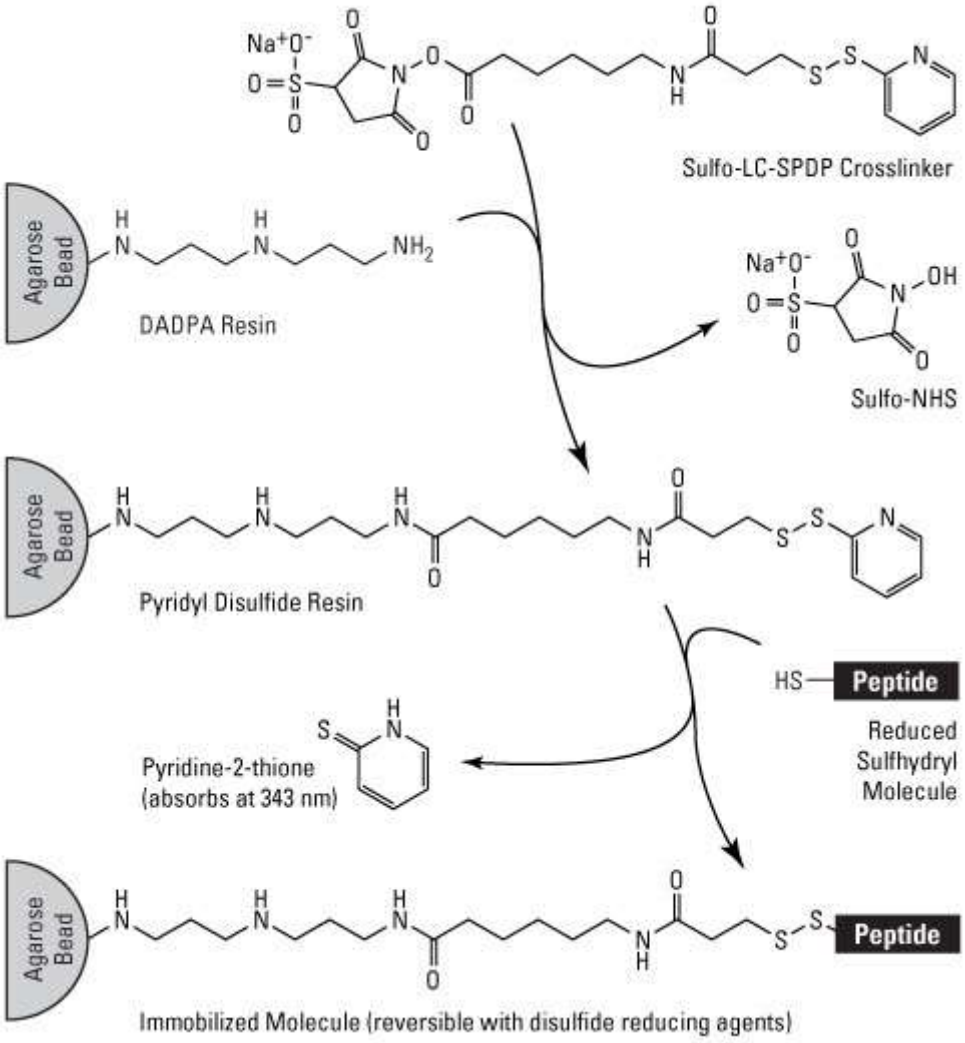


Figure 5: Sulfo LC-SPDP reaction chemistry. In our application the agarose bead would represent the Silicon thin film. Instead of DADPA resin we would have a primary amine.

One particular advantage of Sulfo LC-SPDP against the other two crosslinkers is that upon interaction with a thiol group, Pyridine-2-thione is released as a byproduct. This molecule has an absorbance peak at 343nm which would allow to add a simple checkpoint in the experiment to detect whether or not and to which extent the protein has been immobilized onto the surface.

The full functionalization process for the three crosslinkers is represented in Figure 6.

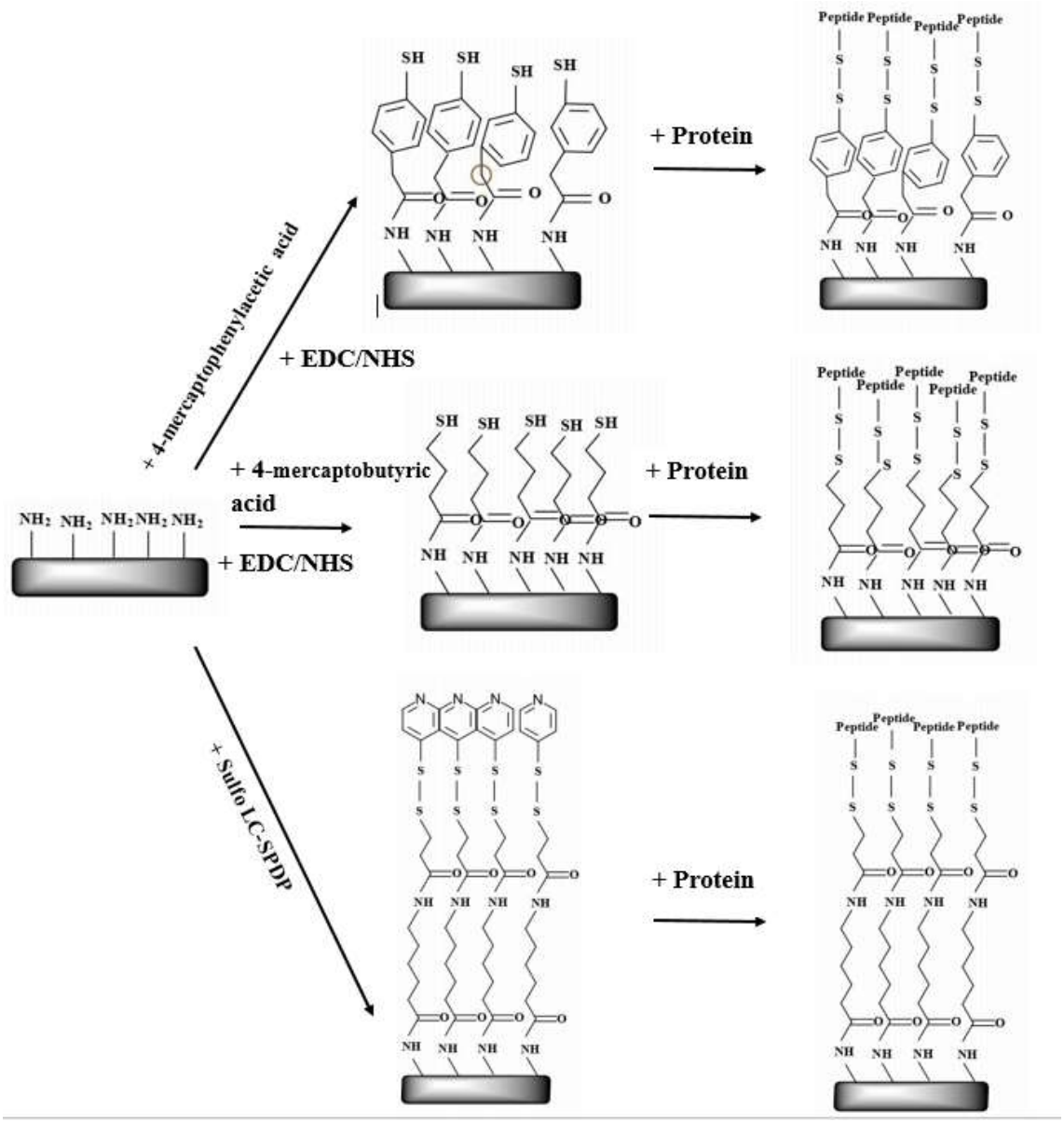


Figure 6: Steps of biofunctionalization for each of the crosslinkers

2.2.2. LDH as a protein model

The protein used in this work is Lactate dehydrogenase (LDH). It is present in mammalian cell cytoplasm. Its function is to regenerate NAD+ from NADH and pyruvate in low-oxygen conditions to keep glycolysis working. This protein has not been proven to have a beneficial effect regarding the compatibility of a material. However, it is a marker of cell death and tissue damage. Nevertheless, the main reason why this protein has been selected is because it is very easy to track its presence by means of spectrophotometry studies. Both NAD+ and NADH have absorbance peaks at 259nm but only NADH

exhibits another peak at 340nm (Figure 8). Therefore, as the reaction takes place and NAD+ is generated from NADH, a decrease in the absorbance at 340nm can be observed.

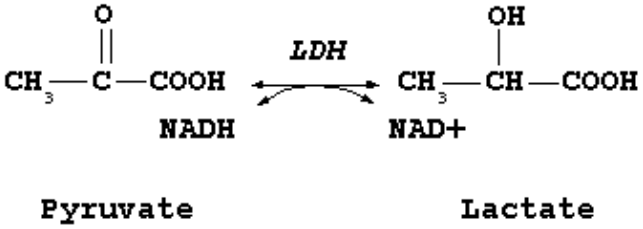


Figure 7: Reaction catalyzed by LDH

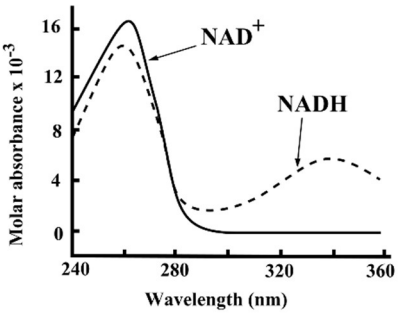


Figure 8: Absorbance of NAD+ and NADH at various wavelengths

3. MATERIALS AND METHODS

3.1. Biofunctionalization of silicon thin films

3.1.1. Crosslinking protocols in solution

In order to test the binding of the different cross-linking agents to the amine groups on the surface of the AVS functionalized silicon films and to optimize the concentrations of each reagent, a model in solution was performed.

This model would consist on a solution of Concanavalin A from Canavalia eusiformis saturated in amine groups. It was assumed that each Concanavalin molecule has 6 amines per molecule (one per arginine) available for binding to the cross-linker, as the constitutive amino group of each amino acid would be involved the peptide bond. A 1mM solution of Concanavalin A (that is, a 6mM solution of amine groups) was aimed for each of the cross-linkers. Since the different cross-linkers require different buffers and procedures, two different protocols were followed.

For each cross-linker and concentration two different assays were performed to study the binding:

→ Functionalization of the protein followed by a dialysis

→ Functionalization of the protein, labelling with fluorescein 5-maleimide and finally dialysis.

In both cases the absorbance of the sample was measured with a spectrophotometer both before and after dialysis at the following wavelengths:

- 280nm: to measure protein concentration (concentration of concanavalin)
- 343nm: absorbance of pyridine-2-thione (a byproduct of the reaction of Sulfo LC-SPDP when reacting with a sulfhydryl group)
- 494nm: absorbance of fluorescein 5-maleimide

The size of the pore of the dialysis membrane (3500 Da) was smaller than the size of concanavalin but significantly bigger than the size of all the cross-linkers and of fluorescein. Only the cross-linker bound to the protein would be retained inside the dialysis column, whereas the unreacted molecules would be eluted. In the fluorescence labelled case, the fluorescein would only attach to those concanavalin molecules that have

been successfully functionalized, as the maleimide group reacts with the thiol group that the cross-linkers leave exposed. (Figure 9)

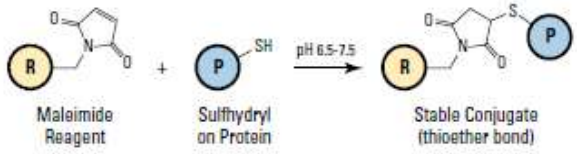


Figure 9: Maleimide reaction chemistry. (R) represents a labeling reagent or one end of a crosslinker having the maleimide reactive group; (P) represents a protein or other molecule that contains the target functional group (i.e., sulfhydryl, -SH). (Crosslinking technical handbook. Thermo Scientific)

3.1.1.1. Cross-linkers requiring EDC/NHS chemistry

The buffer required for this reaction chemistry was MES buffer (0.1M MES hydrate in distilled water pH=3.74). Therefore the concanavalin solution was prepared in this solvent.

On a first stage, two different concentrations of cross-linkers were tested (1X and 10X). The concentration 1X would have ten times of cross-linker molecules than amines, and for the 10X, a hundred times the amount of amines. The concentration for the 1X samples was $6x10^{-5}$ moles/mL and for the 10X samples, $6x10^{-4}$ moles/mL

In the case of 4-mercaptophenyl acetic acid it had to be first dissolved in 0.25mL of ethanol and then transferred to the 0.25mL of the concanavalin in MES buffer solution as it had a very low solubility in MES buffer. 4-mercaptobutyric acid was directly dissolved in 0.25mL of MES buffer and then mixed with 0.25mL of concanavalin in MES buffer solution.

In both cases, the amount of NHS was kept as the same amount of NHS than cross-linker, and the amount of EDC was four times larger than that of NHS (according to the literature, increasing the amount of EDC with respect to NHS stabilizes the reaction).

For the functionalization of the concanavalin, the protein solution and the cross-linker solution were mixed and incubated for 1h. Then 1mL EDC/NHS solution was added and the incubation continued for 4 more hours at room temperature. Then the samples were diluted in MES buffer up to a volume of 10mL and separated for dialysis or fluorescence labelling.

3.1.1.2. Sulfo LC-SPDP

In this case, the buffer required by this chemical according to the technical sheet of the product, was PBS-EDTA coupling buffer (50 mM Phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2). The cross linker solution was prepared by mixing 50uL of a 10mM solution of Sulfo LC-SPDP in distilled water per mL of coupling buffer. It was then added to the protein solution and incubated for 30minutes minimum at room temperature to allow binding. Then the samples were separated for dialysis or fluorescence labelling. This was the only concentration tried for this reactant as it was the one indicated in the protocol of the supplier.

3.1.1.3. Labelling with fluorescein 5-maleimide

For the labelled assays, 1mg of fluorescein 5-maleimide was added to each 5mL sample that was then incubated overnight at 4ºC.

3.1.2. Functionalization of Si thin films

Silicon wafers were cut in 1cm^2 square samples. They underwent a cleaning protocol consisting on sonication in acetone and isopropanol, treatment with hydrofluoric acid, piranha solution and ethanol and were dried with argon. Then they were amine-functionalized in a 20-minute cycle in the AVS equipment.

3.1.2.1. Activated vapor silanization (AVS)

The silanization process was performed in a device designed and constructed entirely by the biomaterials group at Centro de Tecnología Biomédica (CTB-UPM) [7]. A scheme of the device is shown in Figure 10.

Four silicon samples were placed in the AVS device holder that was then introduced in the chamber. 3mL of APTS were placed inside the evaporation chamber. The system was then closed and sealed. Then the vacuum pump was turned on. Once the pressure reached a value in the range of 10^{-2}mbar, dry ice and acetone were poured into a cold trap. The activation furnace was then turned on to 750ºC and the temperature controller of the evaporation chamber was set to 150ºC. The argon tank valve controlling argon flux was opened and the pressure was adjusted to 1.9-2.1mbar. The duration of a functionalization cycle is 20 minutes. This cycle timing yields an approximate amine thickness layer of 200nm. When the system had cooled down, the samples were taken out and allow to cool to room temperature.

Before further functionalization the samples were cleaned again by sonication in acetone and ethanol.

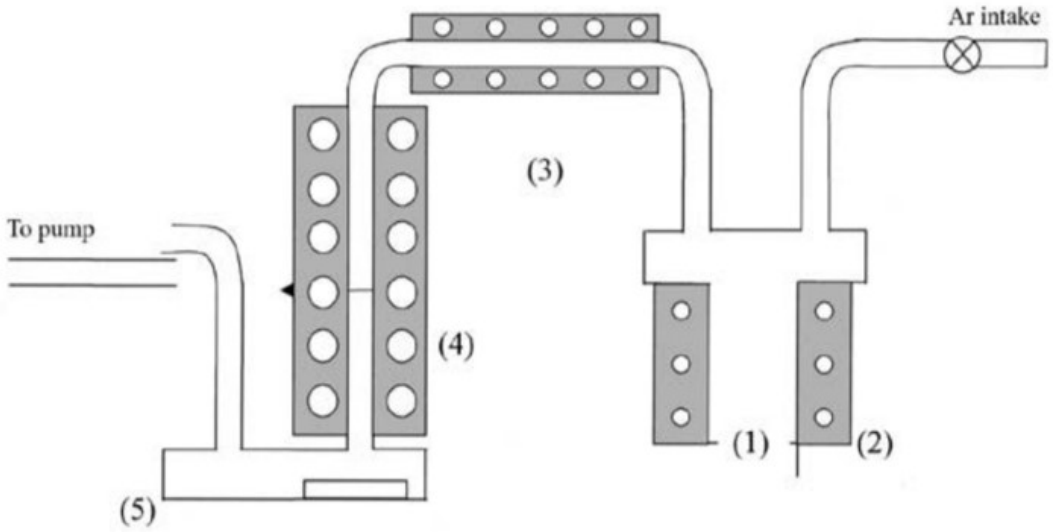


Figure 10: Scheme of an AVS reactor with each of its components. (1) Evaporation chamber, (2) Evaporation furnace, (3) Tape furnace, (4) Activation furnace, (5) Activation chamber. (Martin Palma et al., 2004) [7]

3.1.2.2. Cross-linkers requiring EDC/NHS chemistry

As it was aimed to replicate the conditions of the concanavalin-in-solution experiment, the same concentration of cross-linker ($6x10^{-5}$ moles per mL), NHS (5X amount of crosslinker) and EDC (4X amount of NHS) was employed to prepare the crosslinker and EDC/NHS solutions for functionalization. These solutions were performed in MES buffer (0.1M, pH 3.74).

The clean silicon samples were placed in a multi well, covered with 0.5mL of cross linker solution and incubated at room temperature for 1h. After that, 0.25mL of EDC/NHS solution were added to each sample well and incubated for 4 more hours.

Once the functionalization was done, the samples were washed in MES buffer to eliminate non-reacted products and transferred to a new well.

3.1.2.3. Sulfo LC-SPDP

The clean silicon samples were placed in a multi well, covered with 0.5mL of an analogous Sufo LC-SPDP solution to the one used for the solution experiment prepared

(final concentration 0.5mM) and incubated for 30 minutes at room temperature. Then the samples were washed in distilled water and transferred to a new well.

3.1.2.4. Labelling with fluorescein 5-maleimide

To prepare the surface, each simple was incubated in 1mL of coupling buffer for 1h. Afterwards, coupling buffer was substituted by 1mL of fluorescein solution and incubated overnight at 4ºC. Then the samples were washed in coupling buffer and transferred to a new well.

3.2. Study of the kinetics of protein adsorption on the surface

Cleaning and preparation of the silicon surface for this experiment was found to be critical as the oxide layer on silicon films could interfere with the adsorption process of the protein. The cleaning protocol consisted again in sonication in acetone and isopropanol for 3 minutes, followed by a 10 minute bath in a 10% HF solution (for passivating the surface), a brief wash in piranha solution and a final wash in ethanol to be then dried with argon. Before every enzymatic assay the surface was equilibrated by a 1-hour incubation in Tris-HCl (0.1M, pH 7,1).

3.2.1. Calibration curve of NADH

As the amount of protein on the surface was to be derived from the amount of NADH consumed along time, measured as the change in absorbance at 340nm (peak of absorption of NADH), a calibration curve was needed to relate the absorbance measured in the spectrophotometer and the amount of NADH.

By Lambert-Beer's law it is known that it can be obtained an equation of the form:

$$Abs = m \times [NADH] \qquad \text{(Eq. 1)}$$

Where Abs is the absorbance measured in the spectrophotometer in a.u. and [NADH] is the concentration of NADH on the sample.

A blank solution of Tris-HCl (0.1M, pH 7,1) with sodium pyruvate 2.9mM (the other necessary reactant for the LDH reaction) was prepared and used to set the 0.000 absorbance in the spectrophotometer. From that blank solution a stock solution of NADH 6mM was prepared to then obtain a set of more diluted solutions (0.05mM, 0.1mM,

0.2mM, 0.3mM and 0.4mM). The equipment used for this measurements was a Halo RB-10 (Dynamica) spectrophotometer with a 1cm long quartz cuvette.

A total of 5 measurements were taken for each concentration. After 3 measurements, the cuvette was washed with blank solution and the absorbance with blank solution was checked. In the cases where it was non-zero, the value was kept to correct the following two measurements. Before changing to a new concentration the cuvette was washed again and the blank-correction measurement was performed. The five absorbance values for each concentration were averaged to obtain the calibration curve.

The slope of Eq.1 was obtained by least-squares fitting with Excel.

The calibration curve was performed every week in order to ensure an accurate calibration according to the analyte as it could be degraded along time during storage.

3.2.2. Enzymatic activity of LDH

Another calibration experiment was needed to characterize the enzyme for further analysis of its adsorption on a solid substrate; the quantification of its enzymatic activity for the reaction: Pyruvate + NADH→Lactate + NAD+

We define the enzymatic activity of an enzyme as the amount of substrate converted per unit time per unit mass of the enzyme.

In this case, a solution of 0.18mM of NADH and a 0.1ug/mL LDH solution were prepared and placed in the spectrophotometer cuvette. The absorbance was monitored during 10 minutes by taking measurements every 30 seconds. The same process was performed with a solution of [LDH]=0.05ug/mL.

The enzymatic activity of LDH was calculated using Equation 2:

$$U = \frac{\Delta n_{NADH}}{\Delta t} \times \frac{1}{m_{LDH}} \qquad \text{Eq. 2}$$

Where U is the enzymatic activity in $\frac{\mu mol}{min \times mg}$, m_{LDH} is the amount of enzyme and the term $\frac{\Delta n_{NADH}}{\Delta t}$ can be obtained as the slope from a linear fitting of the measurements of absorbance of the experiment transformed to molar units using Eq. 1 and the value of the slope from the calibration curve of NADH.

3.2.3. Study of adsorption under static conditions

As a first approach, the process of adsorption of a protein (in this case LDH) to a solid surface was studied under static conditions to see the dependence of the time of incubation and of the concentration of the protein on the adsorption process. This study would serve as control to compare it with the results under dynamic flow incubation conditions and to compare the adsorption of the protein on naked silicon versus functionalized silicon.

For this study the silicon samples were cleaned following the cleaning protocol mentioned previously, placed in a multi-well and incubated for 1h in Tris-HCL (0.1M; pH 7.1) for one hour to equilibrate the surface prior to the beginning of the experiment. The concentrations of LDH to be compared were 50ug/mL, 80ug/mL, 110ug/mL and 300ug/uL. For each sample, four different incubation times 30min, 60min, 120min and 240min were studied.

The corresponding LDH solution was prepared in Tris-HCl (0.1M, pH 7.1). Once the equilibration of the surface was done, the Tris-HCl buffer was removed and substituted by 600uL of LDH solution with the desired concentration and allowed to incubate at room temperature for 30, 60, 120 and 240min respectively.

After the incubation time, the LDH solution was removed and the sample was washed in 1mL of Tris-HCl (0.1M, pH 7.1) by carefully pipetting up and down against the wall of the well to remove the unadsorbed protein. Then it was transferred to a new well and covered with 600uL of stock NADH solution (6mM NADH in blank solution). The first spectrophotometry measurement (corresponding to time 0, i.e., starting conditions) was taken by transferring 30uL of solution from the well into the spectrophotometer cuvette containing 970uL of blank solution and measuring the absorbance at 340nm. The cuvette was inverted twice with a parafilm for proper mixing of the 30uL sample with the blank solution in the cuvette. The same measurement was performed every 30minutes for 90 minutes. After every measurement the cuvette was washed with blank solution and a calibration measurement with blank solution was taken for calibration.

In order to account for the reduction in volume in the sample well after each 30uL extraction, a correction of the Beer-Lambert Law from Eq 2. was made using Equation 3:

$$\mu mol\ NADH\ (t_n) = \frac{OD_{340}(t_n) \times V_{cuvette}}{\varepsilon \times l} \times \left(n + \frac{V_{essay}(t_n)}{V_{sample}}\right) \qquad \text{Eq. 3}$$

Where n corresponds to the number of previous samples taken (being n=0 for the time 0 measurement), OD_{340} is the absorbance measured at 340 nm, $V_{cuvette}$ is the volume inside the spectrophotometer cuvette (970uL+30uL=1mL), V_{essay} is the volume remaining in the sample well at time t_n (that is, (600uL-30xn uL)), V_{sample} is the 30uL taken for each measurement, l is the length of the cuvette (1cm) and ε is the molar extinction coefficient in $\frac{1}{mM \times cm}$. In practice, the term $\varepsilon \times l$ is replaced by the slope of the calibration curve of NADH for more accurate conditioning of the experiment.

Knowing the NADH decay along time (which follows a linear relationship) and assuming that the enzymatic activity is the same in solution than when the enzyme is adsorbed on a substrate, the amount of adsorbed protein molecules can be extrapolated using Eq 2. Also, since the size of the silicon samples is kept constant at 1cm^2 and knowing the molar mass of LDH (36595 Da) one can calculate the density of protein molecules adsorbed on the substrate.

3.2.4. Study of adsorption under dynamic conditions

As a more realistic model of the biological environment, another setting that allowed the flow of the LDH solution over the silicon substrate was prepared. The aim of this experiment was to observe how the volumetric flow rate (which in turn results in a shear strain rate) affects protein adsorption when compared to the static conditions described in the previous section.

The experimental setting consisted on a pump connected to two plastic syringes (one for the inlet and another one for the outlet of the fluid) connected in turn to a metallic circular platform used normally as substrate holder in atomic force microscopy. The syringes and the platform were connected with plastic tubes. The silicon sample was stuck to the platform with vacuum grease. The platform with the sample was then covered with a petri dish to minimize evaporation of the protein solution during the process. A photograph of the experimental setting can be seen in Figure 11.

Before the beginning of the experiment, the circuit was filled with protein solution avoiding as much as possible the formation of bubbles as these could hinder the adsorption process by creating a turbulent flow.

Figure 11: Set-up for the dynamic enzymatic study. Inlet and outlet syringes are connected to a syringe pump. The syringes are connected to the pool containing the silicon sample with plastic tubes.

In view of the results obtained in the static conditions, it was decided that the optimal concentration to have a good time vs adsorption compromise was 110ug/mL. As a first approach, the experiment was performed with this concentration for different incubation times (30 and 60minutes) for a volumetric flow rate of 16.67uL/min. Then, also for a [LDH]=110ug/mL, the volumetric flow rate was halved (8.34uL/min).

The cleaning and surface equilibration procedures were the same as for the static conditions experiment. Once the dynamic incubation was over, the sample was also treated similarly to the protocol followed for the static conditions study: It was washed, incubated with 600uL of NADH stock solution and spectrophotometry measurements were performed every 30min for 90min.

4. RESULTS

4.1. Preliminary results of the crosslinking protocols in solution and optimization of the concentrations

Both 10X concentrations were over saturated. They precipitated in the tube and formed granules. They were therefore discarded.

The absorbance results for the concentrations 1X and Sulfo LC-SPDP can be observed in Tables 2 and 3.

TABLE 2. ABSORBANCE (A.U.) OF THE UNLABELLED SAMPLES

UNLABELLED		4-MERCAPTOPHENYL ACETIC ACID (1X)	4-MERCAPTOBUTYRIC ACID (1X)	SULFO LC-SPDP
280NM	BEFORE	3	1.305	3
	AFTER	-0.102	0.274	0.101
343NM	BEFORE	3	1.305	3
	AFTER	0.119	0.078	0.023
494NM	BEFORE	1.223	0.013	2.387
	AFTER	0.068	0.053	0.017

TABLE 3. ABSORBANCE (A.U.) OF THE LABELLED SAMPLES

LABELLED		4-MERCAPTOPHENYL ACETIC ACID (1X)	4-MERCAPTOBUTYRIC ACID (1X)	SULFO LC-SPDP
280NM	BEFORE	3	1.818	3
	AFTER	3	0.816	3
343NM	BEFORE	2.097	2.854	0.518
	AFTER	3	0.484	1.231
494NM	BEFORE	1.353	0.516	2.854
	AFTER	1.755	0.487	2.42

As another way of visualizing the results after functionalization and labeling, the labelled dialyzed samples were put in a multi well to be examined under a fluorescence microscope (Figure 12).

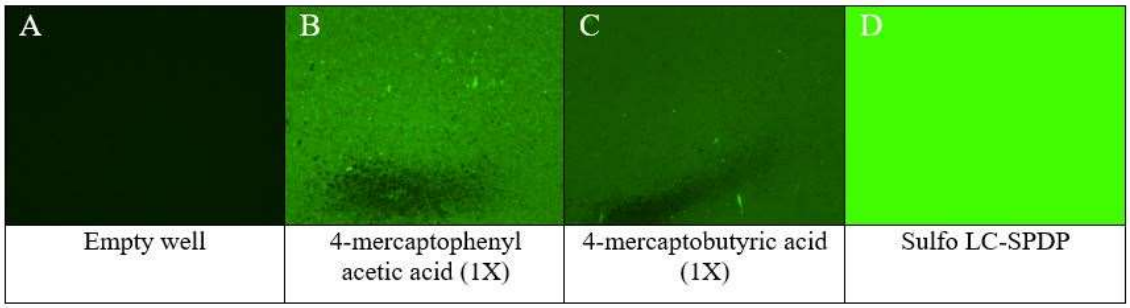


Figure 12: Fluorescence microscopy images of the labelled samples after dialysis

Considering that the number of amines in solution was noticeably greater than that achievable in the 1cm^2 silicon films, it was decided that the results obtained for 4-mercaptophenyl acetic acid and 4-mercaptobutyric acid would not yield satisfactory results when applied in silicon films. The fraction of functionalized amines would be too low.

Therefore the experiment was repeated changing the EDC and NHS concentrations. As the reaction follows a 1:1 stoichiometry for EDC, NHS and cross linker it was speculated that having a 1:1 proportion of NHS and cross linker was limiting the reaction. The new proportions proposed were 5X NHS than cross linker and 4X EDC than NHS (that is, 20X EDC than cross linker). The protocol followed was maintained the same.

The absorbance results are displayed in Tables 4 and 5 and fluorescence in Figure 13.

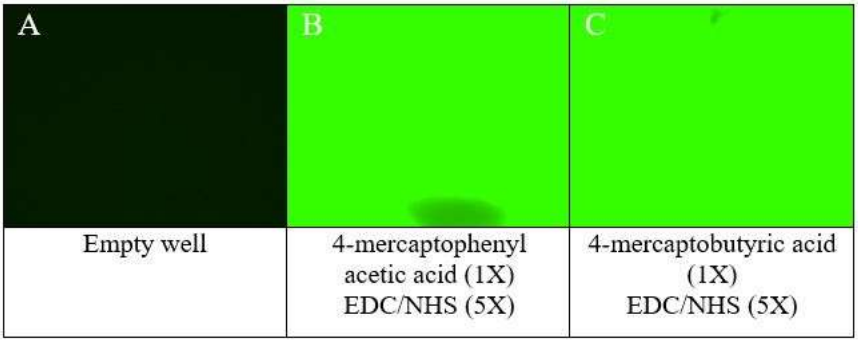


Figure 13: Fluorescence microscopy images of the labelled samples after dialysis with new EDC/NHS concentrations.

TABLE 4. ABSORBANCE (A.U.) OF THE UNLABELLED SAMPLES FOR NEW EDC/NHS CONCENTRATIONS

UNLABELLED		**4-MERCAPTOPHENYL ACETIC ACID (1X) EDC/NHS (5X)**	**4-MERCAPTOBUTYRIC ACID (1X) EDC/NHS (5X)**
280NM	**BEFORE**	3	3
	AFTER	2.678	2.208
343NM	**BEFORE**	2.398	0.811
	AFTER	1.656	0.678
494NM	**BEFORE**	1.44	0.353
	AFTER	1.031	0.319

TABLE 5. ABSORBANCE (A.U.) OF THE LABELLED SAMPLES FOR NEW EDC/NHS CONCENTRATIONS

LABELLED		**4-MERCAPTOPHENYL ACETIC ACID (1X) EDC/NHS (5X)**	**4-MERCAPTOBUTYRIC ACID (1X) EDC/NHS (5X)**
280NM	**BEFORE**	3	3
	AFTER	2.797	2.268
343NM	**BEFORE**	3	1.371
	AFTER	2.208	1.047
494NM	**BEFORE**	1.995	1.176
	AFTER	2.347	1.128

These new EDC/NHS concentrations were established as optimum and were the ones used for silicon functionalization.

4.2. Functionalization of Si thin films

After functionalization with the different cross-linkers, silicon samples were examined under the fluorescence microscope. The results of the functionalization can be seen as green fluorescence. Two negative controls were prepared for comparison: naked silicon and silicon treated with AVS but no further cross linking. In both cases they underwent the same cleaning and labeling protocols. Results can be observed in Figure 14.

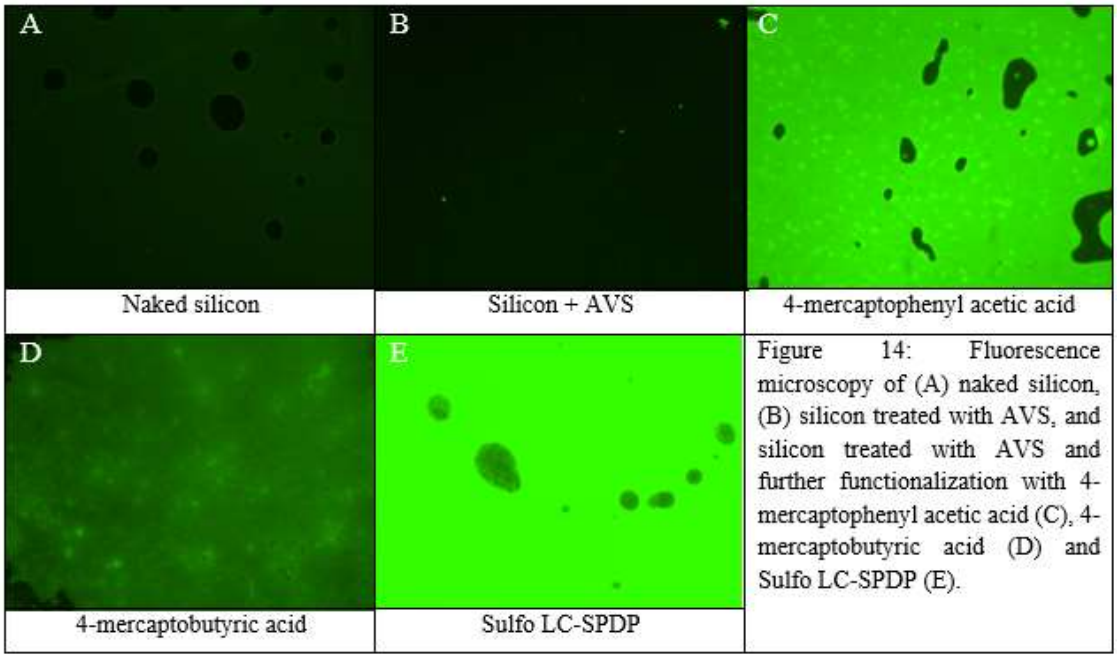


Figure 14: Fluorescence microscopy of (A) naked silicon, (B) silicon treated with AVS, and silicon treated with AVS and further functionalization with 4-mercaptophenyl acetic acid (C), 4-mercaptobutyric acid (D) and Sulfo LC-SPDP (E).

4.3. Study of the kinetics of protein adsorption on the surface

4.3.1. Calibration curve of NADH

An example of the data and the graphs obtained in a calibration curve experiment can be seen in Table 7 and Figures 15 and 16. As it can be observed, the variability in each of the measurements was negligible and, as expected, there was a linear relationship between the absorbance and the concentration of NADH (obtaining in every case high R^2 values).

TABLE 7: EXAMPLE OF THE DATA OBTAINED IN A CALIBRATION CURVE EXPERIMENT

	0.05mM	**0.1mM**	**0.2mM**	**0.3mM**	**0.4mM**
MEASUREMENT 1	0.369	0.982	1.288	2.078	2.994
MEASUREMENT 2	0.37	0.986	1.288	2.078	2.994
MEASUREMENT 3	0.371	0.984	1.288	2.078	2.994
BLANK SOLUTION	-0.001	-0.001	-0.002	0	0.005
MEASUREMENT 4	0.371	0.971	1.287	2.076	2.995
MEASUREMENT 5	0.371	0.973	1.291	2.076	2.995
BLANK SOLUTION	-0.003	0.001	-0.002	0.006	
ARITHMETIC MEAN OF CORRECTED VALUES	0.3704	0.9792	1.2884	2.0772	2.9944

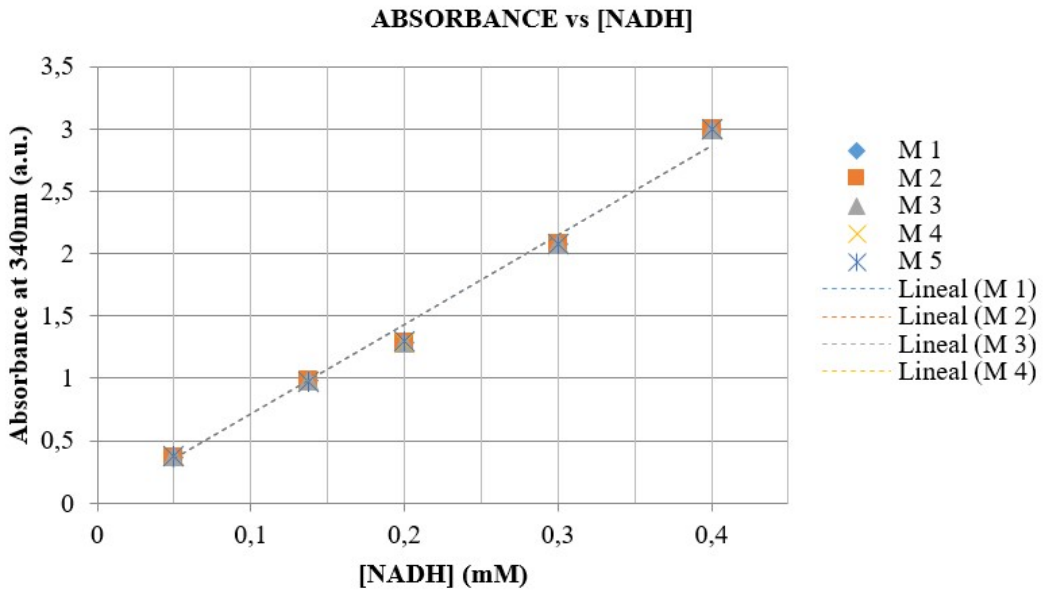


Figure 15: Example of a calibration curve for absorbance vs [NADH]. The five measurements for each NADH concentrations with its corresponding linear estimations are shown.

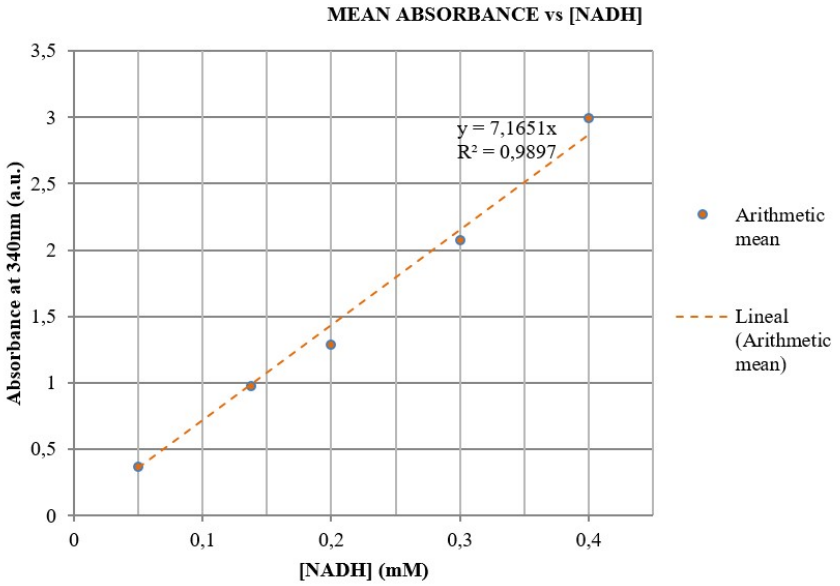


Figure 16: Example of a calibration curve for absorbance vs [NADH]. The mean of the five measurements and its linear estimation is shown.

4.3.2. Enzymatic activity of LDH

An example of the graphs obtained from the enzymatic activity experiment and used for the calculation of the enzymatic activity of LDH is shown in Figure 17.

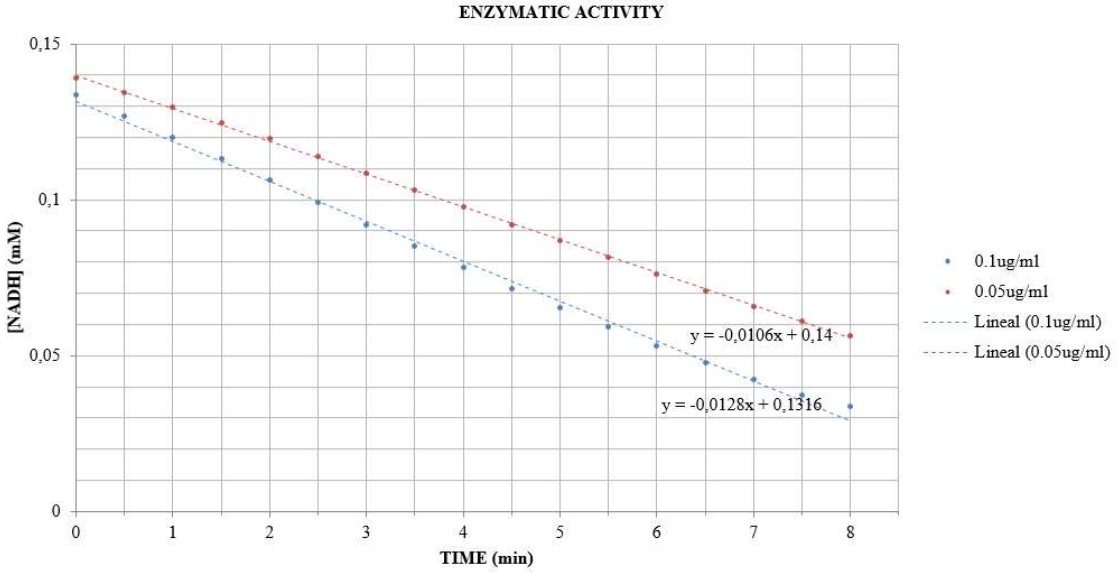


Figure 17: Example of a calibration experiment for enzymatic activity of LDH. Only the linear region is shown as it is the one considered for the calculations.

The amount of NADH due to the activity of the enzyme follows an exponential decay, so only the linear region of the experiment was considered for the linear fitting (which was usually the first 6 to 8 minutes of the experiment).

The value obtained in this particular case by averaging the activities obtained with the data of both LDH concentrations was 169.7 $\frac{\mu mol}{min \times mg}$. The theoretical range of enzymatic activity for this enzyme is between 117.3 and 225.2 $\frac{\mu mol}{min \times mg}$, so the value obtained (as it was the case in every repetition of the experiment) falls within these theoretical values. This test was performed periodically to check the possible degradation of the enzyme upon storage.

4.3.3. Study of adsorption under static conditions

Figures 16 and 17 show examples of the information obtained from the enzymatic assays. After several repetitions it was observed that the experiment was highly variable, as there was always a set of data points that diverged from the expected results. These deviations were random, not being related to a particular concentration or incubation time. It was therefore concluded that it was the consequence of experimental mistakes in the relatively sophisticated experimental procedure. When the experimental errors were removed, the tendency was clearly observed during the several repetitions of the experiment; The longer the incubation time, the more protein adsorbed on the substrate and therefore steeper a decay of NADH was observed (Figure 18); and the higher the protein concentration, the better the adsorption for the same incubation time (Figure 19).

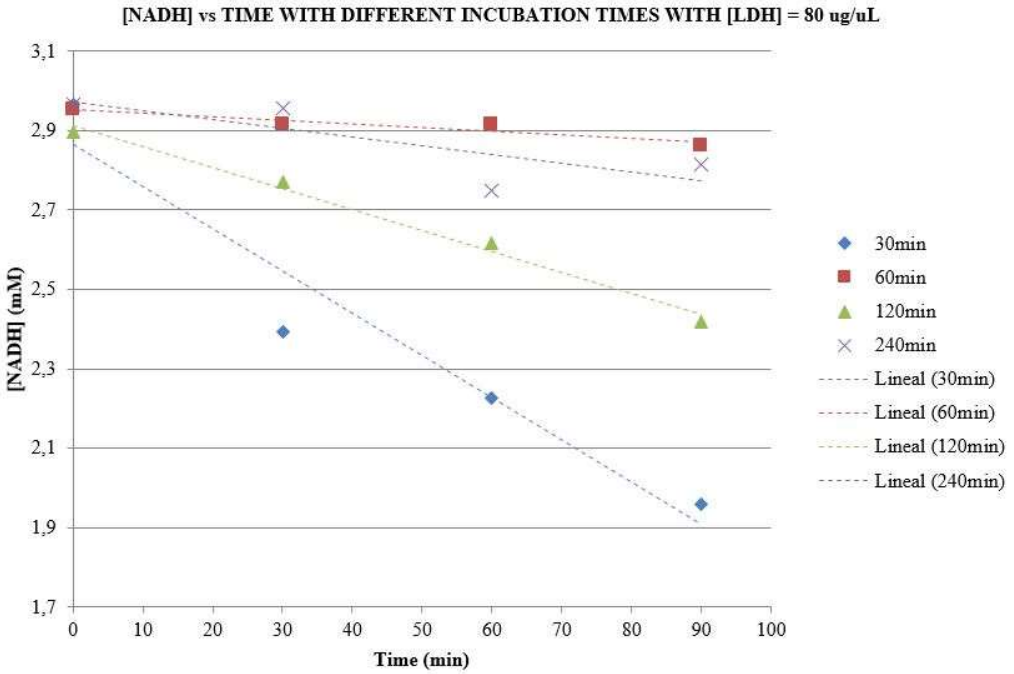


Figure 18: Enzymatic assay in silicon for a fix [LDH]=80ug/uL for different incubation times.

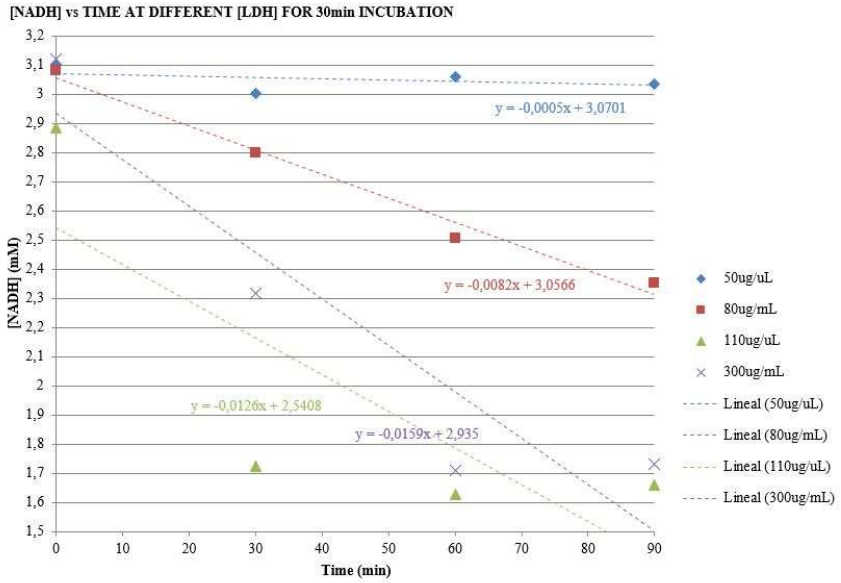


Figure 19: Enzymatic assay in silicon for a fix incubation time (30min) and different concentrations of LDH

4.3.4. Study of adsorption under dynamic conditions

The results obtained for the dynamic conditions show no evidence of adsorption on the substrate neither for longer incubation times nor for slower volumetric flow rates. As it can be seen in Figure 20, there is not a linear decay of NADH as it was the case under static conditions. It was concluded that there was no LDH adsorbed on the silicon substrate and that the slight variability of the absorbance was due to experimental errors.

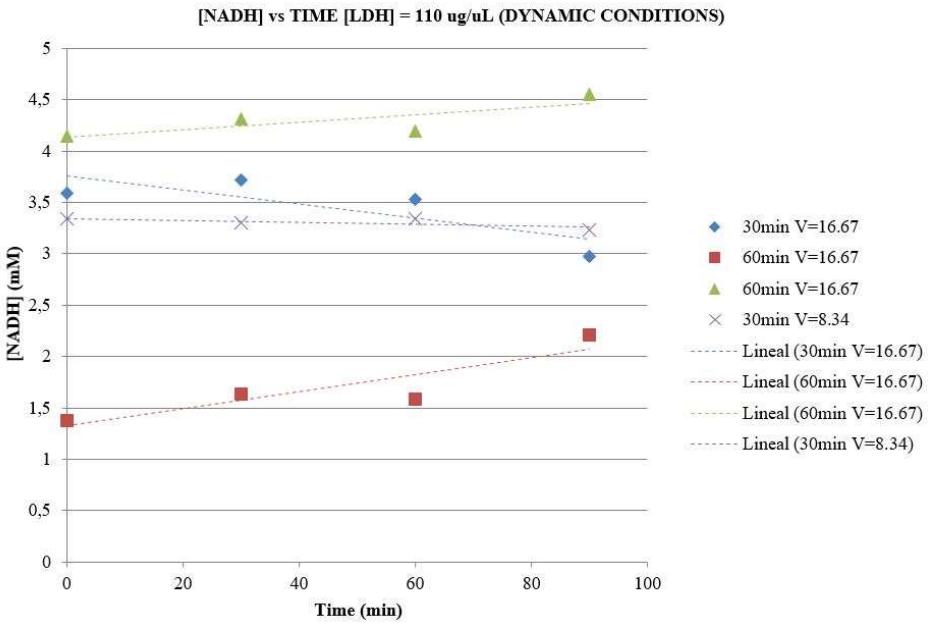


Figure 20: Enzymatic assay in silicon in dynamic conditions for a fix [LDH]=110ug/uL and two different incubation times (30min and 60min). Two repetitions of each incubation time were performed.

In order to guide the experimental procedure, a mathematical model was evaluated to see if the range of volumetric flow rates chosen for the experiment were appropriate to allow protein deposition.

4.3.4.1. Mathematical model of adsorption in dynamic conditions

In order to provide a better understanding of how the system of protein adsorption under dynamic conditions was working, a mathematical model describing the condition was elaborated. The model was adapted from the one proposed by Ortega-Vinuesa et al. [10] where they compare the process of protein adsorption of blood proteins in static versus dynamic conditions, with different shear rates. They concluded that the parameter that most affected the protein adsorption process was the concentration of the protein, whereas shear rate had almost no effect. Only a slight difference was observed between the completely static conditions and the dynamic conditions. They claimed that while in static conditions the only phenomenon acting is diffusion, when they introduced a fluid velocity, convection also starts to play a role. They define two different cases:

- Diffusion limited adsorption: The rate of transport of the protein to the surface is slower than the rate of adsorption of the protein to the surface. This would occur mainly for low shear rates and more diluted solutions.
- Kinetic limited adsorption: It is the opposite case; when the rate of transport of the protein to the surface is faster than the rate of protein adsorption. Mainly applies for faster shear rates or highly concentrated protein solutions.

The adsorbed amount of protein will vary between both cases.

It is reported in the bibliography that there are two key parameters that affect the system and are specific of each protein and for each buffer and surface; those are the sticking coefficient and the diffusion coefficient. These specific values can be extrapolated from the experimental results in under static conditions following a mathematical model based on experimental data published by Daniel R. Weaver and William G. Pitt [12].

They elaborated a graph that relates the dimensionless observed slope of protein adsorption over time (which can be obtained from the enzymatic assays for different incubation times for the same concentration. See results section) with the dimensionless reaction rate. Once the dimensionless reaction rate is calculated, the sticking coefficient can be easily obtained for our specific condition following Equation 4. The experimental

slope obtained in the experiments can be transformed into the dimensionless observed slope using Equation 7.

$$\Phi = \frac{q \times P \times v_a \times C_m}{D \times C_b} \qquad \text{Eq. 4}$$

Where q is the probability of a step from the outlet towards the surface due to random walk (which is set to 0,5), P is the sticking coefficient, v_a is the average velocity of random walk in cm/s, C_m is the concentration of a monolayer in mg/cm^2, D is the diffusion coefficient of our protein (LDH) for our specific surface (silicon) in cm^2/s and C_b is the initial bulk protein concentration in mg/cm^3.

As we performed all the dynamic conditioned experiments with an LDH solution of [LDH]=110ug/mL, C_b is set to 0.11 mg/cm^3. C_m was calculated using data obtained from previous experiments of LDH adsorption in silicon films using atomic force microscopy [1], where it was estimated that the diameter of an adsorbed LDH molecule (after the well-known flattening of the protein in contact with the surface) was 2.19nm. Assuming that the area a molecule of LDH occupies is of a round shape, it was found that the maximum concentration of a monolayer is 1.6x10^{-6} mg/cm^2. v_a is calculated using Equation 5.

$$v_a = \sqrt[2]{\frac{kT}{Mw}} \qquad \text{Eq. 5}$$

Being k the Boltzmann constant in erg/moleculexKelvin (1.381x10^{-16}), T is the absolute temperature in Kelvin (assuming room temperature, T=298) and M_w is the molecular weight of the protein in gr/molecule (6.1x10^{-20}). The obtained value for v_a was 822.8 cm/sec.

For the calculation of the diffusion coefficient (D) it is assumed that a LDH molecule is spherical and therefore the Stokes-Einstein relation can be applied (Equation 6).

$$D = \frac{10^4 kT}{6\eta r_{sol}\pi} \qquad \text{Eq. 6}$$

With k=Boltzmann constant in J/K (k=1.38x10^{-23}), η the dynamic viscosity of the buffer at the temperature at which the experiment took place in kg/m*s (assuming the viscosity of water at room temperature gives a value of 0.008921) and r_{sol} is the radius of the molecule in solution in m (extracted from the PDB, r_{sol}=4.35x10^{-9}).

For the dimensionless observed slope:

$$Q = \frac{\sqrt{t}\frac{dC_s}{dt}}{C_b\sqrt{\frac{D}{\pi}}} \quad \text{Eq. 7}$$

Where $\frac{dC_s}{dt}$ is obtained from the enzymatic assays in silicon for the different incubation times.

In accordance with the model by Ortega-Vinuesa et al. [10], if we find that in our system $\frac{D}{L} \ll \frac{v_a P}{4}$ it will mean that it is diffusion limited, whereas if we find that $\frac{D}{L} \gg \frac{v_a P}{4}$ it will be kinetic limited. Here L is defined as the distance of the boundary layer to the surface and was calculated with Equation 8.

$$L = \frac{2}{3}\sqrt[3]{\frac{2Dz}{\gamma_w}} \quad \text{Eq. 8}$$

Being z the distance from the inlet and γ_w the shear rate at the surface. For pipe flow this is calculated with Equation 9, where X is the volumetric flow rate in L/s and R is the radius of the pipe in cm.

$$\gamma_w = \frac{4X}{\pi R^3} \quad \text{Eq. 9}$$

Once having identified if the system is diffusion limited or kinetic limited, one can calculate the adsorbed amount of protein for a given time in each case.

For a diffusion limited system:

$$\Gamma = 2C_b\sqrt{\frac{Dt}{\pi}} \quad \text{Eq.10}$$

And for a kinetic-limited system:

$$\Gamma = \frac{v_a P}{4} C_b t \quad \text{Eq.11}$$

The graph from which the dimensionless reaction rate (Φ) was to be obtained from the experimentally obtained value of the dimensionless observed slope (Q) extracted from the model of Weaver and Pitt [12] is presented in Figure 21.

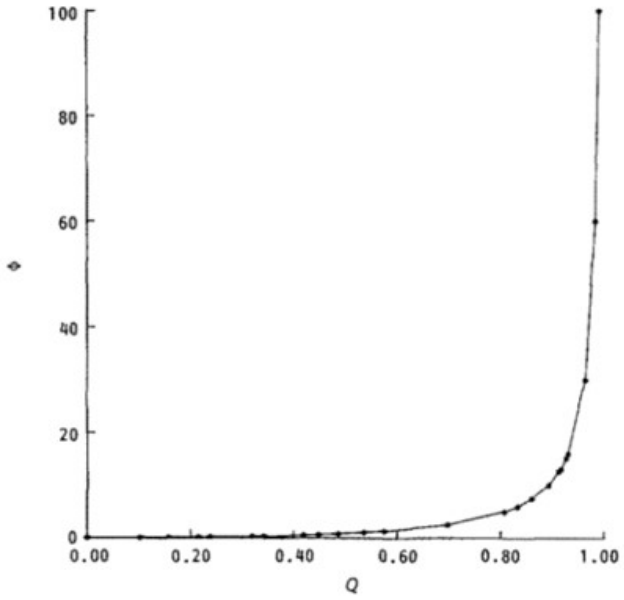


Figure 21: Dimensionless reaction rate (Φ) as a function of the dimensionless observed slope (Q). (Weaver and Pitt) [12]

However the final value obtained for Q from the experiments in static conditions with [LDH]=110ug/mL was $Q=4.37\times10^{-3}$. Given the scale of the graph it was impossible to provide an estimate for Φ and therefore to obtain the sticking coefficient (P) for our specific condition. Weaver and Pitt assert in their paper that the difference between a system with $\Phi=1$ and one with $\Phi=2\times10^{-5}$ cannot be discerned with experimental data [12].

The value obtained for the diffusion coefficient (D) was $D=5.62\times10^{-7}$ cm^2/s which is in the range of the values used by Ortega-Vinuesa et al [10] to generate their results (Albumin (6.7×10^{-7}) and fibrinogen (2×10^{-7})), and also in the range that Weaver and Pitt take as a typical value for a protein system ($\approx2.5\times10^{-7}$) [12].

A comparison of the different values studied by Ortega-Vinuesa et al [10] for Albumin and Fibrinogen and the ones obtained in this work for LDH are presented in Table 8.

It can be observed that the shear rates that have been tested in this work are considerably lower and the protein solutions noticeably more diluted, which would be in principle in accordance with a diffusion limited system. The limiting case between kinetic and diffusion limited adsorption for our system ($\frac{D}{L}=\frac{v_a P}{4}$) occurs for $P=1.99\times10^{-6}$ for $\gamma_w=2.83$, and for $P=3.94\times10^{-7}$ for $\gamma_w=1.41$.

TABLE 8: SOME PARAMETERS FOR THEORETICAL CALCULATION AND CHARACTERIZATION OF THE SYSTEM

Sample	**γ_w (1/s)**	**D (cm^2/s)**	**v (m/s)**	**Avg L (um)**	**D/L (m/s)**	**vP/4 (m/s) P=10-6**	**vP/4 (m/s) P=10-8**
Albumin (1mg/mL)*	225	$6.7x10^{-7}$	10	12.8	$5.2x10^{-6}$	$2.5x10^{-6}$	$2.5x10^{-8}$
Fibrinogen (0.3mg/mL)*	225	$2x10^{-7}$	4	8.6	$2.3x10^{-6}$	$2.5x10^{-6}$	$2.5x10^{-8}$
LDH (0.11mg/mL)	2.83	$5.62x10^{-7}$	8.23	55.01	$1.02x10^{-4}$	$2.05x10^{-4}$	$2.05x10^{-6}$
LDH (0.11mg/mL)	1.41	$5.62x10^{-7}$	8.23	69.29	$8.11x10^{-5}$	$2.05x10^{-4}$	$2.05x10^{-6}$

*Data extracted from Ortega-Vinuesa et al. [10]

However, taking into account that it was impossible to determine Φ due to a too low value of Q that would in turn give a very low value of Φ (which is directly proportional to P), and considering that no adsorption was observed for the dynamic conditions, it was concluded that $P<<3.94x10^{-7.}$ If that was the case, then the adsorption of LDH on silicon in this conditions would be kinetic-limited, and the absence of adsorption could be explained by an unbalance between the rate of transport of the protein to the surface and the rate of adsorption of LDH to the surface. It is also worth mentioning that the incubation times for adsorption studies found in the bibliography are considerably lower than the ones proposed in this work, which can lead to thinking that the affinity of this particular protein to this specific substrate is not very high.

5. CONCLUSIONS

5.1. Objectives accomplished

In view of the successful results obtained using 4-mercaptobutyric acid, 4-mercaptophenylacetic acid and Sulfo-LCSP it was concluded that they are good possibilities for crosslinking of proteins onto aminated surfaces in a reversible fashion by the use of disulfide bonds. The conjugation can also be applied in solution if the application requires so. Optimum conditions for the biofunctionalization reaction have been explained in this work along with the advantages that each of the molecules provide.

It has been checked that protein adsorption (specifically of LDH) takes place under static conditions in bare silicon films and it is dependent on the incubation time and the concentration of the protein. However, no adsorption was observed when the conditions were dynamic. A mathematical model was used to understand the system workflow and estimations for the sticking coefficient and diffusion coefficient were obtained. By comparing these values and other experimental parameters with the ones used by different authors in the bibliography, it was concluded that either the experimental set-up and selection of the parameters was not suitable for the reaction to take place, or the affinity of LDH for silicon films was too low to detect adsorption at such low concentrations. On the other hand, since the enzymatic assays were very experiment-dependent due to its sensitivity to contamination, inaccurate measurements of the low amount of reactants or timing of the experiment, and the low number of repetitions of the experiment, it could have been a consequence of experimental error.

5.2. Future lines of work

The conjugation of protein to the surface as the final step of the initially proposed biofunctionalization procedure was not carried out due to lack of time, so as future work this should be finished to obtain as a final product a fully biofunctionalized silicon thin film able to interact with proteins in the environment. Enzymatic assays would have to be performed on these biofunctionalized silicon thin films and compared to the ones from bare silicon to confirm that protein adsorption is improved and therefore biocompatibility is enhanced.

Then, the reversibility and repeatability of disulfide bond formation between the protein and the surface in a stable and efficient manner should be proven.

For the study in dynamic conditions, more repetitions of the experiment would be necessary to unveil if the non-adsorption in dynamic conditions is the product of experimental error. If that is not the case, concentration of the protein should be increased and faster shear rates should be introduced in the protocol. It would be interesting to find a set up such that the sticking coefficient could be determined to introduce it into the model. Some other proteins that have reported results in the literature, like albumin or fibrinogen, could be investigated for the study of the protein adsorption process.

6. SOCIO-ECONOMIC FRAMEWORK

6.1. Budget

On the following tables (Tables 9, 10, 11 & 12) it is presented the cost of each of the materials used throughout the project in order to get an estimate of its overall cost. Nevertheless, during the five months the project has lasted, not the full vial nor equipment packs were finished, so this data serves just as a rough approximation.

TABLE 9: COST OF LABORATORY REAGENTS

PRODUCT	SUPPLIER	PRICE (€)	VIAL	REFERENCE
L-Lactic Dehydrogenase	Sigma Aldrich	510	12.5KU	L1006-
Sodium pyruvate	Sigma Aldrich	14	5g	P2256
NADH	Sigma Aldrich	136	500mg	N8129
Sulfo-LC-SPDP	Sigma Aldrich	579,59	50mg	803316
Silicon wafers	Institute of electronic materials technology	400	25uds	S13949
Concanavalin A	Sigma Aldrich	115	100mg	L7647
4-mercaptobutyric acid	Sigma Aldrich	60	100mg	CDS004545
4-mercaptophenylacetic acid	Sigma Aldrich	63,9	1g	653152
EDC	Sigma Aldrich	127	5g	E7750
NHS	Sigma Aldrich	19,2	5g	130672
Fluorescein 5-maleimide	Thermo Fisher Scientific	194	25mg	62245
MES hydrate	Sigma Aldrich	49,3	25mg	M8250

TABLE 10: COST OF LABORATORY MATERIAL

MATERIAL	AMOUNT	COST (€)
Pipette tips (big)	Pack 1000	30,25
Pipette tips (medium)	Pack 1000	9,85
Pipette tips (small)	Pack 1000	21,8
Falcon tubes 15ml	Pack 500	392
Multiwell plates	Pack 75	157
Dyalysis membrane	1x30.5m	197,98
Gloves	Pack 100	22,5
Syringes	100uds	5,4

TABLE 11: COST OF EQUIPMENT

EQUIPMENT	COST
Dynamica Halo RB-10 Spectrophotometer	Already amortized
Fluorescence microscope	Already amortized
Computer	Already amortized
Power supply	118
Pump	Already amortized
Digital Sonifier 450	Already amortized

TABLE 12: COST OF HUMAN RESOURCES

CATEGORY	COST OF HUMAN PER MONTH (€)
Laboratory technician	1150
Bioengineering student	1000

6.2. Legal framework

Before reaching the market or a final application in living cells, tissues or organisms, a medical device must comply with a certain set of standards and regulations. Being biocompatibility a necessary requirement for not damaging the living environment the device interacts with nor for eliciting undesired responses in the patient (including possible side effects or the release of hazardous byproducts), many of these regulations demand biocompatibility and characterization tests in the first steps. Of course, depending on the final application (implants, tissue engineering, laboratory material…) the tests to be underwent and its level of demand will vary.

The ISO standard regulating biomedical devices is the ISO-10993 (Biological and Clinical Evaluation of Medical Devices) and the main tests regarding material biocompatibility, characterization and degradation products are [23]:

- Part 4: Selection of tests for interactions with blood
- Part 6: Tests for local effects after implantation
- Parts 13, 14 & 15: Identification and quantification of degradation products
- Part 18: Chemical characterization of materials
- Part 19: Physico-chemical, morphological and topographical characterization of materials

Another important international regulatory agency is ASTM. Some of its most general standards for new biomaterials and their interactions with the living world are ASTM F748 - 16 (Standard Practice for Selecting Generic Biological Test Methods for Materials and Devices) and ASTM F813 - 07(2012) (Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices). Nevertheless there are more precise standards depending on the final application. [22]

6.3. Socio-economic impact

The aim of this work is to contribute to develop a new biofunctionalization technique that can have several applications in the biomedical industry providing as a novelty an easily reversible protein cross-linking system. In addition, it intends to contribute to understand the protein adsorption process, a key factor on biomaterial outcome and its interactions with the living world.

All the medical equipment and devices must comply with the ISO10993 standard for FDA approval consisting on several biocompatibility tests, as rejection can have serious consequences on the patient in whom the biomaterial is being used. Regarding the diagnostics applications low biocompatibility can turn into biased and low quality results or difficulties during experimental practice.

Such is the case that in the last 25 years there has been a skyrocketing increase in the number of papers citing the term “Biocompatibility” in their keywords (Figure 22). This trend is exhibited not only at a worldwide scale but also specifically in Spain (Figure 23).

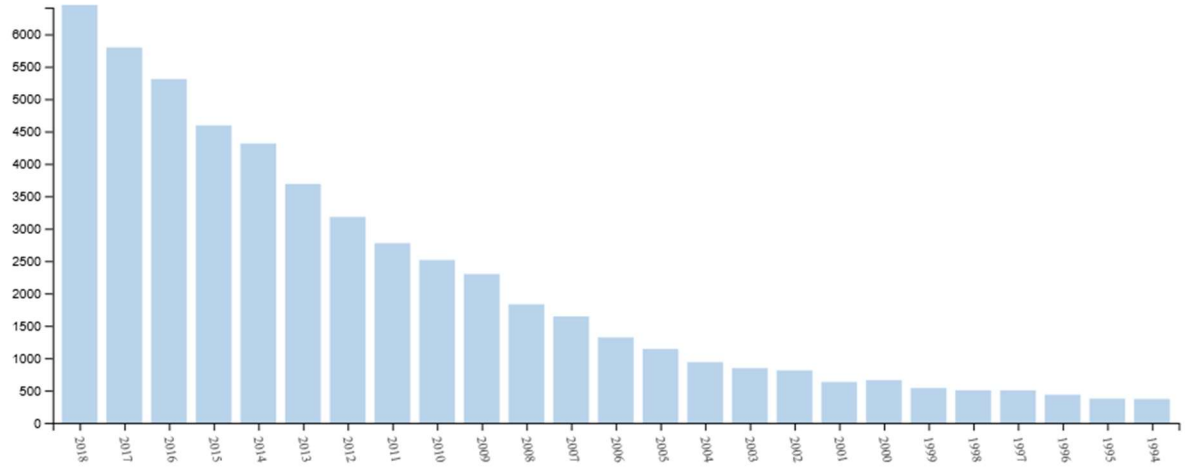


Figure 22: Number of papers published regarding the term "biocompatibility" worldwide in the last 25 years. (Data extracted from the Web of Science Citation Reports)

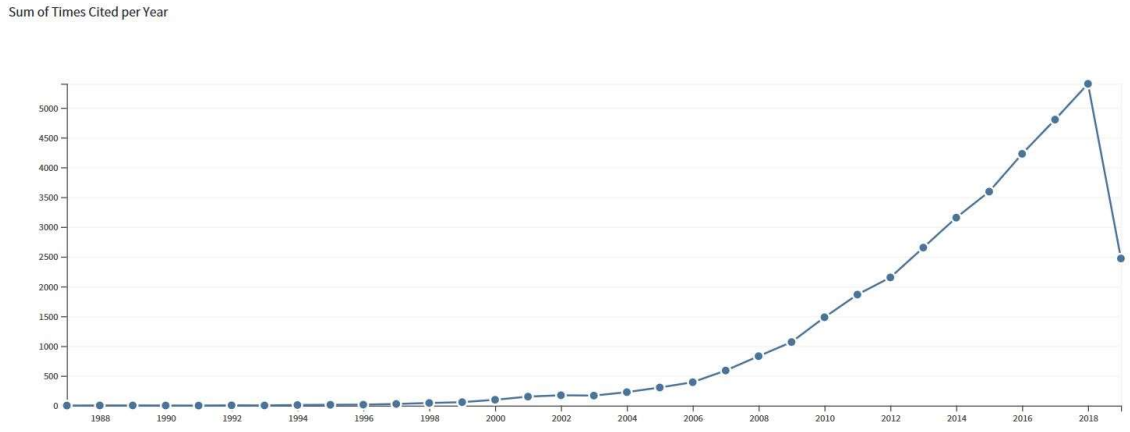


Figure 23: Number of papers published regarding the term "biocompatibility" in Spain in the last 25 years. The last point corresponds to data from May 2019, so it is expected to be at least doubled by the end of the year. (Data extracted from the Web of Science Citation Reports)

Therefore it can be concluded that this work is of scientific relevance as it provides an insight into new techniques inside a promising field as it is the biomaterial industry.

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