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Nanosensors for therapeutic drug monitoring

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1 Summary

In this project porous silicon has been used to work as an electrochemical sensor with the aim to help in developing cost-effective and simple tools able to discriminate the electrochemical detection of various antibiotics in serum and monitor their levels almost in real time. First, a brief introduction to therapeutic drug monitoring focused on antibiotics is made, and then follows the discussion on porous silicon, introducing its fabrication process. Some methods used nowadays for the monitoring of antibiotics in clinical and research laboratories are also discussed. Finally, using various electrochemical techniques we have studied the detection of various antibiotics on screen-printed electrodes and porous silicon fabricated samples, and we have compared the results obtained by both platforms.

2 Introduction

First, it is necessary to understand the meaning of pharmacokinetics. Pharmacokinetics is defined as the study of the time course of drug absorption, distribution, metabolism, and excretion. And as a sub-classification of pharmacokinetics, we find clinical pharmacokinetics.

Clinical pharmacokinetics emerged in the mid-1970s as a new guide of pharmacokinetics. According to Gerhard Levy, considered the founder of the biopharmaceutics field and the "father" of pharmacodynamics [2], **clinical pharmacokinetics** is defined as: "**a discipline of health sciences that deals with the application of pharmacokinetics to the therapeutic, safe, and effective care of the individual patient**".

With that main objective of improving therapy in individual patients through pharmacokinetic criteria, the functions of clinical pharmacokinetics are various. Since clinical pharmacokinetics is focused on a specific patient, their possibilities and applications are diverse depending on the patient himself. They are generally summarized in three:

1. **Design of dosage regimens**
2. **Provide information of the current health status of the patient**
3. **Consultation in the face of therapeutic problems that have pharmacokinetic basis and individual monitoring to control drug concentrations in the patient's blood**

The idea that drug concentration measurement in patients could be used to improve or control pharmacological treatments was proposed for the first time in 1950 and applied to the treatment of cardiac arrhythmias with quinidine. Physicians had appreciated the differences, both quantitative and qualitative, in the dose-response relationship. When it was possible to measure the concentrations, it was shown that most of these differences were caused by intern variables in the kinetic processes of absorption, distribution, metabolism, and excretion that drugs experiment in the body. Consequently, for some drugs, a stronger correlation between concentrations - response was established, which was more efficient than the correlation volume of dose - response, and therefore, the possibility to modify drugs concentrations for the optimization of pharmacological treatments began to be studied.

2.1 Introduction to therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is a test that measures a chemical parameter, which will directly affect medical drug prescriptions. It is done to ensure that the amount of prescribed medicine taken by patients is safe and effective.

Sometimes, to administrate a dose for certain drugs is hard and requires special testing, because it is necessary to know which dose provides enough drug to treat a condition without any dangerous side effects for the patients. TDM is helpful by health providers when searching for the proper drug doses to administer to each patient.

TDM is essential for drugs that have to be administrated for a lifetime. These drugs must be administrated at steady concentrations year after year while the patient goes through different life events that may alter the patient's therapeutic dose, such as pregnancies, infections, emotional and physical stress, accidents, or surgeries. Through time, these patients can also acquire more chronic conditions that require lifetime dosages, which may affect the processing of their monitored drugs.

With an appropriate TDM, these changes with dosages are adapted to the treatments. It is also possible to detect if patients are not following medications regularly as prescribed by the physicians and the possible adverse effects when different medications interact, as it can lead to unexpected changes in drug concentrations. In these cases, TDM has the objective to adjust the treatment with personalized dosages, adapting it to each patient's specific need.
This brings us to precision medicine, the healthcare model that focuses on the customization of treatments, practices, or products to specific groups of patients instead of doing a general model.

Concluding, TDM is used to find and optimize the best dosages for patients when they are treated with certain types of medicines, which are hard to dose by physicians.

TDM monitors blood levels of specific drugs to ensure they are in a narrow range in which the drug is adequate but not toxic. In addition, some drugs require to be supervised because the amount of dose administrated to the patient is not the same that reaches the blood afterward. The way a drug is absorbed and metabolized varies from one patient to another patient, and also the physical health of the patient can directly affect the drug level in blood.

After years of testing, the optimum therapeutic ranges for specific drugs have been determined. These ranges have been tested so that most patients will be effectively treated with minimum or ideally without any side effects caused by their toxicity. When a drug is given to a patient, physicians adjust the dose, upwards and down, by testing its blood concentration continuously until it reaches a stable level, where it is efficient and safe. Adjusting the dosage level is usually a process that takes some days or even a few weeks.

Actually, to improve the accuracy of the monitoring process and minimize the toxicity in a more extensive range of drugs with broad therapeutic indices, both pharmacokinetics and pharmacodynamics should be taken into account. Pharmacokinetic of the drugs (PK) studies drug reactions undergone *in vivo* during the whole cycle starting when administrated to the patient. On the other hand, pharmacodynamic (PD) studies the responses of the patient's body induced by the drug. The interaction between PK and PD (PK/PD) defines the optimal drug activity, useful to optimize the drug concentration in the affected zones of the patient's body. TDM studies are trying to find out values for PK and PD for different drugs on different treatments.

2.1.1 Classification of monitored drugs

There are many drugs that are used for all kinds of treatments in different fields within medicine. While some have been used for years, others are still under study to identify any potential long-term adverse effects or toxicity. Therefore, research laboratories and physicians have to determine the optimal therapeutic ranges in which every drug works without causing any toxicity symptoms or negative effects to the patients. The work involved in deciding these ranges and other variables to take into account for drug prescription and treatment requires time and skilled personnel.

For this reason, there are some lists for physicians published by recognized health organizations around Europe, where they can consult and follow protocols for the administration of certain drugs. These lists contain all the authorized drugs to work within clinical fields. Drugs published on these lists have all kinds of general information, from drugs names, their active substances, and their dosage forms, to essential tips to follow for their administration, such as the optimal therapeutic ranges, frequency of doses, and duration of the treatments. When health specialists add new drugs approved or change any parameter of previously included drugs, these lists are updated.

If we look at some of these lists, we can see all kinds of drugs, which we have classified into different categories. Specifically, we searched on the list of the monitored drugs from the Canadian ministry of health and Long-Term Care [3] and the list of medicines under additional monitoring from the European medicines agency [4].

Table 1. Classification of monitored drugs [38]

Drug Class	Clinical Application	Examples
Cardiac Drug	Congestive heart failure, angina, arrhythmias	Digoxin, Amiodarone, Quinidine
Antibiotic	Infections with bacteria that are less resistant to less toxic antibiotic	Aminoglycosides, Vancomycin, Meropenem, Daptomycin
Antiepileptic	Epilepsy, prevention of seizures	Phenobarbital, Phenytoin, Zonisamide, Valproic acid
Bronchodilators	Asthma, chronic obstructive pulmonary disorder, apnea	Theophylline, Caffeine
Immunosuppressant	Prevent rejection of transplanted organs, autoimmune disorders	Cyclosporine, Tacrolimus, Sirolimus, Mycophenolate mofetil
Cancer treatment	Psoriasis, rheumatoid arthritis, osteosarcoma, different kinds of cancers	Methotrexate, all cytotoxic agents
Psychiatric	Bipolar disorder, depression	Lithium, Valproic acid, antidepressants

2.2 Monitoring of antibiotics

In general, TDM of antibiotics consists of quantitative measurements of one or several antibiotics directly in the plasma of patients to minimize the toxicity risk or long- and short-term adverse effects.

The target population for TDM of antibiotics is patients in critical and acute care situations and those requiring long-term hospitalization.

An excellent example of these situations is a patient with sepsis, which is an extreme body response to an infection. This medical emergency needs immediate treatment, usually by continuously administering antibiotics to the patient's blood flow.

2.2.1 Antibiotic monitoring methods

Here we will discuss some viable techniques used nowadays for antibiotic monitorization in specialized laboratories. We will also discuss their advantages and disadvantages.

Techniques for antibiotic monitoring need to be accurate, highly sensitive, simple, easy to use, requiring minimum laboratory equipment, and being able to detect more than one antibiotic in a complex sample. Cost-effectiveness is also essential, as antibiotic treatments are frequent in almost every clinical unit. The time required for the analysis is also crucial as patients are constantly under the supply of these antibiotics, so the time should be short enough to provide results to allow analysis and planning before the next dose.

For antibiotic detection systems developed in a laboratory, their implementation in the clinical field requires the system's users to have a minimum of knowledge of the system and also scientific formation.

- **Microbiological assays**

The most common method for detecting antibiotics relies on microbiological assays, where some species of microorganisms, commonly bacteria, sensitive to the targeted antibiotics, are used to detect the antibiotic in the patient's plasma or any other kind of biofluid.

These assays relate the inhibition of a microorganism's growth to the concentration of an antibiotic.

Commercial assays are based on two different techniques to detect antibiotics in the biological sample [5].

1. **Cylinder plate:**

It consists of the antibiotic (from the sample to analyse) diffusion from a vertical cylinder through a solidified agar layer in a plate, where an added microorganism is growing. It studies how antibiotic diffusion affects the growing microorganism, which will stop at a certain point around the cylinder where the antibiotic solution is placed.

2. **Turbidimetric:**

This method consists of different tubes containing different concentrations of antibiotics in a liquid culture medium with the presence of the tested microorganism. After doing an incubation, we can plot a curve displaying the different concentrations in function of the visible light transmitted through the tubes.

Microbiological assays usually need complex equipment and sophisticated sterile laboratory devices. Moreover, they are optimized for specific antibiotics, which means that they are not suitable for on-site field analysis.

Over the last years, the development of microfluidics paper-based methods has expanded the range of these assays, widening the range of target antibiotics. These methods involve devices made by paper, or other porous membranes, which are low cost and easy to fabricate.

- **Physical and chemical assays**

Physical and chemical assays work with specific properties of targeted molecules, such as size, charge, binding characteristics, or reactive properties, to identify the presence of antibiotics in a complex sample.

A physical essay requires purification to isolate the pure antibiotic from other impurities in the studied sample. Purification consists of several fractionation steps to aliquot the sample into smaller quantities in which the composition varies in function of a gradient. The final aliquots are spectrophotometrically analyzed, providing detection limits in the low ng/mL range [6].

Liquid chromatography – Mass spectrometry (LC-MS) has also been adapted to detect antibiotics in serum [8]. It has also been used to analyze field samples, assessing matrix effects [9].

Both purification strategies and spectroscopic techniques require experienced personnel and complex and high infrastructure, which is not easy to adapt to the work field. As a result, these techniques are mostly found in completely equipped research laboratories.

Alternatively, Surface-enhanced Raman spectroscopy (SERS), another technique used for molecular detection and characterization, allows high sensitivity and fast detection in less than 10 minutes. Nonetheless, SERS also requires sample purification to isolate antibiotics from other solutes and contaminants, which also needs a high knowledge level.

- **Immunoassays**

The affinity and selectivity of antibodies against their respective antigens have been used to develop a wide variety of analytical techniques which can be applied for the detection and quantification of antibiotics in human samples. The most common immunoassay is the Enzyme-Linked Immunosorbent Assay (ELISA), which labels the antigen-antibody interaction with an enzyme. The enzyme allows the amplification of the binding signal involving the antibiotic present in the sample. Detection occurs through color development or the presence of precipitated material, among others. Another type of immunoassay is the Fluorescence Polarization Immunoassay (FPIA), usually used in clinical laboratories to quantify analytes found in a deficient proportion in biological fluids such as hormones, vitamins, amino acids, or therapeutic drugs. This type of immunoassay relies on the competition between the target analyte (e.g. antibiotic) present in the sample and a fluorophore (e.g. fluorescein)-labeled analyte for their binding to the antibody added in solution. Knowing the fact that the labeled analyte in free state has higher turnover rates than bound to the antibody, and that binding increases the likeliness of emitting polarized fluorescence, the intensity of polarized light in the assay is proportional to the amount of bound labeled analyte and inversely proportional to the amount of analyte in the sample.
The selectivity of the antibody, followed by the transduction and amplification of the detection, achieves in these methodologies sensitive, selective and rapid techniques for the immunodetection and quantification of antibiotics. We also have to take into account the limitations to these techniques, such as the limited availability of primary antibodies and the high-cost equipment necessary to develop these techniques.

- **Biosensors**

Biosensors contain immobilized biological material which can interact with a compound and produce physical, chemical, or electrical measurable signals. They involve the quantitative analysis of various substances by converting their biological actions into quantifiable signals.

We can classify biosensors in function of their physical component (signal transduction) and biological component (biorecognition element). The biorecognition element interacts with an analyte to produce a signal, which can be detected by the transducer.

1. Signal transduction classification:

- **Electrochemical**

Reactions between the immobilized material and the analyte produce or consume ions and electrons, which change electrochemical properties. These electrochemical properties, such as potential or electric current are measured by electrochemical biosensors.
On one hand, we have the **amperometric** biosensors. [40] These biosensors measure the current resulting from the oxidation or reduction of an electroactive species providing specific quantitative analytical information. They have high sensitivity and are practical for multiple measurements.
On the other hand, we have the **potentiometric** biosensors, which measure the change in potential between the working and reference electrodes. Here the analyte is not consumed like in the amperometric sensor.

- **Electrical**

There are **ion-sensitive** electrical biosensors, which are based on ISFET (ion-sensitive field-effect transistor), able to measure ion concentrations in a solution. They have an ion-sensitive surface that changes their potential in function of the interactions between ions and the transistor.

- **Optical**

Here the output measured signal is light, as this type of biosensor is based on various optical properties, such as fluorescence and optical diffraction. **Fluorescence** has high sensitivity, so it is widely used in biosensing. It detects changes in the frequency of electromagnetic radiation emission caused by the absorption of radiation.

- **Piezoelectric**

Also called mass-sensitive biosensors, they are based on the changes (mass, density, viscosity) in the bioelement measured by a piezoelectric component. Examples of a piezoelectric component are a quart-crystal or aluminum nitride, which has this piezoelectric effect. Therefore, this biosensor allows label-free detection of molecules.

2. Biorecognition element classification:

Based on the bioreceptor element used to modify the sensor, which will show affinity for the target analyte.

- **Enzymatic:**

Enzymes can be used as bioreceptors. The analyte can be either an enzymatic substrate or a molecule that inhibits the enzymatic activity. They rely on measuring the enzymatic activity, mainly by following consumption of one of the enzymatic substrates or cofactors needed or production of enzymatic products.

- **Immunosensors**

These biosensors rely on the antigen-antibody interaction. The recognition element here is composed by antibodies, fractions of antibodies, antigens or haptens.

- **Aptamer-based sensors**

We have sensors which use aptamers as the bioreceptors. They have affinity for a wide range of analytes, such as bacteria, viruses, proteins, hormones and other small molecules. This affinity depends on the 3D conformation of the aptamer.

- **DNA sensors**

DNA sensors use DNA as bioreceptor, and the analyte is the complementary sequence to this bioreceptor. They rely on the hybridisation of both DNA strands.

- **Whole-cell-based sensors**

In this case the bioreceptor is a living cell. They work in a similar way to the enzymatic sensors, but here the enzymes are protected inside the cell. They are more stable in this environment, but there might be other reactions interfering.

2.3 Porous silicon as a sensing material

Due to the unique properties of porous silicon (pSi), it is used for all kinds of sensing techniques and devices. Its interest in lots of research areas has been increased recently, such as optoelectronics, and biomedical applications.

There are a lot of advantages that pSi offers over other materials, such as unique electrical and optical properties, its good results in biosensing, photonics, biomedicine, and its compatibility with other existing silicon technologies.

We can get chemical sensors exploiting the electrical, optical, and chemical properties of the pSi structure. Various pSi variations have been developed, changing the morphology and chemistry of the surface, and making it perfect for different biomedical applications.
Some studies have also tried to optimize cell adhesion of pSi, loading capacity, and its biocompatibility for drug delivery applications.

2.3.1 Introduction to porous silicon

Porous silicon is solid silicon with void pores inside, and it's one of the most used porous materials, as it has a wide range of applications, from fuel cells to drug delivery.

The fabrication of pSi is quite inexpensive and straightforward. It can be performed by different methods depending on the desired structure and properties. Moreover, it has highly adjustable properties that we will explain later.

2.3.2 History of porous silicon

The material was discovered in 1956 by Arthur Uhlir Jr. While he was doing electropolishing experiments on silicon wafers using a hydrofluoric acid-based electrolyte. He found that under some specific conditions of current, composition of the solution, and its concentration, several holes were forming in the same direction of the silicon wafers instead of being dissolved uniformly.

Later in the 1980's decade, the interest in pSi increased due to its surface area, which was found to be useful for models of crystalline silicon surface in spectroscopic studies as a precursor to generating thick oxide layers on silicon, and as a dielectric layer in chemical sensors.

In the 1990's decade, Leigh Canham published his results about red luminescence based on pSi, making it a promising material. As a result, the interest of researchers in pSi and other porous semiconductor materials and the number of publications dedicated to porous materials have constantly increased.

During these last years, the optical properties of pSi have become an interesting area of research, as well as the interest for its mechanical and thermal properties, its compatibility with silicon-based electronics, and its low-cost fabrication.

2.3.3 Properties of porous silicon

Porous material structures are classified by their pore dimensions. In pSi, we talk about microporous silicon when pore dimensions are below 2 nanometers, macroporous silicon when pore dimensions are above 50 nanometers, and mesoporous silicon when pore dimensions are in the middle of the two above. Due to the wide variety of pore size, shape, orientation, and distribution, the material's morphology is not a quantifiable aspect of this material. As a result, we can give pSi all kinds of properties and use them in several fields.

For example, we can work with some structural properties playing with pSi's porosity, density, surface area, or pore size (diameter). We can also go for mechanical properties by looking at different mechanical aspects such as Young's modulus or hardness, optical properties (bandgap, color, reflectivity), electrical properties (resistivity, dielectric constant), thermal properties (conductivity, melting point), biochemical properties (medical biodegradability), etc.

Some examples of fields where pSi has a potential application:

- Optoelectronics: pSi may work as LED for efficient electroluminescence or as a waveguide for the modification of the refractive index.
- Biotechnology: Here, pSi may work as a biosensor for enzyme immobilization or tissue bonding material to test different chemical reactivities.

- Environmental monitoring: pSi may work as gas sensor to test ambient sensitive properties.

2.3.4 Porous silicon fabrication

The fabrication process of pSi is easy compared to the fabrication of other porous materials. Although there are up to almost 20 methods for pSi fabrication, we will talk about the one used in our laboratory, **anodization**. This is an electrolytic passivation method used to increase the thickness of the natural oxide layer on the surface of the semiconductor. [39] The part being treated is the anode in the electrolytic cell, explaining the technique's name. The process changes the microscopic texture of the pSi surface and the crystal structure near the surface, increasing corrosion resistance and preventing degradation in front of threaded components.

Usually, an electrochemical cell is used to introduce pores in silicon, using the wafer as the anode, immersed in a hydrofluoric acid (HF) electrolyte. A commonly used material for the cathode is platinum. The corrosion of the anode (silicon) is produced by running an electric current through the anodization cell. It has been observed that the running of constant direct current is usually implemented to ensure a constant concentration of hydrofluoric acid, which results in a more homogeneous porous layer.

2.3.4.1 Anodization parameters

Porous silicon fabrication by anodization has been used by several studies, getting a list of parameters that influence the formation process of the material. The essential parameters that modify properties of pSi are the following:

- Doping: Introducing impurities to a semiconductive material modulates its optical, electrical, and structural properties.
- Current density: Changes in the morphology and size of pores.
- HF concentration: Determines the range of current density values.
- Solvent where HF is diluted: PSi is hydrophobic, and the use of ethanol gives homogeneity to pore’s growth due to the improvement in surface's wettability.
- Etching time: Affects the thickness of the layers.
- Illumination: Important for photosensitive substrates.

The porosity, thickness, pore diameter, and microstructure of pSi depend on these anodization conditions. In addition, there are other conditions that also have an effect, such as temperature, ambient humidity, and drying conditions.

2.3.5 Porous silicon stabilization

Once pSi has its defined morphology and properties, it needs to be stabilized as freshly etched pSi easily degrades, quickly forming an isolating silica layer on its surface. Indeed, the most common method to stabilize pSi is thermal oxidation [43], but it leads to structures not suitable for electrochemical sensing. More recently, researchers demonstrated the effectiveness of stabilizing pSi by a thermal treatment in the presence of acetylene [44]. This forms a homogeneous carbon coating, that later was shown to be excellent for electrochemical sensing purposes [41]. A similar carbon stabilization process relies on coating the Si wafer with furfuryl alcohol (FA) which is polymerized using oxalic acid as a polymerizing agent and later carbonized to make pSi conductive [45].

3 Working hypotheses and objectives

Once these concepts have been introduced, we give way to the practical part of the work where we will apply our knowledge and equipment of the laboratory to contribute on the research of therapeutic drug monitoring methods used, in our case, for antibiotics, and extract some results that we can afterwards evaluate and discuss.

As we explained before, it is necessary to have accurate and efficient methods for antibiotic monitoring in the clinical field, with the aim to contribute to the treatment of patients at critical and acute care situations, or those who require a long-term hospitalization. These medical emergencies require constant monitoring and evaluation of the patients, and therefore, optimal techniques, tested previously, which can continuously measure the levels of antibiotics directly from the patient's plasma, in order to optimize their treatments.

We have been able to test two different sensing platforms:

- First, we used **carbon screen-printed electrodes**. It is a type of commercial sensor, made for the electrochemical analysis, practical, and usually used for detections in microvolumes at a low-cost.
- Second, as is to be expected, we worked with **porous silicon electrodes**, fabricated and modified in the laboratory as a new viable material for the electrochemical-based TDM of antibiotics.

We want to study how these two types of electrodes handle their passivation, a problem usually observed when doing multiple consecutive amperometric/voltammetric measurements, which is something required in TDM. If electrode passivation occurs, electrodes can be initially modified to avoid such passivation, or treated after passivation to remove the species adsorbed on the electrode surface. We would also like to explore their sensitivity, as well as their feasibility to discriminate among various antibiotics in a mixture.

Regarding our expectations, pSi electrodes might provide certain advantages, such as an increased sensitivity as a result of its large surface area, and the excellent electrochemical performance previously reported when compared to commercial carbon-based electrodes [41].

Our objective is then **the experimentation with the two sensing platforms exposed previously, find out their advantages and disadvantages to study their viability in the TDM of antibiotics, and make a comparison between them.**

Our hypotheses are the following:

1. **Porous silicon electrodes can show higher sensitivity than screen-printed electrodes due to their larger surface area of detection.**
2. **The feasibility to discriminate via voltammetric techniques various electroactive compounds showing close oxidation potentials [41], can be extended to the simultaneous detection of multiple antibiotics in a single measurement of a liquid sample using porous silicon electrodes.**
3. **Electrode passivation is expected to be different on the two types of sensing platforms to be tested.**

4 Methodology

4.1 Variables and resources

4.1.1 Chemicals

Our principal solutions on which we based all the experiments were **antibiotics with electroactive properties**. We studied different antibiotics currently available in the clinical field, used on various treatments. These antibiotics are the following:

Table 2. Description of the antibiotics used in the experiments.

Antibiotic	**Description** [20]
Meropenem	Used to treat severe infections of the skin or stomach, as well as bacterial meningitis.
Vancomycin	Oral taken antibiotic that fights bacteria in the intestines caused by *Clostridium difficile* and *Staphylococcus* infections that can cause inflammation.
Daptomycin	It is used to treat bacterial infections of the skin and underlying tissues, and infections that have entered the bloodstream. Complicated infections, including methicillin-resistant *Staphylococcus aureus* (MRSA) infections.
Cefepime	Cefepime injection is used to treat many kinds of bacterial infections, including severe or life-threatening forms.
Azithromycin	It is used to treat many different infections caused by bacteria, such as respiratory infections, skin infections, ear infections, eye infections, and sexually transmitted diseases.
Azactam	It is used to treat severe infections of the blood, urinary tract, lungs, skin, stomach, or female reproductive organs.

All the antibiotics were prepared at high concentrations in Phosphate-Buffered Saline Buffer, and then diluted in the same buffer to the desired concentrations. Solutions were covered with aluminum foil to prevent any light, as they are photosensitive, and finally, they were stored at 4ºC to avoid their degradation. New solutions were prepared daily, as the solutions needed to be fresh for the experiments to avoid any degraded compounds.

We used **Phosphate-Buffered Saline (PBS)** [21] as our buffer solution, which helped us maintain a constant 7,4 pH value. Also, it was compatible with our experiment as the ion concentrations and osmolarity of the PBS are close to human plasma values [36][37].
We used a powder format PBS solute, dissolved in milli-Q water and stored at room temperature.

Another essential chemical used was the **ferrocyanide-ferricyanide**, a pair of electroactive species often used as redox indicator. The concentrations were 2 mM of $K_4[Fe(CN)_6]$ and 2 mM of $K_3[Fe(CN)_6]$ prepared in PBS at pH 7,4.

4.1.2 Equipment

Our experiments required a **potentiostat** and an electrochemical cell to perform the electrochemical measurements.

The potentiostat used for most of the project was the Ivium-n-Stat [32]. The potentiostat offers a wide range of electrochemical techniques. Moreover, it works with a specific software, IviumSoft [33], where we can use standard electrochemical techniques, explained later.

The system we used for the experiments is the conventional electrochemical measuring system, composed of a **three-electrode set**, which is formed by the following elements [34]:

- **Working electrode**: The working electrode is the essential component of the electrochemical cell, as it is where the electrically driven chemical reaction and electron transfer happens.
- **Counter electrode** (Auxiliar): It is used to close the circuit with the working electrode so that electrochemical reactions can happen on the latter's surface.
- **Reference electrode**: Acts as a reference in measuring and controlling the working electrode potential without any current flow.

This three-electrode system was used either using screen-printed electrodes or carbon-stabilized pSi electrodes as the working electrode. We performed voltammetric measurements, applying potential and then measuring the current generated by the oxidation of certain electroactive species on the working electrode surface.

Screen-Printed Electrode (SPE) setup:

These are commercial electrodes and thus will be used as a model system. SPEs incorporated themselves the three electrodes; the way is shown in Figure 1. Our SPEs were based on a carbon working electrode, carbon counter electrode, silver reference electrode, and electric contacts made of silver.

We optimized the volume of solution required to get accurate results, and we covered the three electrodes, on the blue surface, with the solution to ensure electrons can flow between working and counter electrodes. Each electrode also had its respective connection connected to the potentiostat using an adapted wire for the SPEs.

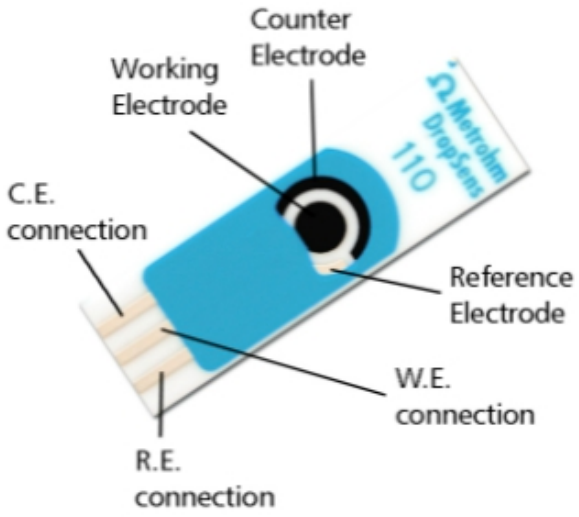
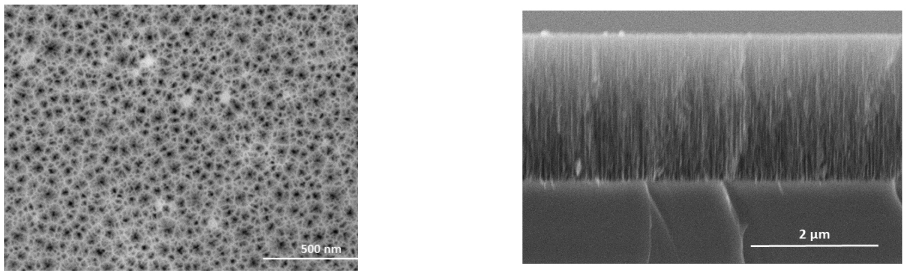


Figure 1. Screen-Printed Electrode scheme [35]

Porous silicon (pSi) setup:

For the pSi, we used mesoporous samples fabricated by anodization. Pore size was 18 - 20 nm, with thickness of 2.2 µm, coated with 20 % furfuryl alcohol which were polymerized using oxalic acid as a polymerizing agent, and later carbonized at 700°.

In Figure 2, images of the samples are shown, obtained by field emission scanning electron microscopy (FESEM).



A B

Figure 2. FESEM image, of a 20 - 24 nm pore size pSi sample from the surface (A), and cross section (B).

For the three-electrode system, we used platinum as the counter electrode, which is suitable for its inertness, Ag/AgCl as the reference electrode, and pSi as the working electrode. Aluminum foil was placed under the pSi sample aiming to achieve a high quality ohmic contact.

Then, the sample was placed inside a Teflon cell, and the electrodes were placed on this cell, as we can see in Figure 3.

Working, counter and reference electrodes were connected directly to the potentiostat to perform our experiments with the required electrochemical techniques.

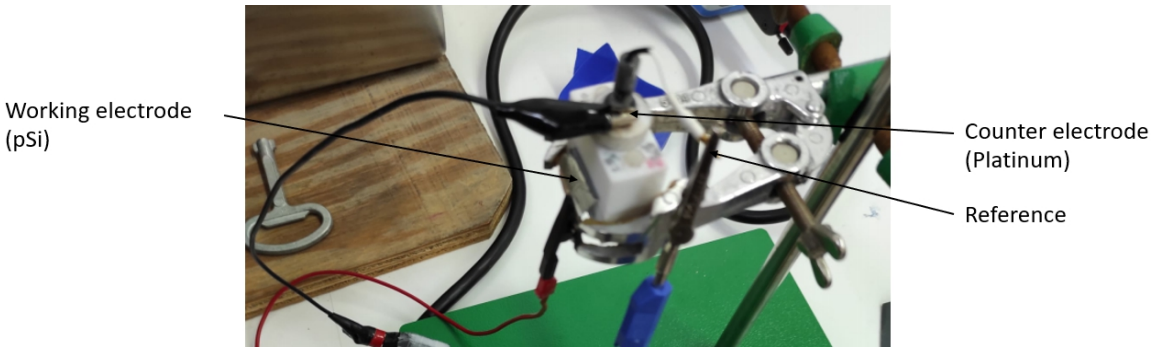


Figure 3. Porous silicon setup photo

4.1.3 Electrochemical techniques

We used several electrochemical techniques, which helped us to get both quantitative and qualitative results.

First, we have **cyclic voltammetry (CV)**, used to study oxidation and reduction of electroactive species in solution, such as the antibiotics used in this study.
The technique applies a triangular sweep potential to the working electrode, measuring the intensity of the current through this electrode in function of the potential. For reversible or quasi-reversible redox systems involving one electron, cyclic voltammograms show a cathodic and an anodic peak with their respective potentials (Epc, Epa) and current intensities (Ipc, Ipa), as we can see in Figure 4.

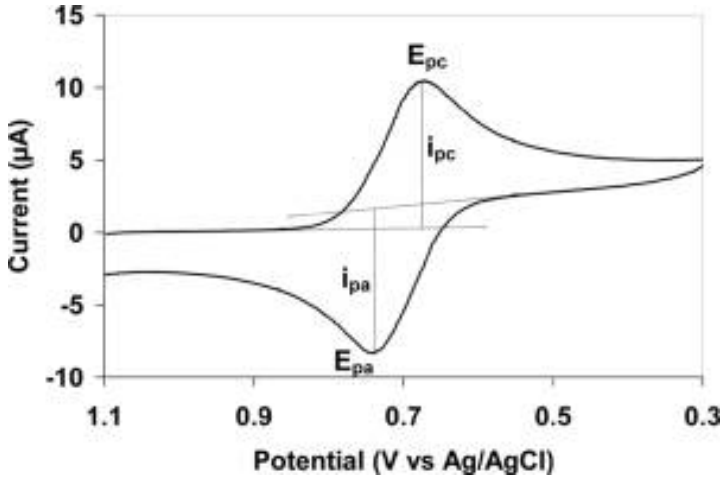


Figure 4. Cyclic voltammogram for a reversible system involving one electron showing the important parameters. We see oxidation and reduction potentials (Epc, Epa) and the respective intensities (Ipc, Ipa) [22]

Another technique is **square-wave voltammetry (SWV)**, a pulse technique with higher sensitivity than linear sweep methods. As a pulse technique, it applies the potential forming staircase series of forwarding and reverse pulses.

As we can see in Figure 5, both SWV and CV are similar as the potential is ramped linearly over the time, the advantage that SWV gives us compared to CV is that it gives a quantified answer, while CV is a qualitative or semi-quantitative point of view helping on the supervision of the stability or the oxidation potentials of our electroactive species.

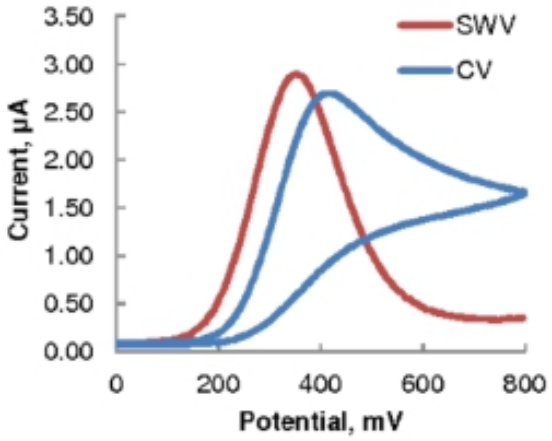


Figure 5. Overlapping SWV and CV with the same solution [42]

We also used **amperometry**, measuring the current through the electrode when a fixed potential is applied, and finally, **electrochemical impedance spectroscopy (EIS)**. With EIS, we worked in a range of frequencies, applying an open circuit potential, and measuring the electrical resistance (impedance) of the electron transfer process on the electrode surface. EIS and CV are complementary electroanalytical methods often used for characterization purposes.

4.2 Experimentation protocols

Initially, we always performed a study of the electrodes by electrochemical **characterization**. It helped us to check the electrode stability over time. This characterization was performed by CV, EIS, and SWV using 2 mM $[Fe(CN)_6]^{3-/4-}$ dissolved in PBS. CV measurements were adjusted from -0.2 to 0.6 V at a scan rate of 100 mV/s, and EIS used a range of frequencies from 100 mHz to 10 kHz.

Characterization was also used to identify whether there was passivation of the electrodes after antibiotic detections, or monitoring the effects of the procedures used to reverse this passivation.

These procedures consisted in **cleanings** of the working electrode surface by applying a fixed potential using a cleaning solution. The adjusted parameters for this method varied in function of the used solution.

And finally, for antibiotic **detection**, we first did CV and SWV using PBS, the buffer solution used for preparing the antibiotics. We adjusted CV from 0 V to a specific potential in function of the antibiotic, at a scan rate of 100 mV/s, and SWV also from 0 V to the same potential as the CV and with the same parameters we used on the characterization. Measurements with PBS were used to obtain a baseline. Then, detection of antibiotics was performed again by CV and SWV. The parameters were adjusted as before also with their specific potentials.

5 Results and discussion

We checked the stability of the SPEs and pSi samples before doing any experiment and we only used those who were stable enough to give accurate results.

5.1 Study of SPEs for the detection of antibiotics and activation of the electrodes to improve the results

First **SPEs** were tested: we did several antibiotic measurements, and **we studied the electrode passivation**. The conventional protocol consisted in doing multiple measurements of an antibiotic at a fixed concentration, and checking the SPE passivation between measurements by electrochemical characterization via CV in a 2 mM ferro/ferricyanide solution. The oxidation current of the ferrocyanide in solution was obtained from the cyclic voltammograms, which was expected to remain constant in the absence of electrode passivation.

We assessed passivation when there was a significative decrease in the measured oxidation current which could interfere the next measurements, seen in Figure 6. When it happened, we **cleaned the electrode using** 0.05 M **carbonate buffer** at pH 9.60, and applying 1.2 V, during 45 seconds. We did several cleanings until the electrode showed the same oxidation and reduction peaks in the CV characterization as before being passivated.

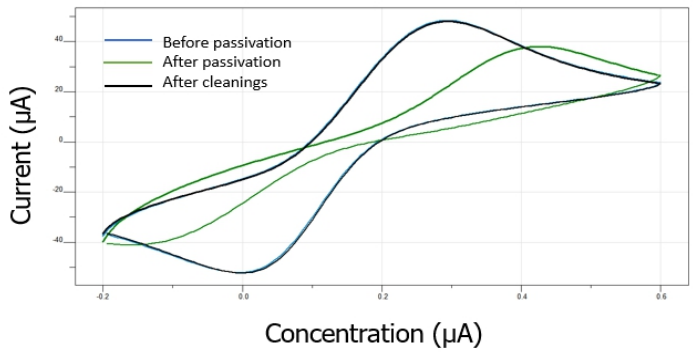


Figure 6. CVs with ferrocyanide-ferricyanide using the same electrode. Comparing before and after measuring with Meropenem 250 µM solution, and after cleaning it with carbonate buffer. Cyclic voltammograms before passivation and after cleaning are almost equal.

For most of the cases, the first characterization presented a big decrease of the oxidation peak and thus several cleanings were required until the current intensity was recovered. After several measurements, either no cleanings were necessary, or just one cleaning was enough to recover the intensity.

After several cleanings we realized that there was a point where the electrode response to ferrocyanide oxidation was not changing anymore, and sometimes the measured signal was even increasing in the last measurements. As a result, we applied consecutive cleanings before measuring the antibiotic, **activating the electrode at the beginning**.

We tested the efficiency of this activation protocol with SPEs to be used in the detection of cefepime, meropenem and vancomycin. For all of them, we used two SPEs, and performed consecutive CVs and SWVs in 250 µM antibiotic solutions: one SPE was initially activated as detailed above prior to the antibiotic measurements, while for the other SPE, cleanings were applied between antibiotic measurements. CV was used for characterization purposes, to identify the oxidation potential for each antibiotic. Due to its high sensitivity, SWV was used to quantitatively analyze the antibiotic solutions. The potential range for CV and SWV for each antibiotic was determined as follows: cefepime from 0 to 1 V, meropenem from 0 to 1.3 V, and vancomycin from 0 to 1 V.

We compared the first CV measurement in antibiotic for both activated and non-activated SPEs to compare the magnitude of the oxidation current:

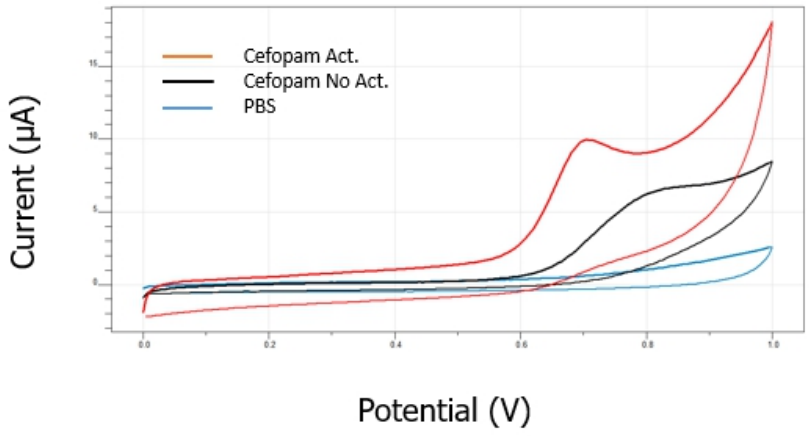


Figure 7. CV for 250 µM cefepime solution prepared in PBS. First cyclic voltammogram comparing activated SPE and non-activated SPE, with PBS reference.

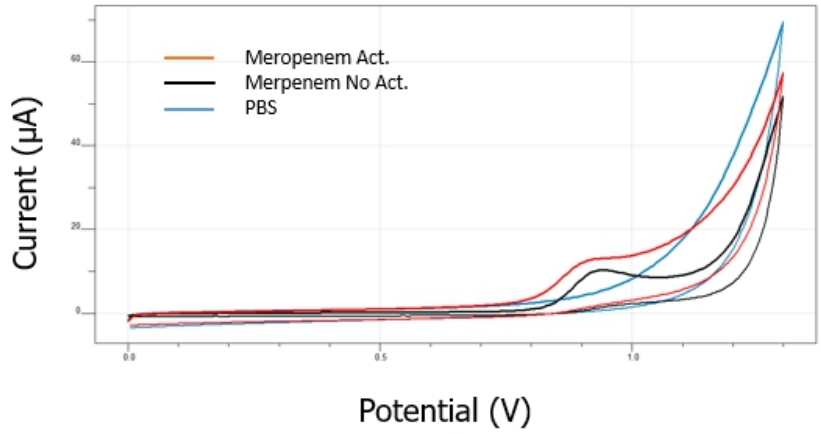


Figure 8. CV for 250 µM meropenem solution prepared in PBS. First cyclic voltammogram comparing activated SPE and non-activated SPE, with PBS reference.

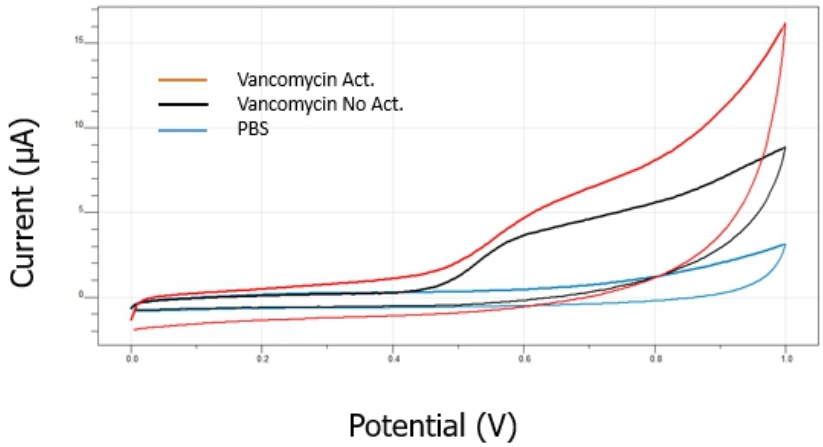
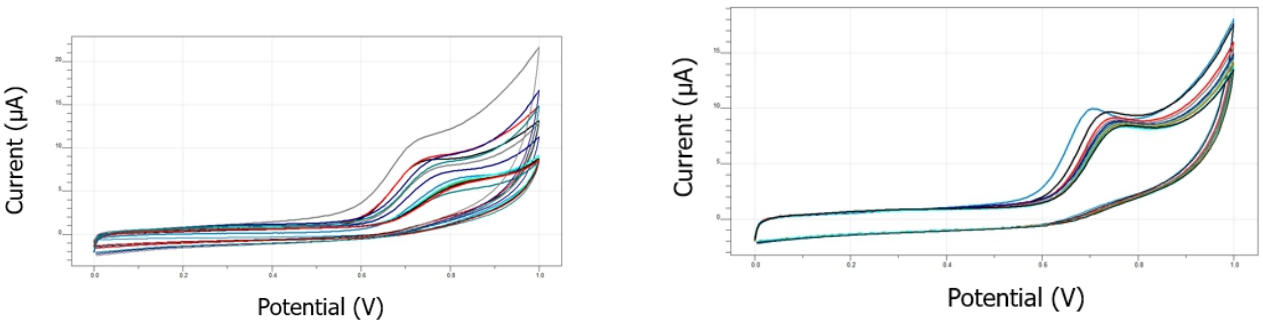


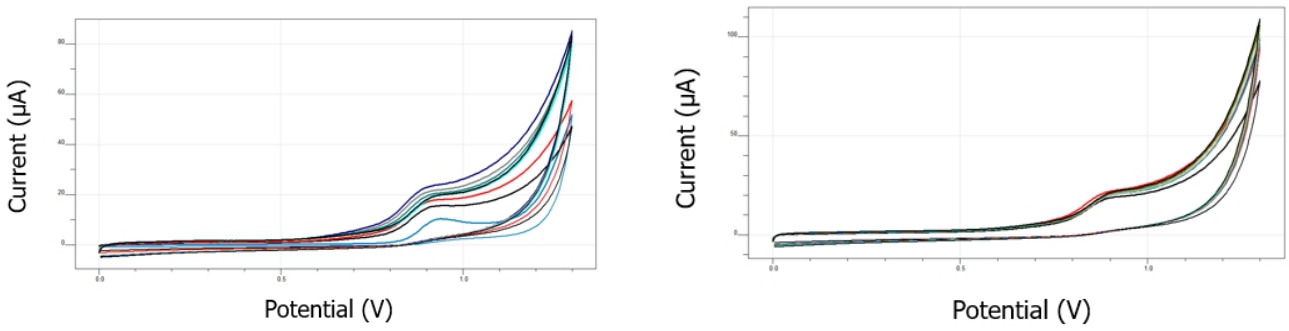
Figure 9. CV for 250 µM vancomycin solution prepared in PBS. First cyclic voltammogram comparing activated SPE and non-activated SPE, with PBS reference.

We saw that the intensity of the oxidation peaks significantly increased for the activated electrodes. We also observed that consecutive measurements were more consistent and stable for the activated SPEs as shown in figures 10, 11, and 12. We used always 250 µM solutions, and we did 10 scans for meropenem, 9 scans for cefepime, and 7 scans for vancomycin.



Non-activated **Activated**

Figura 10. Consecutive cyclic voltammograms for 250 µM cefepime for both activated and non-activated SPEs.



Not activated **Activated**

Figura 11. Consecutive cyclic voltammograms for 250 µM meropenem for both activated and non-activated SPEs.

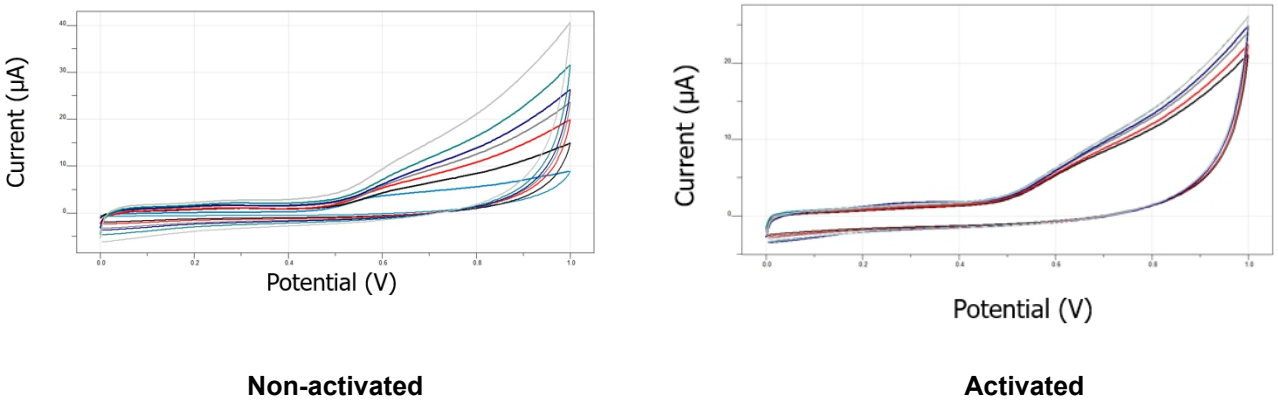


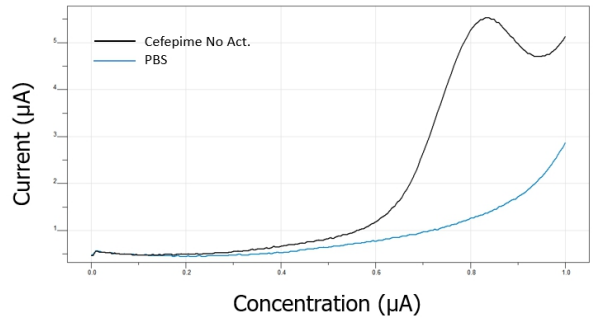
Figura 12. Consecutive cyclic voltammograms for 250 µM vancomycin for both activated and non-activated SPEs.

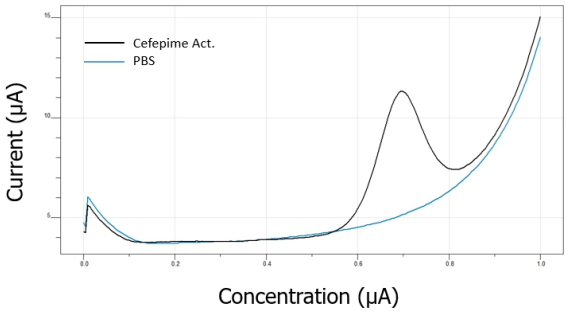
Antibiotic	Non-activated	Activated
Meropenem	86 %	17 %
Cefepime	25 %	17 %

Table 3. Percentage of variation of the measured oxidation current between the first and last scan.

SWV for Vancomycin didn't show any peak; As a result, we can't calculate the percentage of passivation for this antibiotic in table 3.

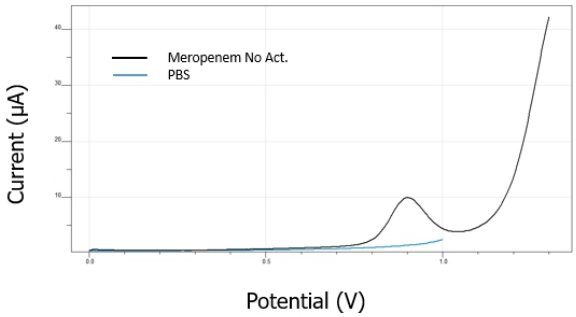
In order to quantitatively assess the activation effect on the antibiotics detection, we performed SWV measurements for each antibiotic solution, once again comparing the results for activated and non-activated SPEs.

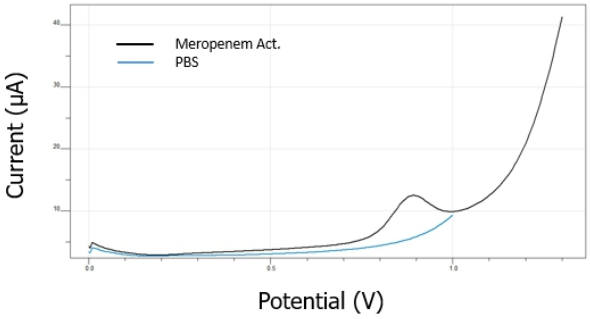




Non-activated **Activated**

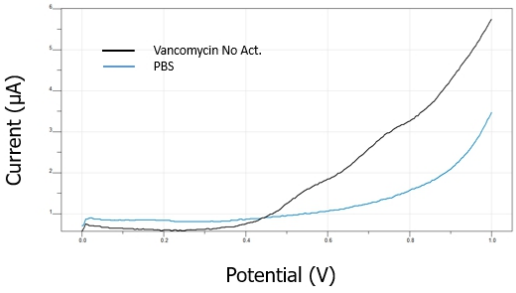
Figura 13. First SWV for 250 µM cefepime for both activated and non-activated SPEs.

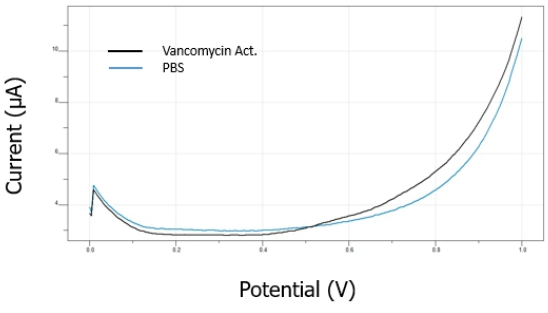




Non-activated **Activated**

Figura 14. First SWV for 250 µM meropenem for both activated and non-activated SPEs.





Non-activated **Activated**

Figura 15. First SWV for 250 µM vancomycin for both activated and non-activated SPEs.

For vancomycin the software used to analyse the electrochemical results could not provide accurate values of intensity current. We tried to increase the concentration and results will be shown and discussed in the next section.

SWV data from previous figures were analysed to extract current intensity values and oxidation potential values for meropenem and cefepime measurements as shown in Tables 2 and 3:

Table 2. Comparison of oxidation potential and intensity values extracted from the SWVs obtained when measuring **cefepime**.

SWV scan	Potential (V) Non-activated SPE	Potential (V) Activated SPE	Current Density (μA) Non-activated SPE	Current Density (μA) Activated SPE
1	0,835	0,695	2,09	5,34
2	0,740	0,710	2,18	3,69
3	0,735	0,715	2,80	3,53
4	0,755	0,720	1,68	3,71
5	0,765	0,725	1,33	3,39
6	0,780	0,725	1,00	3,17
7	0,730	0,730	1,92	2,92
8	0,745	0,730	1,61	2,78
9	0,750	0,735	1,57	2,52

Activated SPEs show a slightly lower oxidation potential that changes slightly over the first few scans before stabilizing. Non-activated SPEs show higher values of oxidation potential, agreeing with a less favored electron transfer on the electrode surface, that vary in a wider range without reaching a stable value. Current intensity values randomly change for non-activated SPEs, while activated SPEs show a trend that leads to more stable measurements with 62 % higher value in the last measurement.

Table 3. Comparison of oxidation potential and intensity values extracted from the SWVs obtained when measuring **meropenem**.

SWV scan	Potential (V) Non-activated SPE	Potential (V) Activated SPE	Current Density (μA) Non-activated SPE	Current Density (μA) Activated SPE
1	0,900	0,885	7,30	5,44
2	0,895	0,875	5,40	5,51
3	0,890	0,865	5,23	7,62
4	0,885	0,870	5,47	7,77
5	0,885	0,875	5,08	6,70
6	0,890	0,870	5,45	6,00
7	0,895	0,870	5,14	7,36
8	0,890	0,870	5,54	7,21
9	0,875	0,870	6,14	6,37
10	0,890	0,875	6,97	6,80

Here the potential is stable for both activated and non-activated electrodes, but activated ones again show a lower oxidation potential underpinning a more favorable electron transfer on the electrode surface.

Regarding current intensity values, both activated and non-activated electrodes show random variations that do not stabilize.

In general, we can conclude that when initially activating the electrodes, then it was not necessary to clean them with carbonate buffer after each measurement, as the passivation was minimum.

Activation of SPEs not only minimizes electrode passivation upon antibiotic measurements, but also increases in some cases the signal measured. The latter, added to the lower oxidation potentials identified for activated SPEs, are believed to be caused by a favored electron transfer when electrodes have been activated initially in carbonate buffer by applying 1.2 V for 45 s.

5.2 Detection of antibiotics on SPE

Once we optimized the protocol to avoid SPE passivation during antibiotic electrochemical detection, we proceed to **study the viability of the platform to detect different antibiotics at different concentrations**.

We fixed a range of concentrations to obtain a calibration curve, which was used to assess the sensitivity and calculate the theoretical LOD for the detection of each antibiotic on SPEs. Replicates were performed to assess the reproducibility.

The antibiotic concentrations used for plotting the calibration curves are: 10 µM, 25 µM, 50 µM, 100 µM, and 250 µM. We prepared a stock solution at 1000 µM, and then performed serial dilutions. We had to avoid errors during diluting the stock solution (i.e. pipetting, weighting, etc...) because they are propagated to the rest.

First, we used CV to identify the oxidation potential of the antibiotics. Next, we measured CV and SWV first in PBS and then at each antibiotic's concentration. Additionally, we electrochemical characterized the SPEs via CV in ferro/ferricyanide to check eventual electrode passivation.

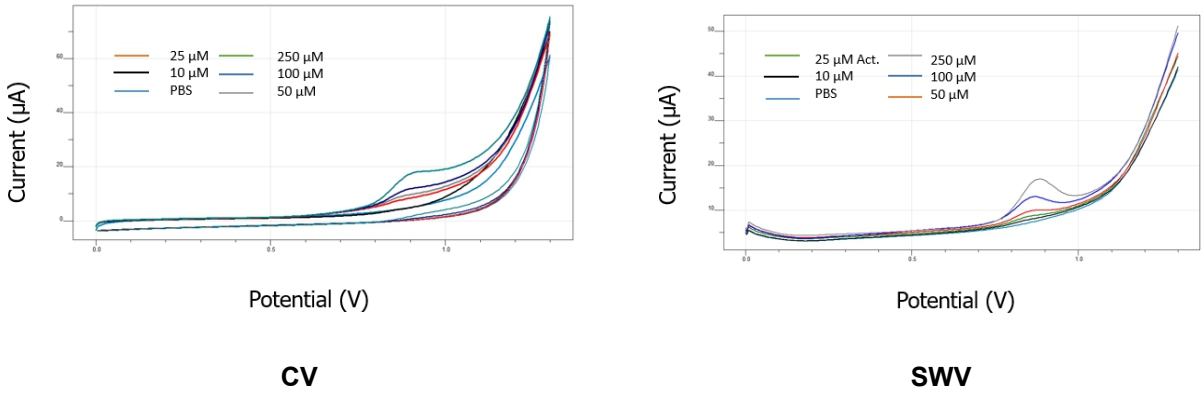
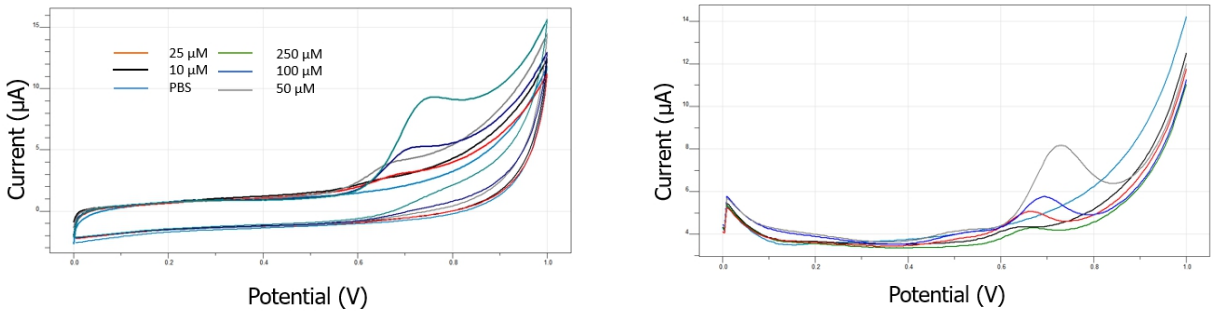
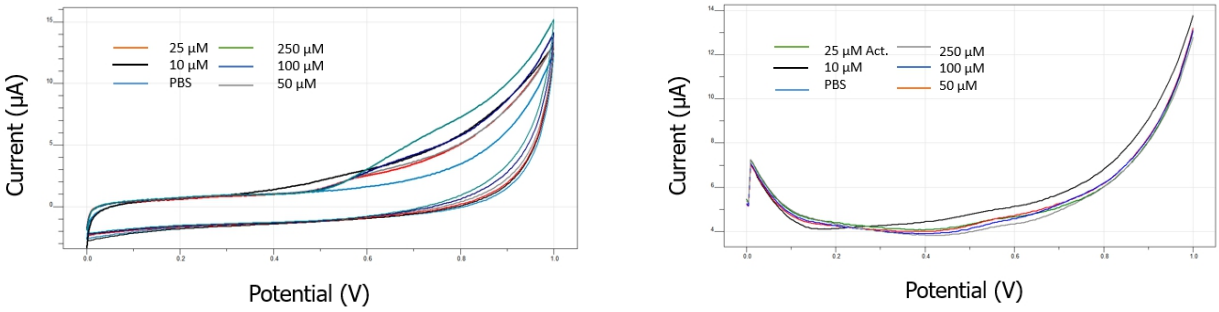


Figura 16. Cyclic voltammograms and square wave voltammograms for various concentrations of **meropenem**, measured from 0 to 1.3 V.



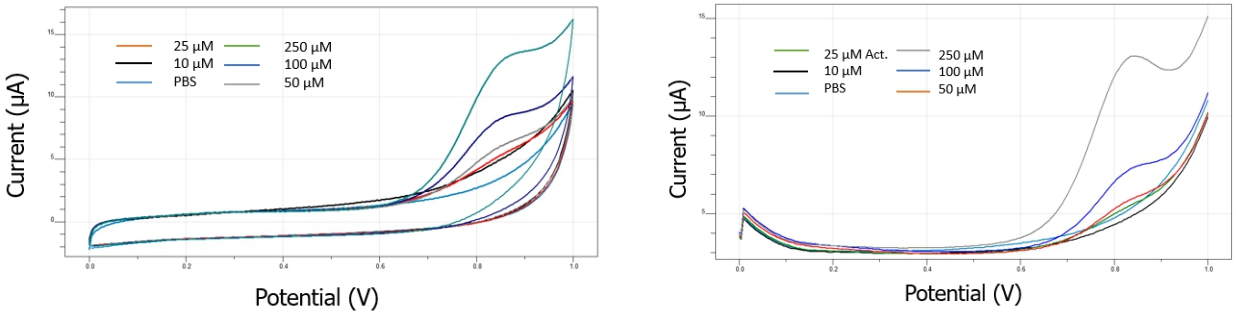
CV **SWV**

Figura 17. Cyclic voltammograms and square wave voltammograms for various concentrations of **cefepime**, measured from 0 to 1.0 V.



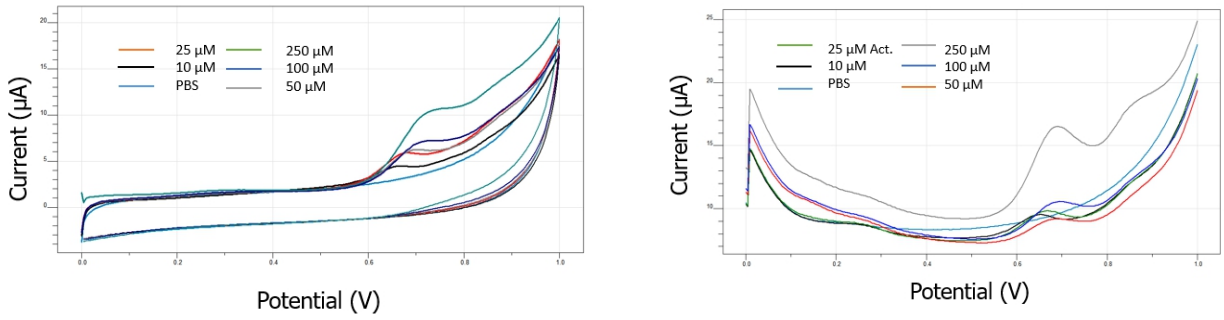
CV **SWV**

Figura 18. Cyclic voltammograms and square wave voltammograms for various concentrations of **vancomycin**, measured from 0 to 1.0 V.



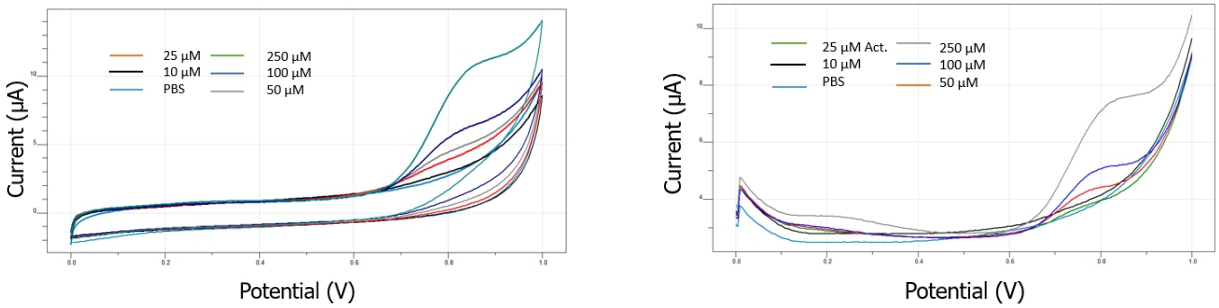
CV **SWV**

Figura 19. Cyclic voltammograms and square wave voltammograms for various concentrations of **azithromycin**, measured from 0 to 1.0 V.



CV **SWV**

Figura 20. Cyclic voltammograms and square wave voltammograms for various concentrations of **daptomycin**, measured from 0 to 1.0 V.



CV **SWV**

Figura 21. Cyclic voltammograms and square wave voltammograms for various concentrations of **azactam**, measured from 0 to 1.0 V.

Analyzing these cyclic and square wave voltammograms we can extract:

1. **Quantified results by finding the peak intensities of SWV measurements.**
2. **Total passivation produced at the end of the measurements.**
3. **Oxidation peaks of the cyclic voltamogramms.**
4. **Calibration curves, with sensitivities and LOD.**

Table 4. Extracted oxidation intensities of the square wave voltammograms for each concentration, and total passivation at the end of the measurements.

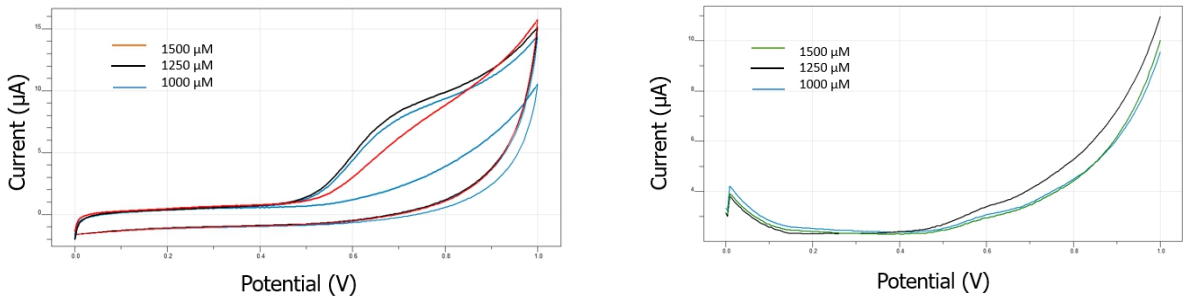
Antibiotic	10 µM (µA)	25 µM (µA)	50 µM (µA)	100 µM (µA)	250 µM (µA)	Total passivation (%)
Meropenem	0	0,88	2,09	3,70	7,53	-4%
Cefepime	0,41	0,66	1,31	1,84	3,18	2%
Vancomycin	-	-	-	-	-	-12%
Azithromycin	0	0,45	0,63	1,34	2,93	8%
Daptomycin	0,92	1,01	1,28	1,96	4,92	-16%
Azactam	0	0,32	0,66	1,19	2,19	2%

Table 5. Oxidation potential values extracted from cyclic voltammograms corresponding to a 250 µM solution of each tested antibiotic.

Antibiotic	Oxidation potential (V)
Meropenem	0.88
Cefepime	0.72
Vancomycin	-
Azithromycin	0.83
Daptomycin	0.68
Azactam	0.80

In table 4 we calculated the **total passivation** by analysing the oxidation peaks for first and last CV, shown in annex 8.1, 8.2; Then, we analyzed the peaks using Origin 9.0[46] software to find out the **intensity values** for each SWV peak, which are used later for calculating calibration curves, once we do the triplicates. Table 5 shows the oxidation potentials for each antibiotic in the highest concentration, 250 µM, which is compared in the next section with the pSi platform.

As it is shown in table 4, some antibiotics did not show any oxidation current for the lowest concentration tested, 10 µM. In the case of vancomycin we could not observe any oxidation current in the range of concentrations tested. Therefore, vancomycin concentrations higher than 250 µM were tested, providing the results shown in Figure 22.



CV **SWV**

Figura 22. Cyclic voltammograms and square wave voltammograms for **vancomycin** at 1000 µM, 1250 µM and 1500 µM.

Although CV shows an oxidation peak for vancomycin at approximately 0.7 V, the intensity current tends to saturate when measuring higher vancomycin concentrations, and thus SWV measurements could not be quantitatively analyzed.

From the SWV measurements performed in triplicate, we can plot the **calibration curves** as follows:

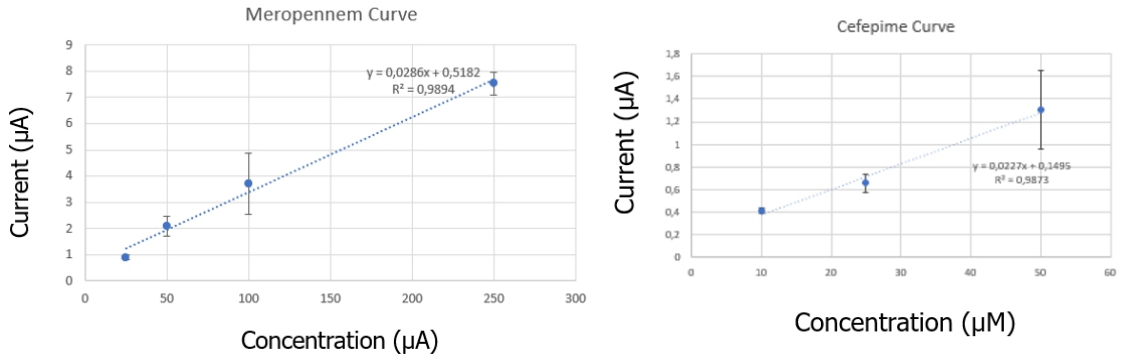


Figura 23. Calibration curves for meropenem and cefepime.

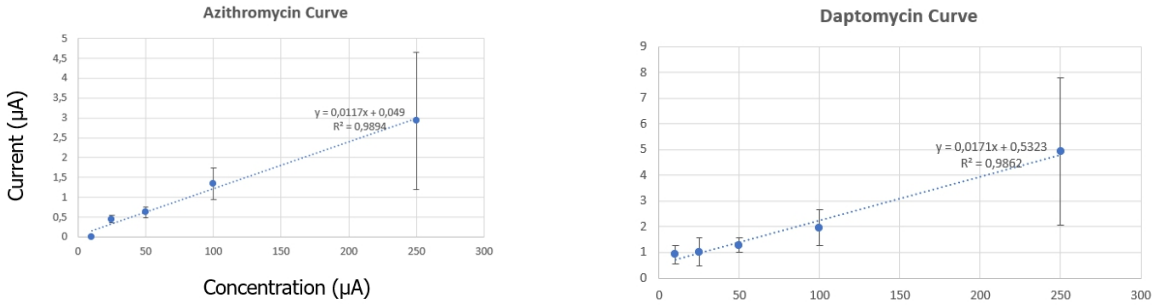


Figura 24. Calibration curves for azithromycin and daptomycin.

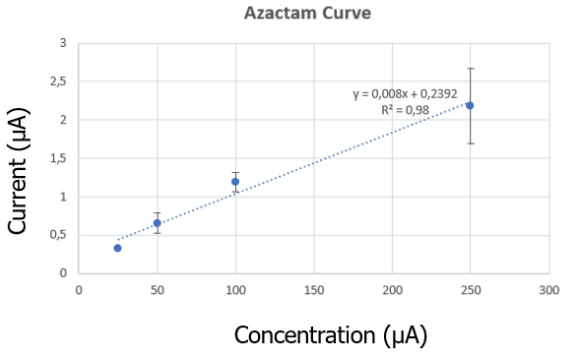


Figure 25.. Calibration curve for azactam.

Table 6. Calibration curves equations and regression coefficients.

Antibiotic	Calibration curve linear equation	Regression coeficient (R^2)
Meropenem	y = 0,0286x + 0,5182	0,9894
Cefepime	y = 0,0227x + 0,1495	0,9873
Azithromycin	y = 0,0112x + 0,1463	0,9967
Daptomycin	y = 0,0171x + 0,5323	0,9862
Azactam	y = 0,008x + 0,2392	0,9800

Finally, we can calculate the LOD of the curves by using the formula:

$$LOD = 3.3 \cdot \frac{\sigma}{S}$$

Where σ is the standard deviation of the response (determined based on the standard deviation of the y-intercept of the linear regression) and S is the slope.

Table 7. Sensitivity, SD of intercept and LOD for each antibiotic.

Antibiotic	Sensitivity (slope) (μA · L / μmol)	SD of intercept	LOD (μM)
Meropenem	0.0286	0.674	78
Cefepime	0.0227	0.189	27
Azithromycin	0.0117	0.192	54
Daptomycin	0.0171	0.322	62
Azactam	0.0080	0.265	109

For all antibiotics, except cefepime, the response is linear; As a result we only ploted the calibration curve of cefepime for the first concentrations: 10 μM, 25 μM and 50 μM, which presented a better linearity.

Linear regression of all these calibration curves was performed and results are shown in Table 6 and 7. Figures 24 and 25 show the standard deviation for the three replicates of daptomycin, azithromycin and azactam at the highest concentration tested were significantly large, what could be attributed to potential effects caused by the subproducts of the antibiotics oxidation on the electrode surface.

5.3 Detection of antibiotics on pSi

Finally, we tested the viability of the pSi platform to detect different antibiotics at different concentrations.

We first performed a unique measurement with a low concentration of meropenem solution 10 µM, and then CV characterization in ferrocyanide-ferricyanide solution to assess the passivation of the pSi upon antibiotic detection.

We realized that the passivation of the signal was high and also a big obstacle when trying to do multiple measurements, as we can see in figure 26. We performed this unique measurement several times with the rest of antibiotics and the same problem was shown for all of them. Consequently we tried to address this electrode passivation by cleaning the surface with different solutions.

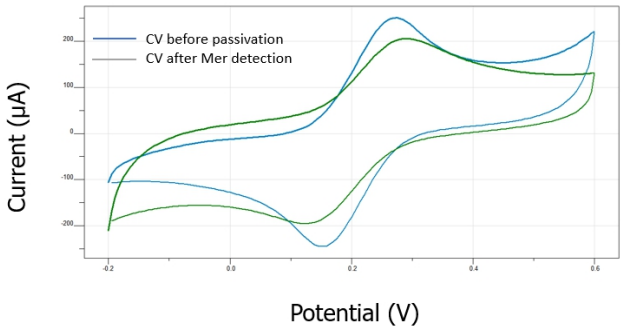


Figure 26. CVs with ferrocyanide-ferricyanide using the same pSi electrode. Comparing before and after detecting a 10 µM meropenem solution.

The solutions we tested to clean the surface and reverting passivation were 0.05 M **carbonate buffer** at pH 9.60 and **0.1 µM HCl in ethanol**.

For carbonate buffer we applied again 1.2 V during 45 s, but the buffer was deleteriously affecting the electrode surface, as we can see in figure 27.

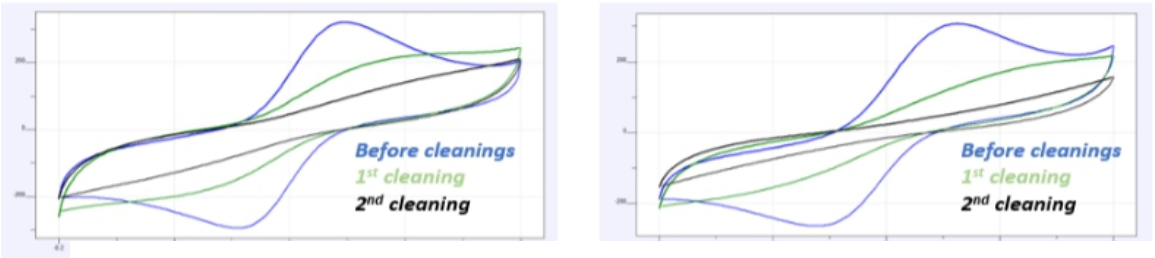


Figura 27. CVs of the cleanings using different pSi samples. Comparing before and after cleaning the sample with 0.05 M carbonate buffer.

Oxidation and reduction current values significantly decreased with each cleaning, so we discarded the cleaning with carbonate buffer.

Cleaning with ethanolic HCl gave confusing results, so we tried different conditions, such as potential values, interval of time for cleaning and number of scans. Also we tried different techniques to clean the surface: CV and amperometry.

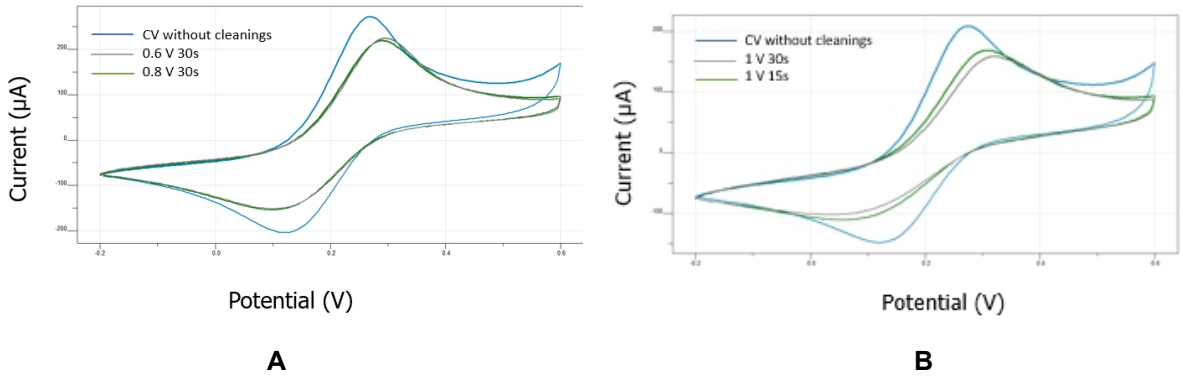


Figura 28. CVs using different pSi samples. Comparing before and after cleaning the sample with 0.1 µM HCl in ethanol, applying 0.6 and 0.8 V during 30 seconds (A), and 1 V during 15 and 30 seconds (B).

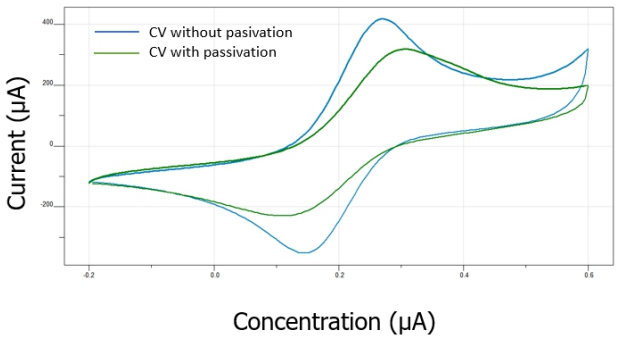


Figure 29. CVs using the same pSi sample with 0.1 µM HCl. Comparing before and after cleaning with cyclic voltammetry technique adjusted from 0 to 1.2 V at a scan rate of 100 mV/s.

Again we can see how the oxidation current decreased after applying both cleaning methods. However, proper analysis was further performed:

Table 8. Oxidation and reduction potentials extracted from CVs of pSi in ferrocyanide-ferricyanide, after applying 0.6 and 0.8 V during 30 seconds compared to untreated pSi.

pSi treatment	Epa (V)	Epc (V)	Ipa (µA)	Ipc (µA)	Peak to peak distance (V)
Non treated	0.270	0.115	29	180	0.155
0.6V 30s	0.295	0.100	202	130	0.195
0.8V 30 s	0.290	0.095	198	129	0.195

Table 9. Oxidation and reduction potentials extracted from CVs of pSi in ferrocyanide-ferricyanide, after applying 1 V during 30 and 15 seconds compared to untreated pSi.

pSi treatment	Epa (V)	Epc (V)	Ipa (µA)	Ipc (µA)	Peak to peak distance (V)
Non-treated	0.275	0.120	172	130	0.155
1V 30s	0.305	0.055	142	75	0.250
1V 15 s	0.320	0.030	131	61	0.290

Table 10. Oxidation and reduction potentials extracted from CVs of pSi in ferrocyanide-ferricyanide, after applying cyclic voltammetry adjusted from 0 to 1.2 V at a scan rate of 100 mV/s, compared to untreated pSi.

pSi treatment	Epa (V)	Epc (V)	Ipa (µA)	Ipc (µA)	Peak to peak distance (V)
Non-treated	0.32	0.040	127	63	0.280
CV 0 to 1.2 V	0.34	0.005	69	50	0.335

The increasing peak-to-peak potential separation when cleaning pSi samples means a less favored electron transfer on the sample surface. Moreover, the intensity decreases for every treatment. We conclude all the conditions applied might degrade pSi and thus we discarded all cleaning protocols tested for pSi.

In this situation, due to the impossibility to avoid passivation, we could not obtain calibration curves, as shown in figure 30.

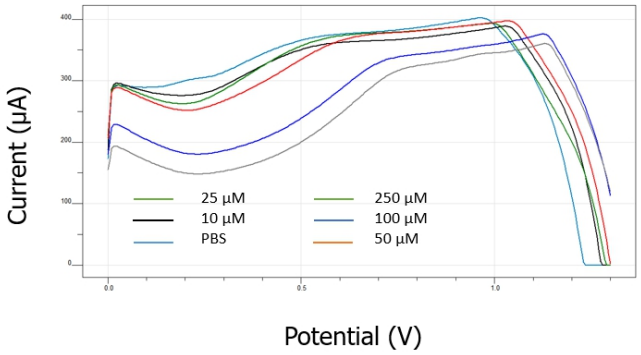


Figure 30. Square waves voltammograms for various concentrations of **meropenem**, measured from 0 to 1.3 V.

As a result, in order to obtain the corresponding oxidation potentials to be compared with those obtained using SPEs, we performed one CV measurement with each sample using 250 µM antibiotic solutions.

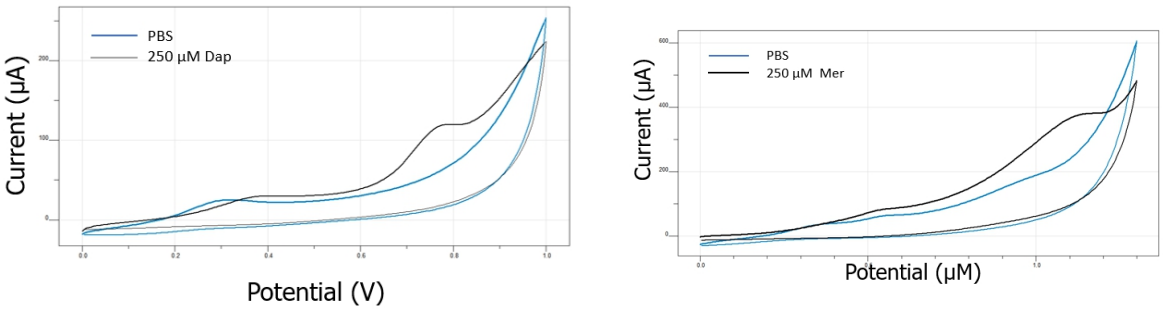
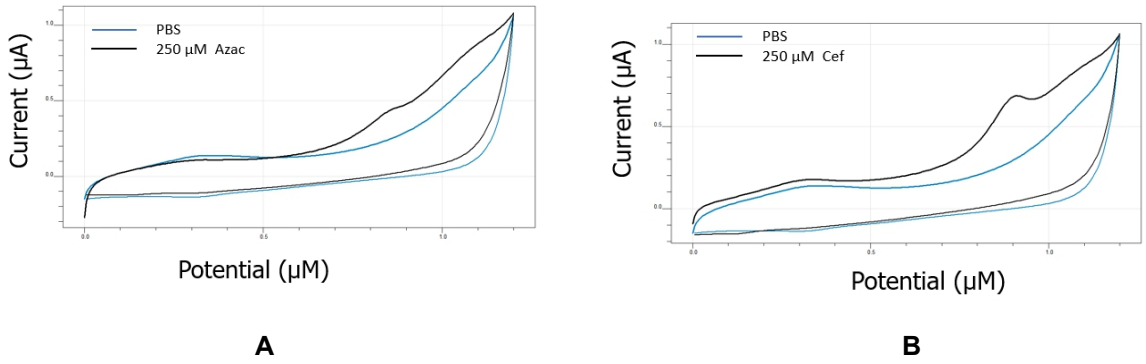
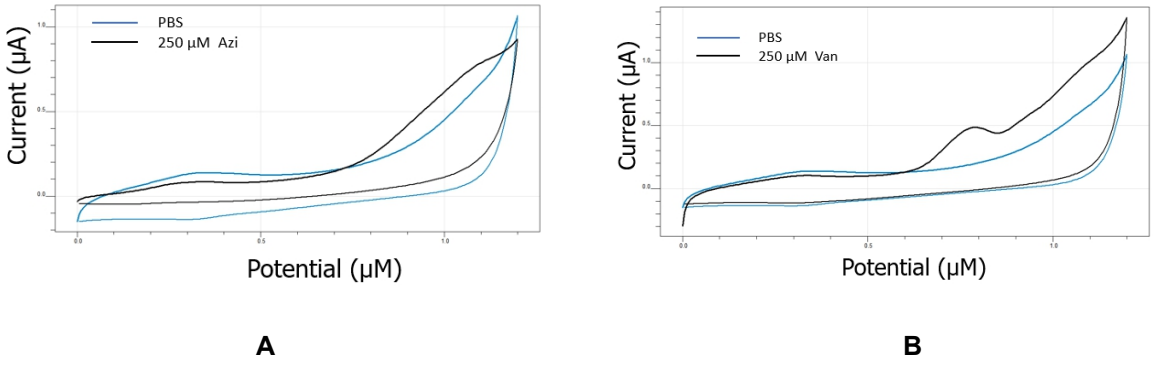


Figure 31. CVs of 250 µM Daptomycin (A), 0 to 1 V, and 250 µM Meropenem (B), 0 to 1.3 V comparing both with PBS CV



A B

Figure 32. CVs of 250 µM Azactam (A) and 250 µM Cefepime (B) comparing both with PBS CV. Potential from 0 to 1.2 V



A B

Figure 33. CVs of 250 µM Azithromycin (A) and 250 µM Vancomycin (B) comparing both with PBS CV. Potencial from 0 to 1.2 V

Table 11. Oxidation potentials extracted from the cyclic voltammograms obtained with pSi measuring each antibiotic at 250 µM.

Antibiotic	250 µM (V)
Meropenem	1.12
Cefepime	0.90
Vancomycin	0.79
Azithromycin	-
Daptomycin	0.78
Azactam	0.85

Except azithromycin, all antibiotics provided clear oxidation potentials in the potential range tested by CV.

Cyclic voltammograms showed a low peak around 0.4 V, which matches with ferro/ferricyanide's oxidation peak. Before the experiments we had to check the stability of the samples using this solution, and it seems that samples were not properly cleaned after that. However, antibiotic's oxidation potentials are higher than 0.4 V, so we could accurately identify them.

Table 12. Extracted oxidation peaks of the cyclic voltammograms at higher concentrations, 250 µM, with both SPE and pSi.

Antibiotic	**SPE** **(** 250 µM)	**PSi** **(** 250 µM)
Meropenem	0.88	1.12
Cefepime	0.72	0.90
Vancomycin	-	0.79
Azithromycin	0.83	-
Daptomycin	0.68	0.78
Azactam	0.80	0.85

We can clearly see how the potentials increase in pSi platform; Also we could identify and analyse the oxidation peak for 250 µM Vancomycin, which was not possible in SPE.

Although potentials for pSi ar higher, they cover a wide range of potentials, which is essential to discriminate among various antibiotics

Table 13. Extracted current intensities of CVs with both SPEs and pSi, for each antibiotic at higher concentration, 250 µM .

Antibiotic	SPE (µA / cm^2)	PSi (µA / cm^2)
Meropenem	62579	14280
Cefepime	110062	7408
Vancomycin	75471	-
Azithromycin	-	10560
Daptomycin	18710	7968
Azactam	69182	9080

We normalized the intensities dividing by the area of both working electrodes: SPE has 4 mm of diameter and pSi 0,9 mm. Calcules in the Annex 8.4.

6 Conclusions

From the discussed results in the previous section we can extract the following conclusions:

1. The activation of screen-printed electrodes using carbonate buffer minimizes electrode passivation when doing multiple measurements with antibiotics, and often increases measured signal. The cleaning also favours the electron transfer at first measurements, as the signal is increased since the beggining.

2. The oxidation of antibiotics leads to subproducts that passivate the electrode surface, causing variations in the signals of following measurements.

3. Porous materials require specific cleaning methods to remove subproducts that passivate the surface.

4. The big area of detection of porous silicon, gives more sensity than commercial SPE electrode, as we can see higher current intensities for 250 µM concentrations.

5. Porous silicon cover a wide range of potentials, which is essential when doing measurements of various antibiotics simultaneously, as we can discriminate them.

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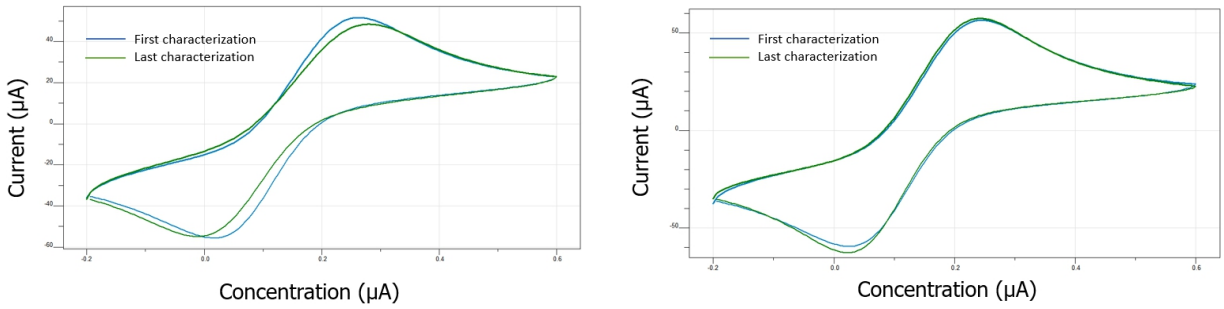
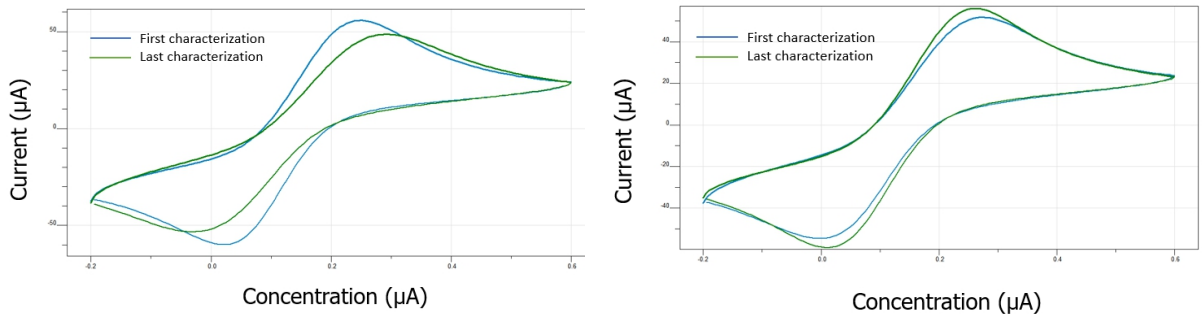
8 Annex

8.1 Cyclic voltammograms at the beginning and at the end of the measurements with antibiotics in SPE



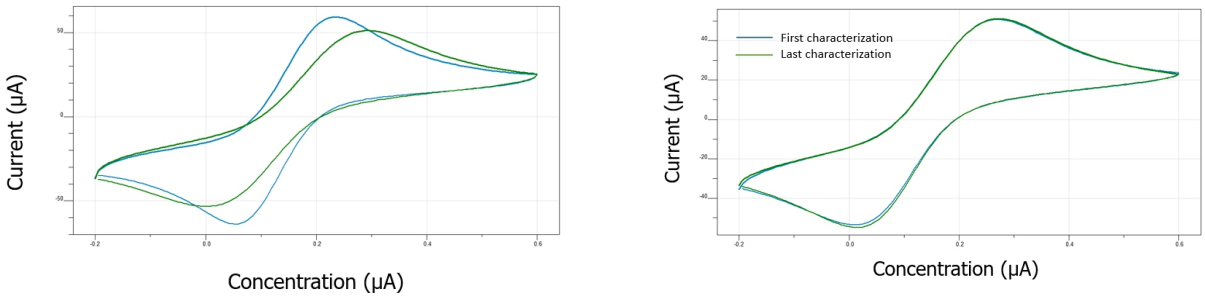
Meropenem **Cefepime**

Figura 34. Cyclic voltammograms for the first and last CV using ferrocyanide-ferricyanide during detections of meropenem and cefepime at different concentrations.



Vancomycin **Azithromycin**

Figura 35.. Cyclic voltammograms for the first and last CV using ferrocyanide-ferricyanide during detections of vancomycin and azithromycin at different concentrations



Daptomycin **Azactam**

Figura 36. Cyclic voltammograms for the first and last CV using ferrocyanide-ferricyanide during detections of daptomycin and azactam at different concentrations.

8.2 Calculation of the total percentages for the total passivation in the detection of antibiotics

Table 14. Extracted oxidation peaks of the cyclic voltammograms for each conccentration.

Antibiotic	Oxidation peak (Before pass.) (µA)	Oxidation peak (After pass.) (µA)
Meropenem	5.15	4.84
Cefepime	5.75	5.65
Vancomycin	5.57	4.85
Azithromycin	5.17	5.60
Daptomycin	5.92	5.11
Azactam	5.08	5.11

We apply the total passivation by using the following formule, where Ii is the peak to peak intensity before the passivation and If is the peak to peak intensity after the passivation:

$$Total\ Passivation = 100 \cdot \frac{Ii - If}{Ii}$$

Table 15. Extracted oxidation peaks of the cyclic voltammograms for each conocentration.

Antibiotic	Total intensity between peaks (Before pass.)(µA)	Total intensity between peaks (After pass.)(µA)	Total passivation (difference of µA)
Meropenem	10.70	10.34	-4%
Cefepime	11.70	11.90	2%
Vancomycin	11.55	10.17	-12%
Azithromycin	10.63	11.50	8%
Daptomycin	12.30	10.44	-16%
Azactam	10.43	10.59	2%

8.3 Extracted oxidation peaks of the cyclic voltammograms for each concentration in SPE

Table 16. Extracted oxidation peaks of the cyclic voltammograms for each conocentration.

Antibiotic	10 µM (V)	25 µM (V)	50 µM (V)	100 µM (V)	250 µM (V)
Meropenem	0	0.85	0.86	0.87	0.88
Cefepime	0.62	0.65	0.67	0.69	0.72
Vancomycin	-	-	-	-	-
Azithromycin	0	0.80	0.81	0.82	0.83
Daptomycin	0.64	0.66	0.68	0.68	0.68
Azactam	0	0.75	0.76	0.78	0.80

8.4 Normalization of the intensities for both SPE and pSi

Table 17. Individual current intensities for each antibiotic in both SPE and pSi without normalization.

Antibiotic	pSi intensities (µA)	SPE intensities (µA)
Meropenem	398	17,85
Cefepime	700	9,26
Vancomycin	480	-
Azithromycin	-	13,20
Daptomycin	119	9,96
Azactam	440	11,35

For 4 mm diameter SPE the area is 0,00125 cm^2, and for 9 mm diameter pSi the area is 0,00636$cm^{2.}$

If we apply the fllowing formule, these areas we get the normalizated values of Table 8:

$$\frac{Intensity}{area\ of\ working\ electrode}$$

Table 18. Normalized individual current intensities for each antibiotic in both SPE and pSi.

Antibiotic	**pSi** (µA / cm^2)	**SPE** (µA / cm^2)
Meropenem	62579	14280
Cefepime	110062	7408
Vancomycin	75471	-
Azithromycin	-	10560
Daptomycin	18710	7968
Azactam	69182	9080