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Eco-Technologies for Immobilizing Redox Enzymes on Conductive Textiles, for Sustainable Development

Eco-technologies pour l'immobilisation d'enzymes redox sur des textiles conducteurs, pour un développement durable

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Abstract

Eco-Technologies for Immobilizing Redox Enzymes on Conductive Textiles, for Sustainable Development

Enzyme immobilization on electrically conductive supports is necessary to improve their bioactivity and stability, for use and re-use in applications depending on bio-electrochemical response, such as in bioelectrodes, biosensors, or biofuel cells. However, the immobilization methods used are still facing many challenges in terms of health hazards and high environmental impact. Thus, it is important to find balanced and eco-friendly approaches to achieve efficient immobilization with minimum harmful consequences.

Hence, within the frame of this thesis, the use of eco-technologies such as cold remote plasma, a bio-compatible conductive (PEDOT:PSS) polymer coating, and a bio-based crosslinker "genipin" which has low toxicity, to immobilize glucose oxidase (GOx) enzyme on conductive carbon fiber-based nonwoven textiles was investigated. These carbon-based textiles, regardless of their hydrophobicity, are robust materials to be used as alternative for expensive rigid metals, since they possess good electrical conductivity and good resistance to corrosion in different media.

The results obtained showed that cold remote plasma treatment with nitrogen and oxygen gas mixture was efficient in functionalizing the surface of carbon felts and PEDOT:PSS coated felts. This increased carbon fiber surface energies, and facilitated the immobilization of GOx by physical adsorption with maintained bioactivity and improved reusability. Furthermore, immobilization of GOx using genipin as a crosslinking agent improved remarkably the stability of performance of bio-functionalized carbon felts. This crosslinker showed to be able to directly crosslink the enzymes without a matrix or hydrogel. Finally, the obtained bio-functionalized carbon textiles were primarily evaluated for use in sustainable applications for wastewater treatment such as Bio-Fenton (BF) and enzymatic Bio-Electro-Fenton (BEF). The results showed that bioactivity and bio-electro-activity of immobilized GOx was promising in color removal of Remazol Blue RR reactive dye and its partial degradation from solution in both treatments, which proved the success of the chosen immobilization methods in producing bioactive textiles that can be used as electrodes for power generation and pollution control.

Keywords: Eco-technology, Carbon felts, PEDOT:PSS, Glucose oxidase immobilization, Cold remote plasma, Genipin.

Résumé

Eco-technologies pour l'immobilisation d'enzymes redox sur des textiles conducteurs, pour un développement durable

L'immobilisation d'enzymes sur des supports conducteurs d'électricité est nécessaire afin d'améliorer leur bioactivité et leur stabilité, pour une utilisation et une réutilisation dans des applications dépendant de la réponse bio-électrochimique, telles que des bioélectrodes, des biocapteurs ou des piles à biocarburant. Cependant, les méthodes d'immobilisation utilisées rencontrent encore de nombreux défis en termes de risques pour la santé et d'impact environnemental. Il est donc important de trouver des approches équilibrées et respectueuses de l'environnement pour parvenir à une immobilisation efficace avec un minimum de conséquences néfastes.

Ainsi, dans le cadre de cette thèse, l'utilisation d'écotechnologies telles que le plasma froid, le dépôt de polymère conducteur biocompatible (PEDOT: PSS) et d'un agent de réticulation biologiquement basé sur la génipine, peu toxique, permettant l'immobilisation de glucose oxydase (GOx) sur des textiles nontissés à base de fibres de carbone a été étudiée. Ces textiles à base de carbone, quelle que soit leur hydrophobicité, sont des matériaux robustes à utiliser comme alternative aux métaux rigides onéreux, car ils possèdent une bonne conductivité électrique et une bonne résistance à la corrosion dans différents milieux. Les résultats obtenus ont montré que le traitement plasma froid avec un mélange gazeux d'azote et d'oxygène était efficace pour fonctionnaliser la surface des nontissés de carbone vierge et ceuxrevêtus de PEDOT: PSS. Une augmentation des énergies de surface des fibres de carbone facilite l'immobilisation de GOx par adsorption physique avec une bioactivité maintenue et une meilleure capacité de réutilisation. En outre, l'immobilisation de GOx au moyen de génipine en tant qu'agent de réticulation améliore de façon remarquable la stabilité des performances des feutres de carbone bio-fonctionnalisés. Cet agent de réticulation s'est révélé capable de réticuler directement les enzymes sans matrice ni hydrogel. Enfin, les textiles de carbone bio-fonctionnalisés obtenus ont été principalement évalués pour une utilisation dans des applications durables pour le traitement des eaux usées telles que la Bio-Fenton (BF) et la Bio-Electro-Fenton enzymatique (BEF). Les résultats ont montré que la bioactivité et la bio-activité électrique du GOx immobilisé étaient prometteuses pour l'élimination de la couleur du colorant réactif Remazol Blue RR et sa dégradation partielle à partir de la solution dans les deux traitements, ce qui a prouvé l'efficacité des méthodes d'immobilisation choisies pour la production de textiles bioactifs. Ces textiles peuvent être utilisés comme électrodes pour la production d'énergie et la dépollution.

Mots clés: Eco-technologie, nontissés de carbone, PEDOT: PSS, immobilisation de glucose oxydase, plasma froid, génipine.

Abstrakt

Eco-Technologies för att immobilisera Redox Enzymer på elektriskt Ledande Textilier, för hållbar utveckling

Enzym immobilisering på elektriskt ledande stöd är nödvändig för att förbättra sina bioaktivitet och stabilitet, till användning och återanvändning i applikationer som är beroende på bioelektrokemiskt respons t.ex. bioelektroder, biosensorer. Däremot den använt immobiliseringsmetoderna fortfarande står inför många utmaningar gäller hälsorisker och miljöpåverk. Det är således viktigt att hitta balanserad och miljövänlig metoder för att uppnå effektiv immobilisering med minimala skadliga konsekvenser.

Därmed den här doktorsavhandling undersöker miljövänlig kall fjärrplasma teknik, en biokompatibel polymerbeläggning (PEDOT: PSS) och ett bio-baserat låg toxicitet tvärbindningsmedel "genipin" för att immobilisera glukosoxidas (GOx) enzym på ledande kolfiberbaserade nonwoven textiler undersöktes. Dessa kolbaserade textilier, oavsett hydrofobicitet, är robusta material och kan användas som alternativ till dyra styva metaller, eftersom de har bra elektrisk ledningsförmåga och bra korrosionsbeständighet i olika medier.

Resultaten visade att kyla avlägsen plasmabehandling med kväve och syreblandning var effektiv vid funktionaliseringen av ytan av kolfilte och PEDOT: PSS-belagd filten. Detta ökade kolfiber ytanergierna och underlättade immobiliseringen av GOx genom fysisk adsorption med förbättrad bioaktivitet och återanvändning.

Vidare immobilisering av GOx med genipin tvärbindare förbättrade stabiliteten i prestandan hos biofunktionella kolfilt på ett märkbart sätt. Denna tvärbindare befanns vara i stånd att direkt tvärbinda enzymerna utan en matris eller hydrogel.

Äntligen de biofunktionaliserade koltextilierna var primärt använt i hållbara tillämpningar för avloppsrening som Bio-Fenton (BF) och enzymatisk Bio-Electro-Fenton (BEF). Resultaten visade att bioaktivitet och bioelektroaktivitet hos immobiliserad GOx lovade färgavlägsnande av Remazol Blue RR-reaktivt färgämne och dess partiella nedbrytning från lösning i båda behandlingarna, vilket visade framgången hos de valda immobiliseringsmetoderna vid framställning av bioaktiva textiler vilka kan användas som elektroder för elproduktion och föroreningsbekämpning.

Nyckelord: Ekoteknik, karbonfilt, PEDOT: PSS, immobilisering av glukosoxidas, kall fjärrplasma, genipin.

氧化还原酶在导电纺织品上的生态可持续固定化技术

中文摘要

生物酶可改善导电材料的生物活性及稳定性，根据其生物电化学反应，经生物酶改性后的导电材料可用于生物电极、生物传感器或生物燃料电池等领域，因此将生物酶固定在导电材料上意义重大。但目前所采用的固定方法对人类生命健康和环境均产生不良影响，仍面临着众多挑战。寻找生态友好的生物酶固定技术以提高固定效率、降低改性所带来的危害极为重要。

因此，本课题主要采用生态友好型技术如低温等离子体远程处理技术(CRP)、生物相容性聚合物聚(3,4-亚乙基二氧噻吩):聚(苯乙烯磺酸)(PEDOT:PSS)涂层技术以及利用毒性较低的“京尼平”作为生物基交联剂将葡萄糖氧化酶(GOx)固定在导电碳纤维基非织造布上。如果不考虑碳基纺织品的疏水性的话，该类材料较为坚固，具有良好的导电性，且对各种介质具有较好的耐腐蚀性，因此可用于替代昂贵的刚性金属材料。

结果表明，通过氮气和氧气混合气氛下的低温等离子体远程处理技术可有效地将碳毡和PEDOT:PSS涂层毡的表面进行功能化改性。该表面改性可提高碳纤维的表面能，促进葡萄糖氧化酶(GOx)在碳纤维上的物理吸附性能，并保持葡萄糖氧化酶(GOx)的生物活性和重复使用性能。此外，利用“京尼平”作为葡萄糖氧化酶(GOx)和碳纤维间的交联剂可明显提高碳毡生物功能性的稳定性。

该交联剂可直接用于酶交联，无需使用基质或水凝胶。最后，将制备的生物功能性碳纺织品用于废水处理如Bio-Fenton (BF)、酶催化Bio-Electro-Fenton (BEF)等可持续应用中。葡萄糖氧化酶的生物活性和电生物活性有助于活性染料雷马素蓝RR溶液的脱色和部分染料的降解，表明通过上述方法制备的具有生物活性的纺织品可用于发电和污染控制等领域。

关键词：生态技术；碳毡；PEDOT:PSS；葡萄糖氧化酶固定化；低温等离子远程处理技术；京尼平

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Hab Nemra/ Syria – July 2019

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List of Publications

- **I.** M. Kahoush, N. Behary, A. Cayla, V. Nierstrasz. Bio-Fenton and Bio-Electro-Fenton as Sustainable Methods for Degrading Organic Pollutants in Wastewater. *Process Biochemistry*, *64* (**2018**), 237_247.
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- **II.** M. Kahoush, N. Behary, A. Cayla, B. Mutel, J. Guan, V. Nierstrasz, Surface modification of carbon felt by cold remote plasma for glucose oxidase enzyme immobilization, Applied Surface Science, 476 (**2019**), 1016–1024.
  DOI:10.1016/j.apsusc.2019.01.155.

- **III.** M. Kahoush, N. Behary, A. Cayla, B. Mutel, J. Guan, V. Nierstrasz, Influence of cold remote plasma on carbon felt coated with conductive PEDOT:PSS polymer for optimized activity of immobilized glucose oxidase, **Manuscript submitted.**

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Introduction

A- Background and research gap

Appropriate methods for enzyme immobilization on conductive materials are necessary to improve the bio-catalytic activity of enzymes for use in applications where electrochemical response is of prime importance, such as in bioelectrodes, biosensors, or biofuel cells.

This field is getting more attention in recent years with applications used on a daily basis at different levels. Glucose biosensors for domestic use facilitate the lives of diabetes patients, while other biosensors make an easy and reliable detection of cholesterol, alcohol and heavy metals, to improve safety and control diseases or pollution. Furthermore, power generation from bio-sourced renewable materials is of great interest for more sustainable processes and services.

According to Web of Science database, the keywords (biofuel and enzyme) are increasingly used in literature over the last 20 years, with a remarkable increase in the last decade (Figure 1). While "enzyme immobilization" topic results in more than 20000 publications in the last 15 years. These statistics reflect the growing interest, relevence and importance of biotechnology in general, and applications of immobilized enzymes specifically.

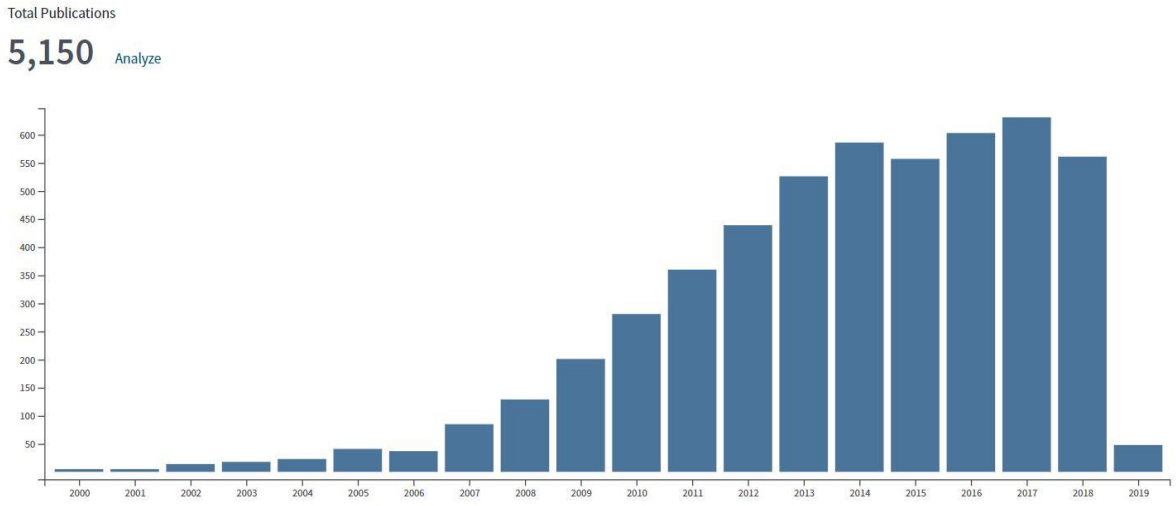


Figure 1 Total publications related to keywords (biofuel, enzyme) in the last 20 years indexed in Web of Science database (collected in February 2019)

Many enzyme immobilization techniques are widely used nowadays, like immobilization on membranes and entrapment in gels or matrix. However, numerous of these methods can be improved in terms of pretreatment requirements and the use of several chemicals or energy consumption.

Numerous of the methods used may suffer from drawbacks in regards to the materials used in preparing the support or textile for the immobilization process. Pretreatments, including the use of acids or oxidant solutions at high temperature for long periods of time are used to achieve an efficent surface modification prior to enzyme immobilization. These wet processes usually contribute to water and power consumption of the overall process, therefore increasing their environmental impact.

Other methods focusing on immobilization enzymes via crosslinking or entrapement within hydrogels, may include the risk of high toxicity to the living orgasims that will be exposed to such materials. Hence, non-toxic or low-toxicity materials are always preffered to improve the safety of work environment as well as the wellbeing of the end-user of these materials.

In this work, immobilization of redox enzymes using eco-friendly approaches were conducted in attempt to reduce the amount of added chemical and power consumption, in addition to minimize waste production within the frame of sustainable development.

Eco-technologies and products such as plasma as dry treatment, biocompatible conductive polymer coatings, and low toxicity bio-based crosslinking reagent, have been used in this thesis to achieve the objectives. Cold remote plasma has been used to activate the surface of carbon textile for immobilization of glucose oxidase enzyme. The immobilization was carried out using either direct physical adsorption method, or bio-based naturally occurring crosslinker (Genipin), which is known for its low toxicity in comparison with conventional enzyme crosslinking agents such as glutaraldehyde.

A potential end-application of the obtained bioactive textiles have been proposed to be within the wastewater treatment field. It is well known that the textile industry is one of the most contributing industries to wastewater production. These waters are usually rich in dyes and additives and can cause serious damage to the environment if released into land fields or surrounding waters. Thus, a proper treatment should be performed to treat these wastewaters to reduce their harmful impacts.

However, many conventional wastewater treatment methods usually require the use of large amounts of chemicals or electrical power to remove the colors, and may result in an incomplete degradation of the organic pollutants. Hence, sustainable approach for wastewater treatment is needed to reduce the environmental impact of wastewater treatment from textile industry.

Therefore in this work, bioactivity and bio-electro-chemical activity of the immobilized enzyme were assessed primarily for a future use as bio-anode in bio/bio-electro-Fenton process, for degradation of organic pollutants. A model pollutant Remazol Blue RR reactive dye has been chosen for these treatments, since it is extensively used in textile industry worldwide, and it is known to be persistent and hard to treat.

The sustainable applications mentioned seem to have advantages over the traditional wastewater treatment methods in regards to environmental impact and safety in work place.

B- Aims and Objectives

The main motivation behind this work was to investigate milder and more eco-friendly methods to immobilize redox enzymes such as glucose oxidase on conductive textiles, and test their potential use in applications such as bio-Fenton and bio-electro-Fenton processes. As previously mentioned, the conventional methods have some drawbacks despite their efficiencies, require large consumption of power or hazardous toxic chemicals, and produce wastewaters in big amounts.

Hence, the proposed methods are focused on resource-efficient approaches to minimize material and energy consumption, produce less waste, prevent health and environmental hazards, and consequently reduce the costs of operations.

Taking into account these criteria, the main objectives of this thesis are:

Objective 1: To use eco-friendly methods to obtain enzyme-functionalized textile-based felts/ electrodes, with maintained enzymatic activity and reusability.

Objective 2: To investigate the possibility of using these felts in sustainable treatment of wastewaters from textile industry.

C- Thesis structure

Chapter I provides a general background of this research and emphasizes the state of Art on enzyme immobilization on conductive textiles and their applications. Furthermore, it highlights the concepts of sustainability and eco-technology, along with the aims and objectives that were the main motivation of the research conducted.

Chapter II includes description of the materials used to achieve the objectives, the methodologies used as to produce the bio-functionalized conductive textile materials, the instruments and characterization techniques to further assess and evaluate the performance of these obtained textiles in terms of bioactivity and bio-electrochemical activity concerning the application proposed.

Chapter III presents the results obtained within this thesis, using three main eco-technologies or approaches to produce textile-based felts/electrodes functionalized with redox enzymes. Furthermore, this chapter also presents the analysis and discussions of the phenomena observed from the obtained results.

Chapter IV presents two possible applications for the obtained samples from Chapter III in regards to textile effluents treatments, which is considered as a main concern regarding textile industry worldwide. These applications fall into the concept of sustainability with primary results about their feasibility and efficiency in color removal and organic pollutants degradation levels.

Chapter V derives the main conclusions of this thesis, with insights into future perspectives within this area of research and the main challenges and limitations facing it.

Therefore, a scheme of the processes used in this thesis is illustrated with annotations (Figure 2).

Graphical abstract

The sequence of the process used in this thesis is graphically summarized with annotations as follows:

1- Carbon felt is used as textile-based support for enzymes; it possesses a hydrophobic tendency that complicates further steps of functionalization with enzymes or reactional medium inflow.
2- Dip-coating with PEDOT:PSS conductive polymer blend to modify surface energy and biocompatibility while maintaining electrical conductivity.

3- Dry pretreatment with Cold Remote Plasma (CRP) for both types of samples to improve surface characteristics.

4- Immobilization of redox enzyme via physical adsorption on carbon-based felts.

5- Immobilization of redox enzymes via naturally occurring cross-linker on carbon-based felts.

6- Testing the bio-catalytic activity of obtain felts in Bio-Fenton process to treat textile wastewater.

7- Testing the bio-electro-catalytic activity of obtain felts as bioelectrodes in Bio-Electro-Fenton process to treat textile wastewater.

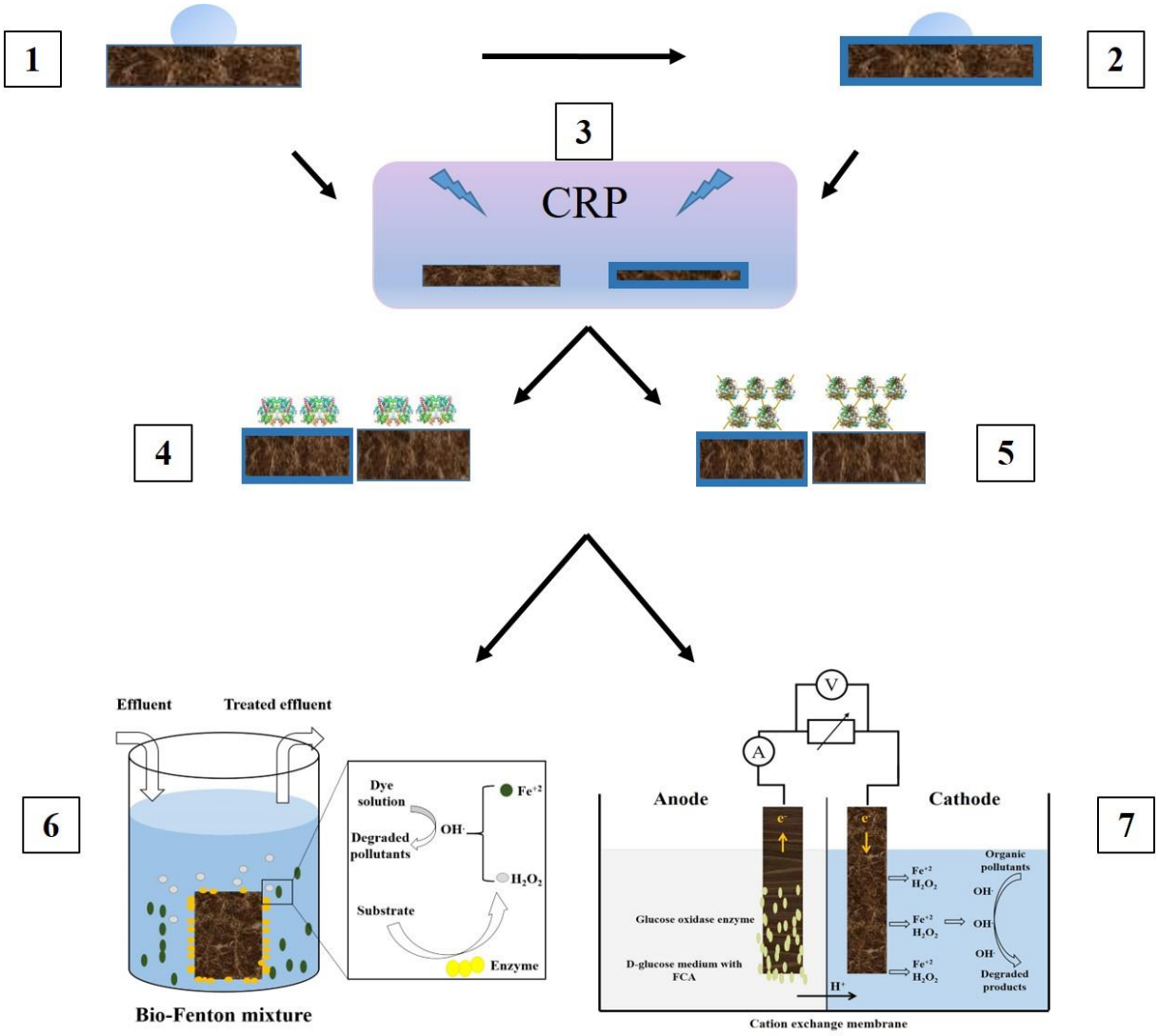


Figure 2 Illustration of the processes presented in this work

Chapter I
State of Art

I- A- State of Art on enzyme immobilization on conductive supports

I- A- 1- Enzyme definition and classification

The enzyme is defined as: "An enzyme derived from an organism or cell culture catalyzes metabolic reaction in living organisms and /or substrate conversions in various chemical reactions" [1].

In general, enzymes are long linear chains of amino acids that fold to produce a 3D structure protein. They are biocatalysts which accelerate specific reaction rate by lowering the activation energy of the reaction [2]. Enzymes have been divided into classes according to the type of reactions they catalyze, and were given systematic naming which describes the involved chemical reactions [3].

There are six categories according to this classification of the Enzyme Commission (EC) which are; oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5) and

ligases (EC 6).

In addition to the six previous classes, very recently in 2018, translocases (EC 7) class has been added according to the enzyme database website [4].

I- A- 1- 1- Sustainability of enzymes

Sustainable development is a multi-domain issue and it has to combine equity, efficiency and intergenerational equity on social, economic and environmental ground. It can be defined according to Brundtland commission as follows: *"Sustainable development* is *the development that satisfies the needs of the current time period without jeopardizing the ability of future generations to satisfy their needs*." [5]. Hence, in order to achieve sustainability, a balance between the social, environmental and economic aspects should be applied.

Enzymes are biodegradable proteins that are derived from renewable sources [6]. They are highly specific towards certain substrates and accelerate the rate of reactions remarkably. Furthermore, CO_2 emissions, water and energy consumption can be reduced in processes catalyzed by enzymes. Consequently, enzymatic processes are usually environment-friendly and cost efficient thanks to modern biotechnology, which make them sustainable biocatalysts [6].

I- A- 1- 2- Enzymes in textile industry

Enzymes have been used in textile industry especially for wet-processing. Furthermore, the high costs of producing these enzymes from their natural resources have been reduced by production using microorganisms, which help in the production at industrial scale and reduction of the prices of these biocatalysts [7].

The integration of enzymes in the different processes in textile industry has shown to be successful in reduction of costs, chemicals, time and energy. Therefore, they are considered as a good sustainable alternative in many processes nowadays. Processes such as desizing and bio-scouring of cotton [8], mild surface modification and finishing of natural and synthetic fibers, discoloration of effluents and decomposition of residual impurities from the fibers are some of the most common applications of enzymes in textile industry [9]. The use of different enzymes such as amylases, cellulases, pectinases, catalases, lipases, proteases in various wet textile processing have been reported in details [10,11]. Furthermore, the focus in recent years has been on the production of biodegradable materials and polymers which are heavily used in textile and packaging, using enzymes such as lipase or laccase [7].

I- A- 1- 3- Enzyme used in this PhD is from the class of Oxidoreductases enzymes (EC 1)

These enzymes are also called redox enzymes and as mentioned previously, they catalyze oxidation/ reduction reactions. This property highlighted the potential of these enzymes in many applications related to bio-electro-chemistry and bio-sensing. This class can be divided into three subcategories according to the mechanism of electron transfer [12].

1- Enzymes with redox center that is weakly attached to the periphery of the enzyme.

2- Enzymes in which at the redox center (or at least a part of it) is located at or near the periphery of the protein shell.

3- Enzymes with a redox center that is deeply bound in a protein shell.

The locations of the active sites or redox center mainly influence the electron transfer process in these enzymes.

However, another classification of redox enzymes according to Nomenclature Committee in 2010 is also available in six main classes: oxidases, dehydrogenases, peroxidases, hydroxylases, oxygenases and reductases [13]. The enzyme used in this study, belongs to oxidases class that is glucose oxidase (GOx).

I- A- 1- 4- Direct and mediated electron transfer

As mentioned, the location of the active site differs among redox enzymes. In the first two types, where the active site is on or close to the periphery of the enzyme, a Direct Electron Transfer (DET) between the enzyme and the other surfaces is possible. However, that is not possible (or too slow) for the third type. When the redox center of the enzyme is deeply bound, a mediator is used as a shuttle to facilitate the electron transfer from the active site of the enzyme to the electrode surface; this process is called a Mediated Electron Transfer (MET). The mediator circulates between the electrode and the active site of the enzyme and produces a catalytic current due to changing from its oxidized to reduced form [14]. It is worth mentioning, that in the last few years, many studies attempted to wire the active site of some redox enzymes from the third type like glucose oxidase directly to the surface of the electrode to achieve DET, using carbon nanotubes or other materials. But, it is yet not clear if the electrical signals are truly due to the wiring, or is it because of loose redox centers that diffused into the surface of the electrode [14,15]. Many types of materials are used as mediators in literature, some of which are ferrocene and many of its derivatives, osmium-based complexes, and quinones [14]. In addition, some redox dyes such as Nile Blue [16] were used as mediators in previous works.

I- A- 1- 5- Glucose oxidase EC (1.1.3.4)

Glucose oxidase (GOx) is an enzyme that falls under the EC 1 class which is the redox enzymes and it is considered as one of the third type of this class (deeply bound active site). It is a biocatalyst of the oxidation of β-D-glucose to produce D-glucono-1, 5 lactone and hydrogen peroxide (H_2O_2), using molecular oxygen [17]. It was discovered in 1928 in the extracts of *Aspergillus niger* fungus by Detlev Muller. This enzyme is a dimeric protein with two subunits that are encoded by the same gene [18]. It possesses an average diameter of 8 nm and a molecular weight of around 160 kDa

(whereas Dalton is defined as $1/12^{th}$ the mass of the carbon atom). It contains one Flavin Adenine Dinucleotide (FAD) per monomer as a cofactor and one iron. The two FAD sites in the two subunits are separated and no redox connection is evident between them (Figure 3). In addition, 16% of the enzyme content is carbohydrate [17].

The FAD cofactor in the enzyme is primer electron acceptor and upon the oxidation of glucose, it will be reduced to the $FADH_2$ form. Eventually, it will be oxidized again by electron acceptors in the form of mediators, which act as shuttles to facilitate the indirect electron transfer process (Eq. 1-4). Since the gap between the active site of the enzyme and the outside is too large due to the thick layer of proteins that surrounds the FAD center, this generates a barrier to direct electron transfer with the electrode surface, for enzyme immobilized on electrodes [18].

$$\text{D-glucose} + GOx_{ox} \rightarrow \text{gluconic acid} + GOx_{red} \quad (1)$$

$$GOx_{red} + 2M_{ox} \rightarrow GOx_{ox} + 2M_{red} + 2H^{+} \quad (2)$$

$$2M_{red} \rightarrow 2M_{ox} + 2e^{-} \quad (3)$$

The sum equation will be:

$$\text{D-glucose} \rightarrow \text{gluconic acid} + 2H^{+} + 2e^{-} \quad (4)$$

Where M_{ox} and M_{red} are the oxidized and reduced forms of the mediator used (oxidation by one-electron mediator).

It is worth mentioning, that even in the presence of different mediators, the reduced form of the enzyme GOx_{red} can be oxidized by oxygen. This oxidation is not desirable in many applications like biosensors since hydrogen peroxide will be a byproduct, which may affect the sensitivity towards the targeted material, and can be toxic in case of in vivo testing.

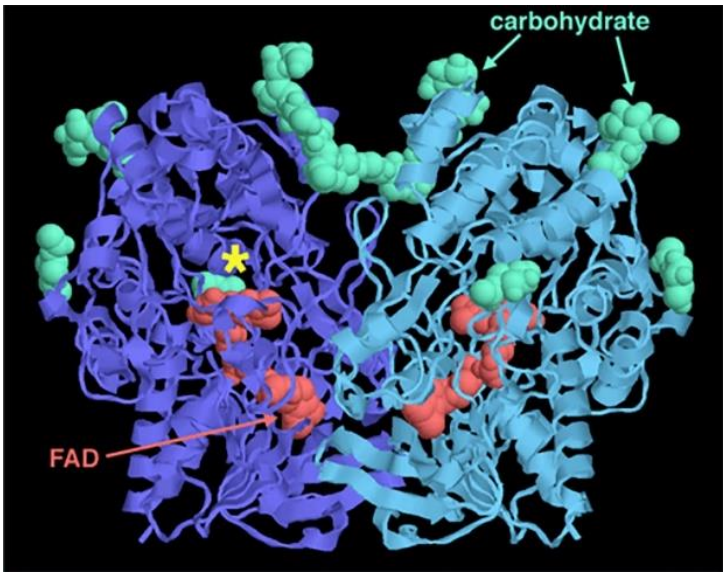


Figure 3 Glucose oxidase 3D structure (Fig. from (https://pdb101.rcsb.org/motm/77))

GOx has a good stability in its freeze-dry form (lyophilized), however, it is not stable at temperatures higher than 40 ºC, nor at pH levels below 2 and above 8 [18].

GOx is inhibited by micro-molar amounts of Ag^{+}, Hg^{+2}, Cu^{+2}, phenylmercuric acetate and p-chloromercuribenzoate [19].

GOx from *Aspergillus niger* EC (1.1.3.4) is considered as the most studied redox enzyme in recent years for electrochemical applications [15]. It gained its popularity, mainly because of its substrate, β-D-glucose. Since diabetes patients are required to monitor the level of glucose in the blood constantly, studies were heavily undertaken to produce biosensing tools of glucose [20–22]. Moreover, the advancement of biotechnology contributed to the importance of GOx, and studies focused on enzymatic biofuel cells to produce energy from bio-sourced materials like sugars and alcohol. GOx was used intensively in the anodic compartments of the enzymatic biofuel cells, along with other redox enzymes, such as laccase [23,24] that can catalyze the reduction reactions in the cathodic compartments.

I- A- 2- Conductive materials and textiles used for immobilization process

I- A- 2- 1- Electrodes and bioelectrodes

Bio-functionalized electrodes or "bioelectrodes" are electrodes that are used in bioelectrocatalytic systems based on redox enzymes, microbial cells or cell organelles. These bioelectrodes have been used intensively in research considering their great potential in applications such as biosensing devices, especially for monitoring changes in biological substances like glucose or cholesterol levels in human body, for treatments of diabetes and cardio-related diseases [25,26]. Other biosensing devices were targeted to detect the pollution levels in several media like soil, air and water, more specifically for heavy metals detection and control [27]. Another important area of research of the bioelectrodes is their use in biofuel cell in both Microbial Fuel Cell (MFC) and Enzymatic Fuel Cell (EFC) forms, to generate electricity from biosourced-materials like simple sugars and alcohol [23,28–33].

The use of expensive noble metals such as gold and platinum as electrodes or bioelectrodes was faced by several difficulties regardless of their remarkable efficiencies for such applications. The high cost of these metals was a crucial factor in their limited commercial use. Moreover, these metallic electrodes never work properly without certain pretreatments which include mechanical polishing followed by electrochemical cleaning to activate the surfaces of these electrodes [34].

Consequently, studies were reported regarding the use of cheaper materials as the base of electrodes or bioelectrodes, followed by the use of a conductive metallic coating as a thin layer to modify surface characteristics to achieve the desired properties according to the end application. These coatings were mainly obtained by galvanic and electrochemical plating methods, which require pretreatments and removal of impurities and oxide layers before the coating to guarantee a proper adhesion level.

I- A- 2- 2- Conductive textile-based electrodes

With the increased attention and importance of wearable/portable, flexible, and sustainable modern technology devices, the search for alternative material to produce electrodes or bioelectrodes has been a mainstream in research in the last decade. Biosensors, wearable sensors, miniature biofuel

cells and wearable electrical storage units are few of the categories that have been studied in both textile materials science and biotechnology. Some products have already hit the market for commercial use.

The health care department witnessed many advancement regarding wearable sensors integrated within the textile, or made from textile-based electrodes for real time monitoring of heart rate [35]. The use of conductive metallic fibers or metallic coated synthetic fibers was reported in mentioned applications. These fibers are mainly produced by "wire drawing" process and were used in protective garments in explosive areas, electromagnetic interference shielding, and infrared absorption [36].

However, recent advances in the conductive polymers and polymers with conductive fillers such as carbon nanotubes (CNTs) highlighted the potential of conductive textiles in their different forms and structures to be used in the biotechnology sector. Desirable properties of these textile-based materials opened the possibilities for use in many biological technologies, replacing the conventional metallic expensive materials due to many advantages, such as:

- **Good electrical conductivity**: conductive textiles possess sufficient conductivity to be used in bio-electro-technology setups (carbon: 10^4 S/m and Poly(3,4-ethylenedioxythiophene) PEDOT: 10^3 S/m).
- **Porosity and high specific surface area compared to their volume**: which make them great choice for immobilization of enzymes in limited and miniature spaces. This property is crucial for mass transfer to guarantee the exchange and flow between the enzymes and their substrates. However, some of these materials such as carbon felts usually possess low surface energy that influence their hydrophilicity and require some pretreatments to improve capillary uptake and permeability to certain liquids.
- **Flexibility and bendability**: unlike other metallic materials that are hard, rigid and heavy.
- **Resistance to corrosion**: in several media including aqueous and solvent based.
- **Cheaper prices**: than metals or noble metals and availability for commercial use.
- Some of them are **biocompatible**.

Conductive textiles or coatings based on carbon fibers, polyaniline, polypyrrole, and PEDOT have been used to produce bioelectrodes with immobilized enzymes for many applications [37,38].

I- A- 2- 3- Carbon fibers

Carbon fibers are among the strongest and stiffest fibrous materials known to date. They became available as early as 1960s, and have low density (around 1.8 $g.cm^{-3}$), thermal conductivity 3 folds higher than copper, high tensile strength (5 folds higher than steel) and they find extensive applications in modern industry and technology [39]. Polyacrylonitrile (PAN) is considered as the most used precursor to produce carbon fibers after treatment in high temperatures between 1000 - 1500 ºC. The produced carbon ribbons contain slight percentage of nitrogen mostly in form of pyridine rings, and the mentioned ribbons have hexagonal structure similar to graphite, and are usually parallel to the axis of fiber. The ribbons fold and interlock among each other which explains their high strength [40]. The manufacturing steps and the structure of carbon fibers are illustrated (Figure 4).

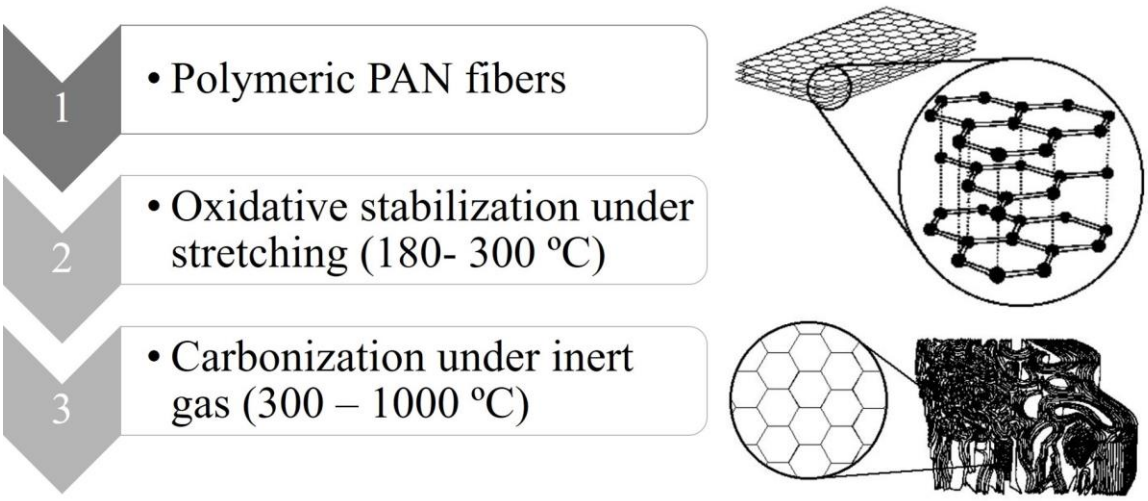


Figure 4 Production and structure of PAN-based carbon fibers *(http://www.chem.wisc.edu)*

Carbon fibers are used intensively in both research and industry nowadays in different forms (yarns, woven and nonwoven structures), to produce composite materials, and to produce electrodes and bioelectrodes for bio/electrochemical applications and reactors [41]. The use of carbon felts was more distinguished in the bio/electrochemical reactors for microbial or enzymatic fuel cells, and in wastewater treatment [42–45]. As previously mentioned, they possess high specific surface area,

which helps immobilization of higher amounts of bioactive materials such as microbial populations and enzymes [46,47].

However, the carbon materials overall lack functionality and tend to be hydrophobic. They require some pretreatments before use to facilitate immobilization of enzymes or to promote the biocompatibility for cell or microbial growth.

To modify these surfaces, several studies have been conducted to treat different carbon materials using variety of methods. Approaches including: chemical, physical or thermal methods have been reported to modify surface energy of carbon materials, and to facilitate treatments for different applications. Materials like rare earth elements such as lanthanum chloride ($LaCl_3$), praseodymium nitrate and ytterbium fluoride (YbF_3) were reportedly used as pretreatment for carbon materials. Furthermore, acids like nitric or acrylic acid, high-temperature solution of hydrogen peroxide, oxy-fluorination treatment, and 4-phenylacetic acid diazonium fluoroborate were commonly used for the same purpose. These treatments showed to be efficient in surface functionalization and modification, in addition to improving adhesion in composite materials, enhancing mechanical properties of nanocomposites, and increasing power efficiency in flow batteries [48–62].

Nevertheless, these methods mostly require wet processing, added chemicals and solvents at high temperatures for long periods, which result in significant environmental impact of these wet treatments, high costs and risks in the working place. Enzymes such as glucose oxidase, glucose dehydrogenase, alcohol dehydrogenase and bilirubin oxidase were reportedly immobilized on carbon fibers [63–65]. Furthermore, the detailed advances in the immobilization of enzymes on carbon fibers were described elsewhere, such as pretreatments with HNO_3 and H_2SO_4 acids followed by immobilization via adsorption, entrapment and covalent bonding [41].

I- A- 3- Methods of immobilizing enzymes

I- A- 3- 1- The importance of immobilization

The interest of enzyme immobilization comes from the need of improving the stability and reusability of the enzymes when compared to their free state. Reusability of enzymes also contributes to the reduction of process costs [6]. In addition, the immobilization process reduces the

risk of contamination of the products with the residues of the enzyme and hence, reduces the risk of allergies and other undesirable side effects [6]. Another advantage of immobilization of enzymes is the ability of extracting the enzymes from media when desired, to stop the reaction resulting in better control of the process, and less additional steps for deactivating or processing the residual enzymes in the media.

The main methods used in literature are covalent bonding, physical adsorption, cross-linking, entrapment in a matrix and encapsulation that are illustrated in (Figure 5).

I- A- 2- 2- Covalent bonding

This method provides a direct chemical bonding between the enzyme and the support material, through functional groups. This method usually produces a strongly attached enzymes with minimized tendencies of leaching into the surrounding mediums [2]. However, this method requires an active surface for the support material, which contains functional groups to bind with the amino acids of enzymes, or else, pretreatments for surface modifications should be performed to the support. One of the challenges facing this method is maintaining the accessibility of the active site of the enzyme after the immobilization in order to maintain the enzymatic activity. Many studies reported the use if this method such as; the covalent bonding of lactase was reported on polyethylene film, β-galactosidase was immobilized on glyoxyl support, pyrroloquinoline quinone (PQQ) monolayer and enzymes were then immobilized by covalent bonds [66,67].

I- A- 2- 3- Physical adsorption

This is the most common method for the enzyme immobilization [68]; it is achieved by weak physical bonding between the adsorbed enzyme and the support material, like hydrogen bonding, and Van der Waals bonds, or stronger ionic bonding. Numerous materials were used for immobilization by adsorption such as metallic oxides, silica gels, chitosan, cellulose and many more [68,69]. This weak bonding however, results in enzyme leaching and makes the process of immobilization reversible, and affects the stability. The glucose oxidase, hydrogenase and laccase enzymes were reported to be immobilized via this method in literature [69–71].

I- A- 2- 4- Cross-linking

This method can be achieved by crosslinking of the enzyme or protein to the other proteins or to a supporting matrix though intermolecular bonding. This method showed to be a good complement to some other methods like physical adsorption, to increase the stability and prevents leakage. However, this approach may influence the enzymatic activity due to diffusion and limited accessibility of the substrate to the active site of the enzyme. Enzymes such as lactate dehydrogenase and glucose oxidase were immobilized via cross-linking using glutaraldehyde, which has high toxicity levels if it is touched or swallowed [72–74].

I- A- 2- 5- Entrapment

It is a method where enzymes are locked up or entrapped in a matrix that is usually permeable towards the substrate, and allows diffusion towards the entrapped enzymes while retaining the enzymes inside the matrix. Hydro-gels and microcapsules can be used to entrap the enzymes [69,75]. Regardless of the efficiency of this method in reducing the leakage of enzymes to the medium and increasing the stability, this method has a considerable disadvantage of mass transfer limitations, since the diffusion occurs inside the biocatalyst in addition to the thin layer of liquid (film) in its surrounding [76], which might be limited when the enzymes are entrapped.

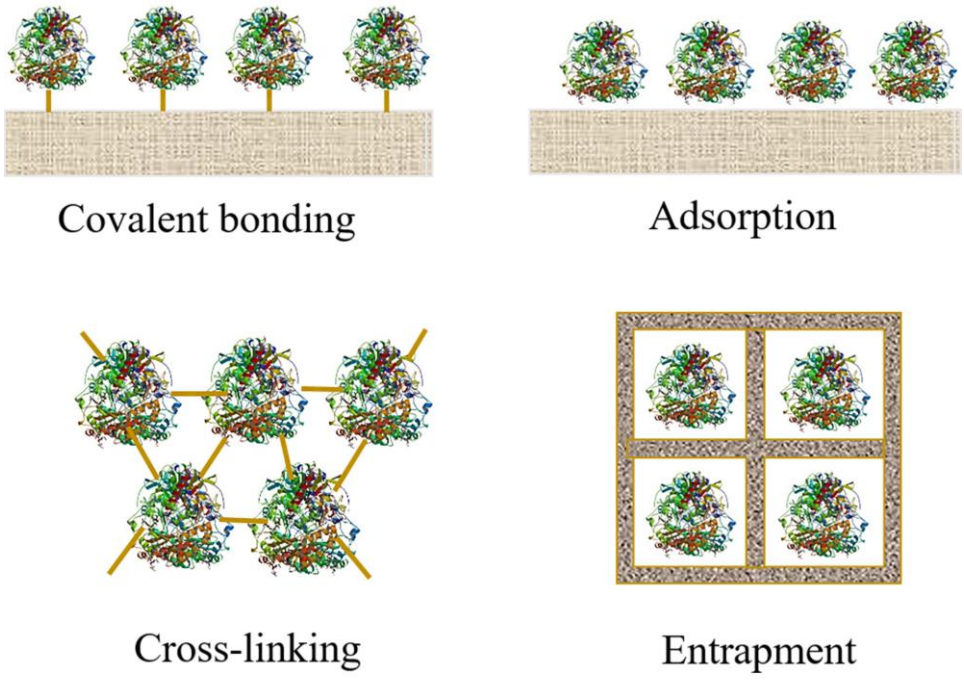


Figure 5 Illustration of immobilization methods of enzymes

I- A- 2- 6- Factors that influence the enzymes activity

Most enzyme function in mild conditions, thus an optimization of the medium conditions should be considered in order to prevent the denaturation of enzymes and the loss of their activity. The most important factors to consider are temperature, pH and the concentration of the enzyme's substrate.

The pH of the medium can affect remarkably both the shape and activity of enzymes. The interactions between the amino acids can change with severe pH change, which leads to the change of the 3D folding of the enzyme, and thus a change in its structure and activity. As for glucose oxidase (1.1.3.4), the optimum pH is 5.5 with broad activity range between pH 4 – pH 7 [77,78].

High temperature accelerates reactions catalyzed by the enzyme to a certain point, after that the activity decreases remarkably. This is mainly due to the denaturation of the enzyme's protein, causing an irreversible loss of the enzyme activity. As the temperature increases, so does the movement of protein molecules that leads to cleavage of the hydrogen bonds that maintain the folded 3D structure of the protein. Consequently, the protein chain unfolds, and the active site is no longer available for interaction with substrate [79].

Unlike simple chemical reactions, in enzymatic activity, the rate of product formation with increasing concentration of substrate, is non-linear. Many enzymes follow the Michaelis - Menten model for the enzymatic kinetics, which can be described according to (Eq. 5).

$$V = \frac{V_{max}\,[S]}{K_m + [S]} \qquad (5)$$

Where V is the initial velocity or rate of reaction (Mole/time), [S] is the molar concentration of the substrate, V_{max} is the maximum velocity attained with high substrate concentration when all the active sites in enzyme molecules are occupied, and K_m is Michaelis - Menten constant [77].

K_m constant is not affected by the amount of enzymes, but rather by the inverse affinity between the enzyme active site and the substrate, or it can be sometimes referred to as the binding index [79].

I- A- 4- Applications

Immobilization of redox enzymes on conductive materials and textiles is a method that can be used to produce flexible equipment in different sizes for various applications. It can be used to fabricate

biosensors or biofuel cells (BFC) with promising applications in many fields like medicine, environment, energy production and pollution control.

Power generation through enzymatic biofuel cells is considered as a main application in this field. Biofuel cells are considered as energy conversion devices based on bio-electro-catalysis like redox enzymes [24]. In classical biofuel cell design, the cell contains basic components that are the anode, the cathode, a proton exchange membrane and external circuit to connect the electrodes. Redox enzymes are immobilized on the electrodes with or without a mediator, and the fuel solution is added.

The fuel is enzymatically oxidized at the anode, producing electrons, which are then transferred from enzyme active site to anode surface and then to the electrical wires connecting with cathode. At the cathode, oxygen reacts with electrons and protons, generating water or other products. Many studies have been focusing on improvement of BFC performance regarding power density and stability which can be found in details elsewhere [23,24,80]. Bi-scrolling process was used to produce conductive polymer-based yarn electrodes that can be woven for immobilization of glucose oxidase and bililrunium oxidase [81]. Another study reported mechanical pressing of the enzymes (glucose oxidase (GOx) + catalase on anode and laccase on cathode) with carbon nanotubes inside Dacron® fabric, and this cell was successfully implanted in rats [82]. Wearable BFC have also been reported, such as a cell with immobilized lactate oxidase which generate power from human sweat [83].

The other major application in this field is biosensors, with remarkable impact in the medical field for detecting and curing diseases like diabetes and cardio-related disease related to cholesterol. Research was driven by the need of accurate, reliable and affordable biosensors for hospitals and domestic use. Detection of pollution was also a force of drive for biosensors development and especially for heavy metals that have toxic and fatal impact on most living organisms. The advances in biosensors are discussed critically in several reviews [20–22,26,27,84–90]. In addition to detection process, the use of redox enzymes in degradation of pollutants is an equally important application, which is discussed in section (**I-B**).

I- A- 5- Limitations of enzyme immobilization

There are some limitations to enzyme immobilization on all carriers including ones with limited conductivity, high cost of some types of enzymes that hinder their use on pilot scale, since they can rise additional cost of operations along with the carriers' costs. Furthermore, immobilization may cause limitation in diffusion, mass transfer and cofactor loss [91]. However, the most specific limitation of immobilization on conductive supports is electron transfer limitations between enzymes and surface of supports which leads to lack of efficiency in applications overall.

Additionally, immobilization of enzymes often results in a slight reduction of enzymatic activity compared to their free state. This can be caused by many reasons some of which are listed below.

- ***Denaturation of enzyme*** can be caused by several factors such as harsh conditions of the immobilization process or the end application. Changes in pH, temperature or pressure can cause damage to the intermolecular bonds between the protein chains of the enzyme, causing deformation of 3D folds that form the active site. With the loss of more active sites, enzymatic activity will be totally or partially lost.
- ***Inappropriate confrontation*** of enzymes of the support material surface, which mainly results in blocking the active sites, and makes the accessibility of the substrate harder or impossible.
- ***Inhibition of enzymes,*** which is mainly caused by certain materials depending on the type of enzyme. Heavy metals in general are considered as main inhibitors for many enzymes including mercury and cadmium. Scientists however took advantage of this phenomenon to produce biosensors based on inhibition of enzyme to detect pollution [27].

In addition to previously mentioned reasons, the loss of enzyme units into the surrounding medium away from the support or carrier may occur due to ***leaching***. Although the leached enzyme may still be active, it will not be in direct contact with the carrier and its recovery from the medium will be hard to achieve. Leaching may cause a considerable loss in output in case of immobilized redox enzymes on electrodes, since electron transfer will be limited in absence of mediators. Leaching occurs due to weak bonding between enzyme and support, such as in the case of physical adsorption. This phenomenon is mainly noticed in case of hydrogen or electrostatic attachments.

I- B- State of Art on use of enzymes for degradation of pollutants

Every year, the demand of fresh water increases due to growing industries and increasing population. Small and medium scale industries, such as textile dyeing and tanneries plants, release toxic organic and inorganic pollutants into local waters in many parts of the world [92]. Dyeing, finishing and wet processing of textile materials require large amounts of both fresh water withdrawal and wastewater disposal [93], which make the textile industry in general one of the most pollution-causing industries to local waters and surroundings especially in developing countries where lack of regulations permits such practices. Studies focusing on wastewater treatment using different approaches, like physical, chemical and biological, have been used separately or combined, on large industrial scale to treat domestic and industrial effluents. These include coagulation, flotation, adsorption on active materials, separation process via membrane, aerobic/anaerobic biological treatment, and combination of physicochemical and biochemical processes [94]. Additionally, different methods of modifying the properties of carbon and bio-sourced waste were reported in order to be used to purify and treat wastewaters from variety of sources. These methods include flushing followed by calcination in humid nitrogen atmosphere for the modification of activated carbon fibers, activating carbon obtained from animal bones using H_2O_2 solution and carbonization at 800˚C for 3 h, and producing composites from banana peels with chitosan [95–97].

The use of different enzymes for degradation of pollutants in wastewater as a bioremediation approach has been studied since these methods might have a lower carbon footprint than physical or chemical approaches [98]. Enzymes (either immobilized or in their free state) such as lignocellulosic enzymes, bilirubin oxidase, laccase, horseradish peroxidase, glucose oxidase, and keratinase were used for this purpose. The removal of color and degradation of pollutants in wastewater from landfill leachate, dyes from textile industry, pharmaceuticals compounds and phenols were reported using those enzymes [99–110].

In the following paragraphs, the combined methods using chemical and biological approaches have been discussed including Fenton-based treatments, with the focus on the use of enzymes and microbial consortiums in these methods, especially for bio-Fenton and bio-electro-Fenton processes.

I- B- 1- Advanced oxidation process through Fenton's reaction

Different treatments based on Advanced Oxidation Processes (AOPs) have been used since 30 years for effluent treatments such as; ozone-based, hydroxyl-based, Fenton-based, UV-based, and sulfate radical-based AOPs [111–115]. Many studies and literature reviews were published focusing on the advancements and different methods used nowadays in industry [44,94,111,116–123].

One of the most reliable methods used in wastewater treatment is Fenton-based treatments. This classic chemical reaction is one of the most efficient AOPs. Fenton reaction occurs between ferrous ions and hydrogen peroxide, which result in the formation of hydroxyl radicals that is a strong oxidant (E^o = 2.8 V) and capable of degrading the organic pollutants (Eq. 6-7).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad (6)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^+ \quad (7)$$

In fact, this reaction occurs in relatively short time, and the radicals are produced without any energy consumption, while the reagents are available at reasonable prices. These advantages made this process heavily used in wastewater plants for a long time. However, this method has some drawbacks like the need of pH control to maintain the acidic range of pH 2-3, in addition to Fe^{2+} ions which may be consumed faster than they are regenerated, slowing down therefore the process [124–126]. Moreover, the problem of iron sludge formation is another issue.

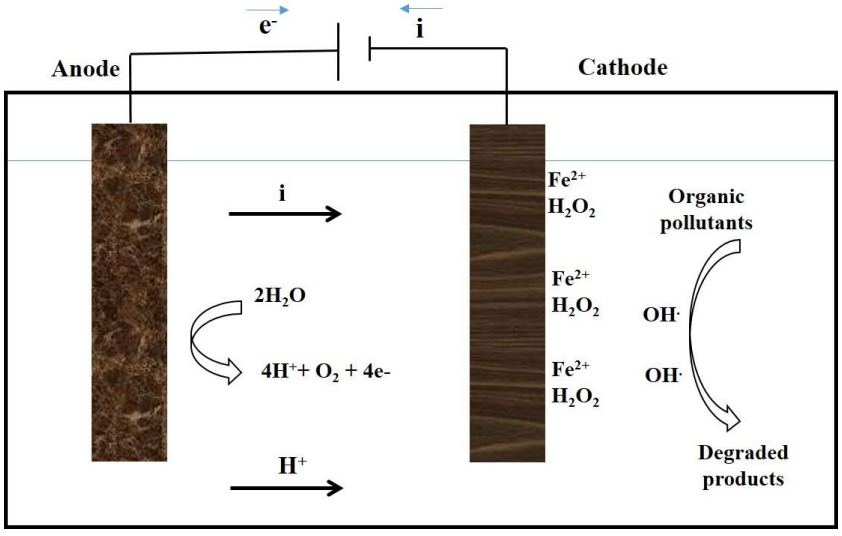


Figure 6 Electro-Fenton reactor setup

This classical reaction was the basis of many varieties of methods used for wastewater treatment like electro-Fenton, photo-Fenton and sono-Fenton, which rely on the electrochemical, radiation or sonication stimulation to produce the hydroxyl radicals, respectively. Many studies and critical reviews focused on these methods [127–134].

In electro-Fenton process, an electrical generator is used in order to facilitate the reduction of dissolved oxygen in the water at the surface of cathode and form H_2O_2 in order to participate in Fenton's reaction [127–129]. Simultaneously water will be oxidized at the surface of anode (Figure 6).

This method has disadvantages of corrosion of the electrodes used with time, and the electrical power consumption (approximate range between 5 – 70 $A.m^{-2}$ [135]). These disadvantages affect the cost of the process significantly. The details for treating different effluents using this method are described [129].

However, the most recent advances in terms of Fenton-based methods for wastewater treatment are bio-Fenton and bio-electro-Fenton, which are more explicitly described in **Paper I** [77].

I- B- 2- Bio-Fenton (BF) method using free or immobilized enzyme

This method is achieved by using enzymes that are able to catalyze reactions to produce hydrogen peroxide as a main or by-product, in the presence of bio-sourced materials and iron ions, Fenton's reaction can occur in the medium (Eq. 6-7). Research work in this field started only about ten years ago, in 2010, using free enzyme or immobilized enzyme. The main advantage of this approach is the sustainable *in-situ* production of H_2O_2. That can decrease the danger of H_2O_2 storing and transport, which reduces the chances of accidents and improves the safety in work environment. Furthermore, this method requires low power consumption, which can contribute remarkably to the reduction of financial expenses.

This method was used recently in wastewater treatment, for treating effluents from textile industry, like dyestuff of Malachite Green and Acid Blue 113 [136,137]. Glucose oxidase enzyme was mostly used in this process due to its ability to catalyze the oxidation of D-glucose, and as mentioned earlier

hydrogen peroxide is released as a by-product of this reaction (Eq. 8). Hence, if ferrous ions are present in the medium, Fenton's reaction takes place.

$$C_6H_{12}O_6 + O_2 + H_2O \xrightarrow{GOx} C_6H_{12}O_7 + H_2O_2 \qquad (8)$$

BF method can be achieved using simple tools, without the need of specific setups. Erlenmeyer flasks were used in literature to contain the BF mixture (enzyme, dyestuff, substrate and iron source) with or without heating and stirring [136–138].

The previous studies were performed using a free-state enzyme or immobilized on a volcanic material as heterogeneous catalyst. Regardless of their efficiency in effluent treatment, additional steps might be necessary to denature the enzymes in the mixture post-treatment. This loss of biocatalysts leads to increase in the costs of operation. Hence, an immobilization process of enzymes is expected to enable reusability and fast extraction of biocatalysts from the reaction medium post-treatment [77].

I- B- 2- 1- Influencing Factors on the efficiency of BF process

Several parameters affect the efficiency of the BF process. Concentration of biocatalyst, of ferrous ions, of substrate and of pollutants will have an important impact on the degradation of pollutants. Moreover, the conditions for best stability, and maximum activity of the GOx enzyme (temperature of 40 °C and pH 5.5), should be taken into consideration. However, the pH suitable for the Fenton reaction should be considered. Fenton's reaction is affected notably by pH values, and according to studies, pH 3.5 is considered as an optimum value [139]. This might be due to the stability of H_2O_2, which is better in highly acidic conditions. On the other hand, at high pH values, $Fe(OH)_3$ starts to precipitate and hence, oxidation rates decrease since less $OH^{\bullet}$ are available [140]. Thus, a compromise between these two values should be held in BF process to obtain the maximum pollutant degradation efficiency. These have been extensively described in **paper I** [77].

I- B- 3- Bio-electro-Fenton (BEF) using bio-anodes

This method is a result of modified electro-Fenton setup for wastewater treatment (see **1- B- 1**). In bio-electro-Fenton (BEF) setups, electrons are released due to the biological activity in anodic compartments (Figure 7). These electrons are transferred afterwards through the conductive anode material to the cathode by an external electrical circuit similar to the conventional Microbial Fuel Cell (MFC), which depends on bioactivity of microbial consortium in the anodic chamber to generate electrical power. In cathodic compartment, hydrogen peroxide is continuously generated by reduction of oxygen on the cathode material, and ferrous ions (Fe^{2+}) are *in-situ* generated by reduction of iron materials existing in the cathode chamber or directly on cathode material (Eq. 13-14). Thus, hydroxyl radicals ($HO^{\bullet}$) are generated sustainably *in-situ* by Fenton's reaction in the cathodic compartment to achieve advanced oxidation for different effluents.

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \qquad (13)$$

$$Fe^{3+} + e^- \rightarrow Fe^{2+} \qquad (14)$$

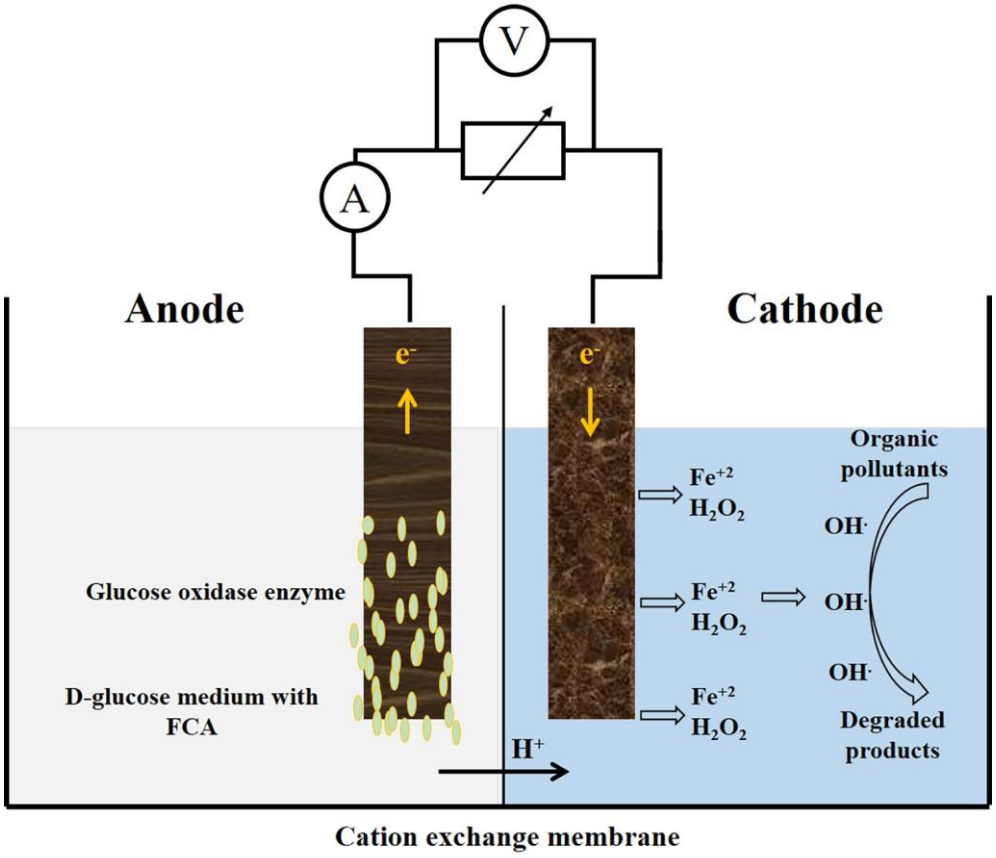


Figure 7 BEF reactor design

In addition to the previous mentioned advantages (see **I- B- 2**), BEF setups permit *in-situ* generation of ferrous ions (Fe^{2+}) needed in Fenton's reaction, which can be included in composite cathodes. This may reduce the amounts of excess metals and chemicals used. Moreover, no power consumption is needed in these setups, since this process is self-dependent and generation of power is achieved by biological activity of enzymes or microbes in anodic compartments [129]. Recent studies and reviews were published about the BEF process for wastewater treatment [77,141–145].

I- B- 3- 1- Materials used in BEF systems

The choice of electrode materials is a crucial step for efficient BEF process. These materials should possess many qualities like, stability, good electrical conductivity and resistance to corrosion in aqueous mediums [139]. Moreover, the chosen material for anodes should be biocompatible to achieve maximum adherence with microbial population or enzymes, and finally it should provide large surface area in order to allow more contact with the bioactive species [43,146].

Anodic compartments

The materials used for anodes in BEF setups were carbon-based materials mostly, carbon felts assisted with granular graphite [147,148], carbon felts [56,140,149–153] , modified carbon felts [146], carbon felts chemically modified using polyaniline deposition or nitric acid activation [154], graphite-based [47,155–158], and carbon brush [46]. These used materials possess good electrical conductivity and stability against electrolytes and solvents. The materials with nonwoven structure possess remarkably high specific surface area when compared to their volume, that helps to increase the contact area with the biologically active catalysts or microbes used in anodic compartments, thus contributes to increasing the electrical output of the setup.

Different types and sources of microbial populations have been used in anodic compartments of MFCs used in BEF setup in literature, such as activated *Shewanella decolorationis S12* [146,149], anaerobic seed/sludge microbial population [147,148,153,155,158], *Saccaromyces cerevisiae* [154], and sludge from dairy wastewater [140,152]. The anodic solutions contained mixtures of nutrients for the microorganisms such as glucose, vitamins and acetate based buffers. Furthermore, pH and

temperature used were in favor of microbial growth, and were sometimes separated from the cathodic compartment via membranes.

Cathodic compartments

The cathodes used in BEF setups have big impact on the efficiency of the cell and it should be made of materials similar to those used for anodes. Reported materials used in literature included graphite rods [156], polypyrrole/ Quinone (PPy/AQDS) modified carbon felt [146], carbon paper and carbon felts [154]. In these previous studies, a source of iron was added to cathodic compartments to achieve the oxidation by Fenton's reaction. However, composite materials bearing iron materials within the electrode itself were reported in other studies. Different combinations were used such as: $Fe@Fe_2O_3$/carbon felt [47,147,148,150,159], carbon felt /ɣ-FeOOH [56], carbon nanotubes/γ-FeOOH [149], graphite coated with pyrrhotite [151], and $Fe@Fe_2O_3$/graphite [157,158]. When these composite cathodes are introduced to aqueous mediums, iron ions start to leach slowly, hence, ferrous or ferric ions are released into cathodic compartment in a sustainable manner. The protocols used to produce such cathodes are discussed in details in **Paper I** [77].

I- B- 3- 2- Reactor design used in BEF process

Different cell designs were used in literature, in attempts to achieve one-step wastewater treatment process via BEF. Unlike BF method, reactor's design showed to be crucial to the success of Advanced Oxidation in BEF process. Mainly, the goal is to generate electrical power from the biological activity in the anodic part of the reactor, then to use this power to stimulate *in-situ* generation of Fenton's reagents Fe^{2+} and $OH^{\bullet}$, to treat different effluents in the cathodic compartment. For this purpose, certain configurations were used.

The most used configuration in literature is a dual-chamber MFC, which is made from different materials including plexiglass, glass, polycarbonate and polymethyl methacrylate [56,140,146–149,151,152,154,156–159]. The chambers were separated by proton exchange membrane in most cases. Moreover, these setups worked mostly in batch mode with an effective volume of each chamber ranged between 75 - 600 mL. Other studies reported the use of different membranes, continuous feeding modes, dual reactor cell with only a single-chamber MFC as a low-voltage power

source, or MFC-assisted-anodic Fenton treatment system, with an anodic Fenton reactor and a two-chambered air-cathode MFC [46,150,153,155,160]**.**

I- B- 3- 3- Main factors affecting the bio-electro-Fenton process (BEF)

BEF setups are complex with many components and elements that are influenced by various conditions. Hence, these factors must be considered when using BEF method to obtain good results in degrading organic pollutants from wastewater.

Presence of H_2O_2

In a similar manner to its influence of BF process, the *in-situ* generation of H_2O_2 is crucial for Fenton reaction to occur, since it can react with Fe^{2+} to form $OH^{\bullet}$, which in turn can oxidize the organic material in effluent solutions. Studies confirmed that the efficiency of BEF setups is mainly affected by this factor, and the degradation efficiency increases with increased concentration of hydrogen peroxide [157,159]. In these studies it was noticeable how the degradation efficiency of Fenton in cathodic compartment decreased as the molar ratio of H_2O_2 to total iron ions decreases, due to lack of active radicals $OH^{\bullet}$. Furthermore, the direct addition of H_2O_2 to the medium enhanced the current density obtained in another study. However, the excessive amounts of hydrogen peroxide may result in undesirable reaction to take place, and a loss of active $OH^{\bullet}$ can be noticed.

Concentration of ferrous ions

Generally, the increase in Fenton's reagents has a proportional increase on the rate of pollutants degradation, due to the increased amount of formed $OH^{\bullet}$. This can be true up to a specific value of Fe^{2+} concentration (can be determined experimentally). Above this point, the unused iron ions will accumulate and contribute even more to the increase of total quantity of dissolved solids in wastewaters [161]. In addition, and as mentioned for the BF process, excessive ferrous can participate in undesired reactions that lead to the consumption of $OH^{\bullet}$.

It is worth mentioning that, to solve these issues related to the use of ferrous catalysts (Fe^{2+}) in BEF reactors, such as instability and iron-chelating, the use of Mn^{2+} as an alternative to Fe^{2+} was reported based on the following reactions occurring in the cathodic compartments (Eq. 15 -17).

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \qquad (15)$$

$$Mn^{2+} + H^+ + H_2O_2 \rightarrow Mn^{3+} + OH^{\bullet} \quad (16)$$

$$Mn^{3+} + e^- \rightarrow Mn^{2+} \quad (17)$$

The study showed that Mn^{2+} ions were not effective in COD removal of the treated effluent with no change detected before and after treatment with Mn^{2+}, compared to 40% of COD removal after 4 h when Fe^{2+} ions were used instead. Furthermore, the power density obtained was much higher in case of Fe^{2+} as a result of ohmic polarization which reduced the effect of Mn^{2+} ions and minimized the movement of electrons [140].

Temperature

Most of the studies reported experiments held in the range between ambient temperature or around 30 °C [46,56,146–149,154]. The temperatures are mainly chosen in favor of microbial growth and biological activity. Moreover, the kinetic constants of Fenton's reaction occurring in the cathodic compartment showed to increase almost 3 times when temperature increased from 15 to 30 °C [162].

pH of the solution in cathodic compartment

As previously mentioned, the optimum pH value for Fenton's reaction is around 3, since the generation of $OH^{\bullet}$ radicals is most effective at acidic conditions as well as the stability of H_2O_2 increases at low pH values [111]. However, the use of neutral pH is interesting because a maximal chance of mineralization of organic pollutants [146]. Furthermore, this reduces the steps of process by eliminating the pH control of the effluents. Many studies were reported working in neutral pH in the cathodic compartments, while other works were conducted under a pH range of 2.7- 3 [47,56,146,147,149–151,156].

Power density

In BEF setups, power density that is generated from the biological activity showed to be an influencing factor on the performance of the reactor overall. With higher power generation, the reduction of oxygen at the cathode increases, leading to increased generation of H_2O_2, and hence higher level of the organic materials degradation is expected. Many studies reported higher rates of pollutants degradation with higher power density running through the BEF setups, in case of effluents that contained Orange II dye, Rhodamine B dye, and Arsenite [56,146,150].

Mass transfer

An interface area is needed between microbial population that are adhered to electrode and the solution with the nutrients in order to achieve the bioactivity and the release of electrons. This process is diffusion-controlled, which results in complex pathways for electron transfer and some limitations related to mass transfer. In batch-mode BEF reactors, a decline in power density could be assigned to the limitations of mass transfer of substrate to reach the microbes on the surface of electrode [163]. Moreover, the depletion of the nutrients in the medium also leads to decrease in the generated power density versus time in the anodic compartments [164]. It is important to mention that proton exchange membranes between the two compartments of the BEF setups also contribute to the mass transfer limitations if they are not fast enough, this may cause gradual change in pH value of the anodic compartment, and affect both the power generation and microbial population [152]. In attempts to minimize the negative impact of mass transfer, it is recommended to use continuous-feeding modes for BEF setups rather than batch-mode, to decrease the electrical resistivity of electrodes used in order to facilitate the electron transfer process, as well as to the sufficient addition of mediator when used.

Other factors

There are other influencing factors on BEF setups and process overall, such as the material used to fabricate the electrodes, since the higher the conductivity the better the degradation of pollutants was reported [146,154]. The external resistance applied to the system. In general, it is preferred to use a moderate-value external resistance to obtain better degradation rates for pollutants [148,156]. This may be because under very low or very high external resistance, the conditions in the reactor become unsuitable for bacterial growth, and this may affect the electron acceptance for the treated pollutant. In addition, removal rate of the pollutants increases with the increased time of the treatment [150,156].

Pollutant degradation has been achieved by heterogeneous catalysis using redox enzymes in their free-state or immobilized on non-conductive carriers, which nevertheless leads to color removal but a non- complete degradation of pollutants with possibility of toxic colorless degradation product

formation [165]. Using immobilized GOx enzyme, in presence of iron ions (bio-Fenton), for Fenton-like degradation of pollutants, is appealing since more reactive oxidative hydroxyl radicals can lead to a more complete degradation of organic pollutants. Lastly, when a conductive carrier is used for the GOx enzyme immobilization, for use as bio-anode in bio-electro-Fenton process, pollutant degradation can take place with simultaneous energy production.

It is thus important to study the use of methods that can bring stability and increased bioactivity to immobilized GOx enzymes for use in bio-Fenton and bio-electro-Fenton processes.

I- C- State of Art on eco-technologies and products used in this study

Eco-technology

Eco-technology is a term that has been use since 1970s, but only recently, a universal definition was proposed by N.R. Haddaway *et al.* to describe this term across disciplines under the general concept of "*at least do no harm*".

It was defined as the following "*Eco-technologies are human interventions in social-ecological systems in the form of practices and/or biological, physical, and chemical processes designed to minimize harm to the environment and provide services of value to society*" [166].

Hence, the latest advances in three eco-technologies that have chosen in this work to facilitate the immobilization of redox enzyme on conductive textiles will be highlighted.

These technologies are plasma treatment, biodegradable and biocompatible coating with PEDOT:PSS polymer blend, and crosslinking with bio-based less toxic agent -genipin.

Each of these technologies contribute to one or more of following attributes: decreasing the amount of added chemical and solvents, minimizing treatment time and energy, reducing health hazards, reducing waste and wastewater production, and minimizing the environmental impact overall.

I- C- 1- Plasma technology

Plasma is the fourth state of matter; it is composed of ionized gas and contains an equal amount of negative and positive charged particles. It consists of ions, free electrons, radicals and UV radiation. Generally, plasma can be created by subjecting a gas or gas mixture to energy such as heat or electric/electromagnetic field leading to ionized gas state formation, which can conduct electrical currents. There are different methods used to create artificial plasma:

1- Plasma created by subjecting gas to high frequency electromagnetic field, such as "Torch plasma".

2-Plasma created by excitation of gas between two electrodes, such as vacuum and atmospheric plasma. These two types of plasma differ according to the material of electrodes used, either metallic electrodes like Corona, or electrodes are covered by dielectric barrier like ceramic or silicon that we can refer to it as Dielectric Barrier Discharge (DBD).

3- Plasma created by excitation of gas by electrodeless microwave discharge with high frequency (2450 MHz), like Cold Remote Plasma (CRP) which is the type of plasma used within the frame of this thesis.

Plasma treatment is considered as an eco-technology, since it is capable of functionalizing different materials in a dry state without added chemicals and in relatively short time. Unlike wet and chemical processing, plasma does not require the use of solvents or water and it is a nondestructive technology overall. By using different elements and gases, different functionalities can be acquired. In the last decade, several types of plasma have been used to treat textile materials including conductive fibers, using a variety of gases to increase or decrease wettability of textiles like PET, cotton, wool, silk, aramid, acrylic and PA6. It was also used to increase fiber-matrix adhesion in composite materials and to improve energy efficiency in flow batteries, through integration of functional groups at their surfaces [58–61,167–174].

The other important use of plasma technology is the synthesis of plasma polymers with unique properties. These materials like plasma polymer films can be attractive in coatings with improved adhesion and highly cross-linked structures. This technique was used in order to produce a surface

rich in free radicals [175], and for deposition of silicon oxide as coatings, which is a good hydrophobic treatment of silk [176].

Many varieties of plasma treatments of textiles were reported in literature such as: non-thermal plasma, atmospheric pressure plasma, arc plasma deposition, Corona, dielectric barrier discharge, low-pressure plasma, cold remote plasma and atmospheric pressure plasma jet [177].

The gas used in these plasma treatments differs according to the required function obtained.

Air, N_2, He, O_2 and Ar were mostly reported in literature for increasing wettability, while gases like CF_4, C_3F_6, $C_6H_{18}OSi_2$ and SF_6 were mostly used for decreasing wettability [177].

Another major application for plasma treatment in textile is modification of surface micro roughness for better adhesion in composite materials and finishing agents. Other uses of plasma in desizing, dyeing, printing and finishing of textiles have been also reported [176,178–180]. In these applications, plasma revealed to be an efficient alternative for chemical pretreatments of textiles that improved outcome properties with lower environmental impact. Considering inkjet printing method for cotton textile, the studies showed an increase in color yield even after washing, in addition to improved outline sharpness, a color fastness and anti-bacterial effects after plasma [181].

Numerous types of plasma were used as pretreatments for textile materials like PET and cotton in order to have better adhesion with electrically conductive coatings like graphene oxides and polypyrrole [182–186]. Another approach was to deposit the conductive layers via plasma technology.

Electrically conductive materials like Ni-Cu textile or films with silver nanowires and graphene were reportedly treated with some types of plasma to enhance conductivity such as reactive-ion etching and atmospheric pressure plasma jet [187,188]. Carbon materials such as carbon fillers and graphene-based fibers were treated with low-temperature and atmospheric plasma in order to enhance the super-capacitive properties and the overall performance of these composites [189,190].

CRP has been chosen in this study for several reasons. Some have the ability to treat electrically conductive materials without the hazards of sparkling, which might happen as in some cases of atmospheric plasma. The sample to be treated is maintained in a treatment chamber far from discharge and species like free radicals will reach the samples without the direct exposure to

electrons. In addition, CRP has the ability of treating thick samples on both sides at the same time. This type of plasma was used efficiently as well with non-conductive materials; it was used as a pretreatment for enzyme immobilization using non-conductive PET textile nonwovens where the enzymes maintained better activity using CRP treatment when compared to atmospheric plasma [191]. The properties of this plasma treatment will be discussed in more details in **Chapter II**.

I- C- 2- Biocompatible and biodegradable conductive coating with PEDOT:PSS

The most interesting breakthrough of poly-thiophenes has been the synthesis of poly(3,4-ethylenedioxythiophene (PEDOT), its properties of transparency, flexibility, good electrical conductivity, solvent stability, biodegradability and biocompatibility made it a landmark in material science [192]. Its widely available form is doped with polystyrene sulfonate known as PEDOT:PSS (Figure 8).

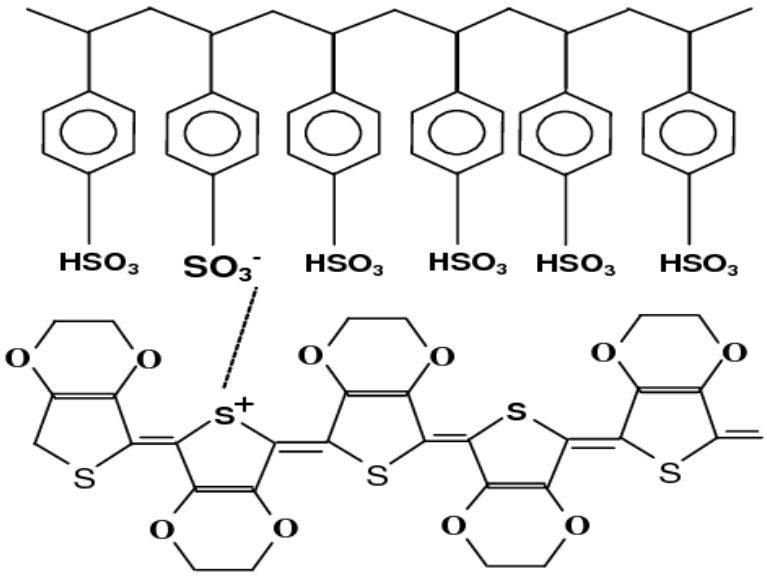


Figure 8 Chemical structure of PEDOT:PSS

This blend of two ionomers consists of PSS as a sulfonated polystyrene, it carries a negative charge because a part of sulfonyl groups is deprotonated, and the second part is PEDOT as a conjugated polymer that carries a positive charge. This blend is mainly available as aqueous dispersion of colloidal gel particles [193]. PEDOT:PSS is mainly used nowadays as a conductive

coating with thermal stability, flexibility and biocompatibility for textiles and other surfaces [194,195].

It is considered as an eco-friendly organic material in its category, due to its biodegradability and biocompatibility that makes it an interesting substitute to conductive metals [196].

According to literature, PEDOT:PSS has a big potential as biocompatible matrix for immobilizing of species with bioactivity or molecular recognition ability, which make it a good candidate for medical and biological devices [192]. Furthermore, its biocompatibility added to its electrical conductivity and capacitance are attractive properties for implantable devices and bioelectrodes [197]. The properties of PEDOT:PSS hydrogels and its behavior in humid conditions were the subject of studies to gain more understanding about its potential [198,199].

The fabrication of a biocompatible conducting micelles, that are self-assembled from PEDOT:PSS and chitosan via electrostatic interaction, has been reported [200]. In addition, a self-assembled tough elastomeric composite hydrogel that is conductive and biocompatible, made from polyurethane with PEDOT:PSS and liquid crystal graphene oxide has also been reported [201].

The use of PEDOT:PSS in the textile has become a mainstream for composite and smart textiles, since it has the flexibility to bend and stretch. For instance, threads have been dip-dyed in PEDOT:PSS to develop a wearable temperature detection sensors [202], and cotton fabric was also coated with this blend in order to exhibit a metallic behavior [203]. Furthermore, the medical applications of textile based materials with PEDOT:PSS are the focus of many research teams. Divinyl sulfone crosslinker was used with PEDOT:PSS to produce wearable textile devices for health monitoring [204], while knitted fabric electrodes coated with PEDOT:PSS and silver were reported for electrocardiography monitoring [35].

Many other applications for PEDOT:PSS coated textiles were reported in literature such as superparamagnetic fabrics, energy harvesting fibers, conductive polyester fabrics, washable textile electrodes, triboelectric nanosensors on a textile [205–209].

Taking advantage of its biocompatibility, PEDOT:PSS was also used for bioelectric applications like biosensors, using immobilized enzymes such as glucose oxidase, urease, cholesterol oxidase and lactate oxidase [210]. Different chemical methods have been reported in order to modify

PEDOT:PSS with fillers including carbon black and carbon nanotubes using dopant materials such as dimethyl sulfoxide, ethylene glycol, and 1-butyl-3-methylimidazolium tetrafluoroborate among other materials [211–213]. Surface modification and oxidation of PEDOT:PSS blend have been also achieved using solvents such as methanol and surfactant to increase the electrical conductivity via chemical oxidative polymerization [214–218]. A recent study was reported for increasing the electrical conductivity of PEDOT:PSS by increased oxidation via electrode potential [219].

I- C- 3- Bio-based crosslinker "Genipin"

Genipin is a naturally occurring crosslinking agent, it can be obtained from *Gardenia jasminoides Ellis* fruit extract (Figure 9). This extract contains geniposide which can be hydrolyzed by bacteria to genipin [220]. Genipin showed to be an effective anti-inflammatory agent, in addition to its use as medicine to treat the type (II) diabetes in Chinese traditional medicine over years [221].

It is a biocompatible material, which makes it a good candidate in many environmental and medical applications. It was reported that genipin is about 5000 - 10000 times less cytotoxic than glutaraldehyde [222]. Its median lethal dosage LD_{50} i. v. is 382 $mg.kg^{-1}$ in mice which makes it so much less toxic than glutaraldehyde [223]. Recently, it has been extensively used in research for crosslinking of materials such as chitosan and gelatin for applications regarding medical and tissue engineering. Details about these applications are provided elsewhere [220].

Figure 9 Gardenia jasminoides Ellis plant and fruits

It was reported that only primary amines can react with genipin to form blue pigments, and that oxygen is essential for the blue pigment formation [224]. It is proposed that a spontaneous reaction between genipin and amino acids occurs which leads to the formation of an aromatic monomer. In a second step, intermolecular crosslinking may occur due to radical reaction [225]. Genipin is also capable of crosslinking proteins by crosslinking two free amino groups of lysine residues on protein macromolecular chains [226]. The chemical formula of genipin is shown (Figure 10).

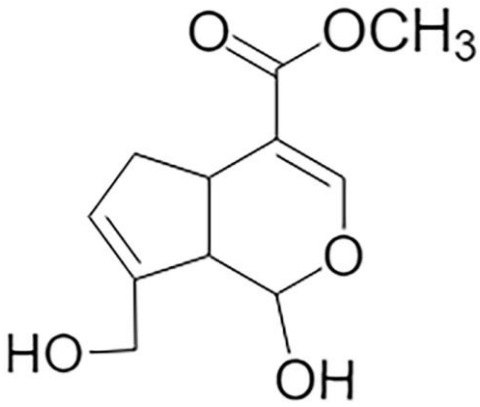


Figure 10 Chemical formula of genipin crosslinking agent

Genipin was used as crosslinking agent for chitosan to form a matrix for β-D-galactosidase immobilization in food industry [227], to crosslink chitosan to form hydrogels used in biomedical and pharmaceutical applications [220], and for extracellular matrix-derived cardiovascular scaffolds for treatment of these tissues [228]. It was reported that it was used as a crosslinker for biomedical purposes using different proteins and carbohydrates like gelatin, collagen, soy protein, alginate and chitosan [229]. Furthermore, it was used to crosslink gelatin to produce bio-adhesive with less cytotoxicity than formaldehyde crosslinked structures [230], and to produce a pH sensitive hydrogel from crosslinking chitosan and alginate for drug delivery applications [225]. A stable hydrogel network was developed using agar-kappa-carrageenan and genipin as a crosslinker for applications related to food industry [231]. Moreover, bioelectrodes were produced using genipin to crosslink chitosan with laccase enzyme in addition to carbon nanotubes and these electrodes showed to be stable and biocompatible in vivo for long-term energy supply from the body [232]. In addition, the

L929 cell adhesion was improved using the hydrogel derived from silk and genipin, which also formed blue pigments [233].The use of genipin is expanding in many industries including textile for its great potential of functionalizing surfaces, producing pigments and crosslinking materials to form tissues or fibers.

Chapter II
Materials and Methods

II- A- Materials

II- A- 1- Carbon Felt

The conductive textile nonwoven used in all chapters of this thesis is a commercial poly acrylonitrile (PAN) based carbon felt and it was provided by Ceramaterials (USA). The high relative surface area of the felt makes it a good candidate for enzyme immobilization process. Moreover, the porous carbon felt has a low-pressure drop and enables easy diffusion of reaction mixture and mass transfer. These nonwovens were baked at 1200 ºC during manufacturing and contain no resins or organic residuals on the surface. The properties of this product are shown in (Table 1).

Table 1 Carbon felt properties as described in product data sheet

Property	Value	Unit
Bulk density	0.15	$g.cm^{-3}$
Carbon content	> 97	%
Tensile strength	0.12-0.30	Mpa
Relative surface area	10-30	$m^2.g^{-1}$
Fiber average diameter	20	µm
Resin or epoxy	0	%
Thickness	3	mm

II- A- 2- PEDOT:PSS

PEDOT:PSS as Clevios™ or poly(3,4-ethylenedioxythiophene):polystyrene sulfonate was provided by Heraeus. This product is provided in the form of commercial blue aqueous dispersion and contains between 1 and 1.5 % solid, and may contain solvents. It is a substituted poly thiophene ionomer complex, with a polyanion (PEDOT) that provides electrical conductivity [234]. This product has a volume electrical conductivity up to 1000 $S.cm^{-1}$, good chemical and thermal stability. It is mainly used for the production of thin conductive flexible coatings as described by the manufacturer (Figure 8).

II- A- 3- Glucose oxidase enzyme (GOx) and activity kit

Glucose oxidase (GOx - EC (1.1.3.4)) from Aspergillus Niger type X-S G7141, phosphate buffered saline (PBS), potassium dihydrogen orthophosphate, iron sulphate hyptahydrate ($FeSO_4.7H_2O$) which is used in Fenton reaction, and Ferrocenecarboxylic acid (FCA) used in cyclic voltammetry test and BEF setup as a mediator for the enzyme were provided by Sigma-Aldrich (Germany). To measure the enzymatic activity of free or immobilized enzyme, an activity kit (K-GLOX 11/16) provided by Megazyme (Ireland) was used.

II- A- 4- Other reagents and products

- Remazol Blue RR dye was provided by Dystar (Germany).
- Genipin was provided by FUJIFILM Wako Chemicals Corp (USA), with purity > 98%.
- Chemical Oxygen Demand (COD) mercury-free vials were provided by CHEMtrics (USA) in the range of 0 - 15000 $mg.L^{-1}$.
- Nafion® proton exchange membrane (N 211) was provided by FuelCells Etc (USA).
- The glass used for the BEF prototype was provided by Adams & Chittenden Scientific Glass (USA).

II- B- Methods

II- B- 1- Dip-coating with PEDOT:PSS polymer dispersion

A diluted aqueous dispersion of PEDOT:PSS (blend of distilled water / PEDOT:PSS as received (1/1 in volume)) was prepared at (22 ±1 ºC) with constant stirring using a magnetic stirrer for 10 min. The (virgin carbon felt) VCF was then immersed in this solution once for 5 min, the samples then were shaken to eliminate excess PEDOT:PSS solution. The samples were left to dry in a dark room for 48 h at room temperature, before any further use. The dilution was necessary to reduce the viscosity, and hence reduce blocking of the nonwoven pores and maintain both the pores and high specific surface area in the original bulk.

II- B- 2- Cold Remote Plasma (CRP)

The setup of Cold Remote Plasma (CRP) used to treat the carbon-based materials is shown (Figure 11).

It is described as far post-discharge of plasma of a gas mixture (nitrogen and oxygen in this thesis). The zone of treatment is free of ultra violet radiations or charged particles. The main reactive species that exist there are atomic oxygen and nitrogen, and electronically and/or vibrationally excited nitrogen molecules. This type of plasma is characterized by a big deviation from thermodynamic equilibrium: the vibrational temperature of vibrationally excited nitrogen molecule is around 2500 - 3000 K , while the gas temperature is about the ambient [235].

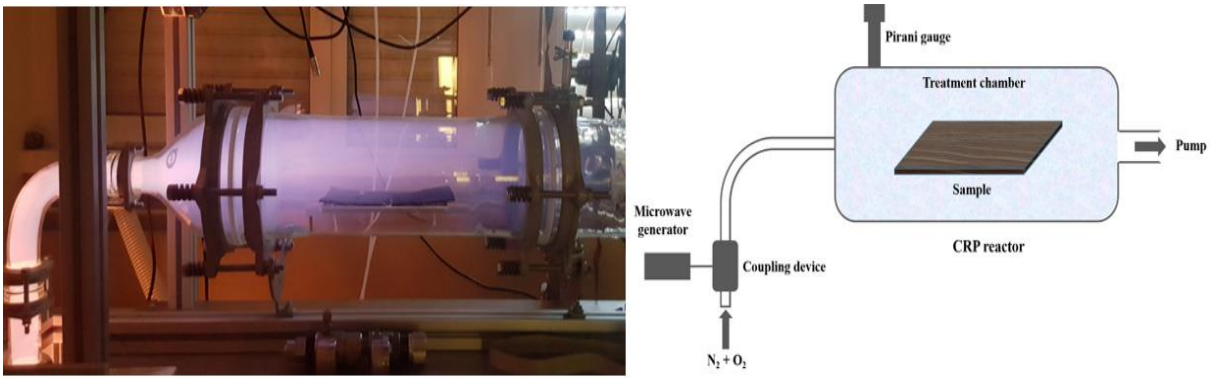


Figure 11 Cold Remote Plasma reactor

The gaseous flow of (N_2 + O_2) mixture, achieved by a continuous pumping by a mechanical rotary pump (33 $m^3.h^{-1}$). The gas flow was excited by an electrodeless microwave discharge (800 W - 2450 MHz) produced in a quartz tube with an inner diameter of 30 mm, coupled to a cylindrical Pyrex treatment chamber with a diameter of 150 mm and a volume of 15 L [236]. The distance between the treatment area and the discharge was 0.9 m. The treatment chamber is far from discharge and species like free radicals will reach the samples without the direct exposure to electrons, which facilitate the treatment of electrically conductive materials without sparkling that may occur in some cases of atmospheric plasma. In addition, the CRP has the ability of treating thick samples on both sides at the same time.

The pressure was measured using a Pirani gauge, and the gaseous flow was controlled by mass flow regulators. The total pressure was 4.5 hPa, and nitrogen flow rate was constant and equal to 2.5 standard liters by minute. The chosen ratios of O_2/ N_2 were 1 or 2 %. The carbon-based samples were settled in the treatment chamber, and treatment period was 15 min with the selected mixture [237]. The plasma treated samples were used after treatment directly, or stored in clean closed containers until use, in order to minimize the access of humidity and light that may accelerate ageing of the plasma treated samples.

II- B- 3- Surface characterization techniques

Fourier Transform Infrared (FTIR)

FTIR analyses were carried out using Nicolet NEXUS 670 FTIR with ZnSe crystal in transmission mode within the range of wavenumbers of 400 - 4000 cm^{-1} (64 scans and 4 cm^{-1} resolution). Background correction for CO_2 from air was performed, along with smoothing where needed to eliminate noise. Spectra are presented in stack mode and arbitrary units. For the crosslinking method, Nicolet 5700 by Thermo Fischer was used in the same settings, using pellets of KBr as carriers to obtain the spectra. In order to collect these spectra, dry powders of both GOx and genipin were used in KBr pellet, and droplet of the solution of (GOx 1 $mg.L^{-1}$ with genipin 60 mM) was placed on the KBr pellet via capillary to obtain the spectrum of the reactional mixture.

Scanning Electron Microscopy (SEM)

To obtain the SEM micrographs, field emission scanning electron microscope JSM-7800F was used with LED detector, accelerating voltage of 6 KV and carbon coating in the case of physical adsorption. While to obtain these micrographs in the case of crosslinking, HITACHI TM3030 was used with accelerating voltage of 15 KV and gold coating.

X-ray Photoelectron Spectroscopy (XPS)

In order to collect the XPS spectra, Kratos Axis Ultra DLD Spectrometer with monochromatic Al KαX-ray source (hν = 1486.6 eV), working at 120 W (12 kV, 10 mA) was used. The base pressure of the analysis chamber was 2×10^{-10} mbar. Survey scans were collected using an analysis area of ≈

300 µm × 700 µm at analyzer pass energy of 160 eV over 1200 to -5 eV binding energy range with a dwell time of 1 s and 1 eV step. High-resolution spectra were obtained with a step of 100 meV at 40 eV pass energy. Spectra were charge corrected to give adventitious carbon C 1s spectral component C- (C, H) a binding energy of 285.0 eV. Iterative measurements were performed as 25 scans for S 2p and 15 scans for N 1s. S 2p1/2 FWHM was fixed equal to S 2p3/2 FWHM and FWHM was limited to 2.6 eV. No smoothening was applied on spectra and a Shirley type background was used to fit the spectra, and OriginLab® was used for presenting the graphs.

II- B- 4- Water contact angle and capillary uptake measurements

In order to measure water contact angle greater than 90°, "Digidrop" was used for sessile drop method from GBX Instrument (France). In addition, for contact angle < 90°, the felts immediately absorbed the water droplets, and thus a wicking was performed with 3S scale tensiometer (GBX instruments), to measure capillary uptake and water contact angle. A rectangular piece of felt was used (3 X 5) cm^2, its top area connected to tensiometer at weighing position, and the bottom area was brought into contact with the surface of distilled water placed in a container (Figure 12).

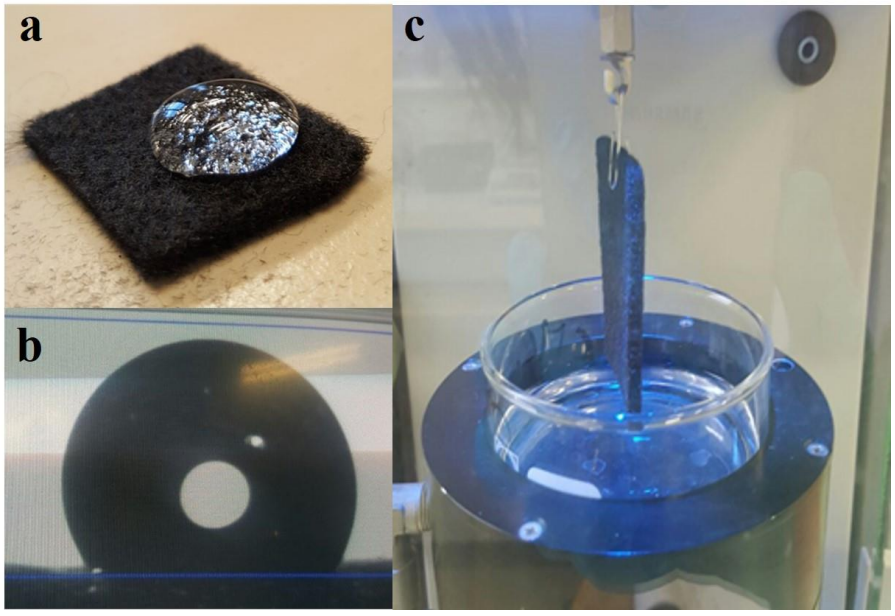


Figure 12 Macrograph of virgin carbon nonwoven felt (VCF) used in this study (a), sessile water droplet at VCF surface for water contact angle measurement (b), and the wicking test set-up (c)

When in contact with water surface, a sudden increase in weight (W_m) was taken, due to meniscus weight formation of nonwoven felts, and water contact angle then was calculated using (Eq. 18).

$$W_m \times g = \gamma_L \times \cos\theta \times P \quad (18)$$

Where W_m is meniscus mass (g), γ_L is surface tension of the liquid / water ($mN.m^{-1}$), P is sample's perimeter that is in contact with liquid (m), and θ is water contact angle of carbon felt (º).

The time of contact with water was set to 3 min. Capillary uptake was measured after removal of felts from water surface work [238]. It is worth mentioning that to determine the exact perimeter of each sample, n-hexane was used as a total wetting liquid with (θ = 0º, thus cos θ = 1).

II- B- 5- Pore size measurement test

To verify the effect of coating on the pores of carbon felt, TOPAS PSM 165 instrument was used. This pore size meter delivers results on the bubble point and pore sizes of different types of materials including nonwovens. Where the bubble point can be described as differential pressure value, at which the wetted sample is starting to become gas permeable, which can be converted to the diameter of the largest pore. While for the dry measuring, permeability is measured to identify the pore sizes (mean and median). The liquid used for wetting the samples as totally wetting liquid is Topor®, the maximum pressure used was 2000 mbar, and flow Rate 50 $L.min^{-1}$. The measurement was repeated three times for each type of samples.

The pore size changes play a significant role in mass transfer rates. It is important to keep the pores open to be able to exchange the substrate of the enzyme and the products of the reaction between surface of the fibers and the reaction medium. Hence, this test is necessary to evaluate the changes after dip-coating process.

II- B- 6- Bulk electrical resistivity measurement

The bulk electrical resistivity of dry samples was measured using the four-probe method. The four probes used for the measurement contacted the samples at points that lie in a straight line with a fixed distance between the probes of 1 cm, with a width of 2 cm and the thickness of the samples of

0.3 cm [239]. The probes were connected to Agilent 34401A Digital Multimeter. For each sample, an average of ten readings were recorded (Figure 13).

Resistivity was calculated as per the equation (Eq. 19), where ρ is the bulk resistivity Ω.cm^{-1}, w is the width of the samples (cm), t is the thickness of the sample (cm), s is the distance between the probes (cm) and R is the resistance measured (Ω).

$$\rho = \frac{\omega\tau}{s} R \qquad (19)$$

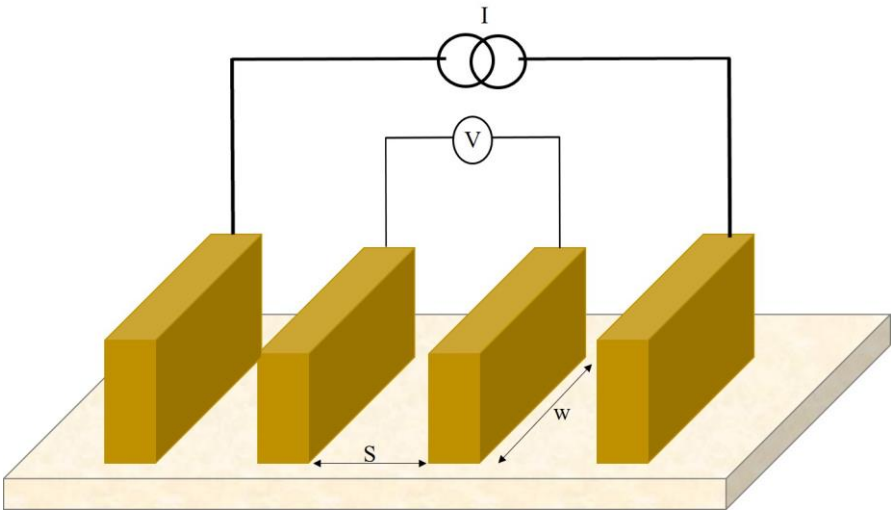


Figure 13 Arrangement of four-probe method used in this study

II- B- 7- Enzymatic activity colorimetric assay

Glucose oxidase enzymatic activity was measured taking into account the enzyme's ability to catalyze oxidation of β-D-glucose. H_2O_2 is released as a by-product of this reaction. The produced hydrogen peroxide enters in another reaction with p-hydroxybenzoic acid and 4-aminoantipyrine, in the presence of peroxidase enzyme (POD) which results in quinoneimine dye complex with pink color at 25 °C and pH 7 (Eq. 20 -21).

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{GOx} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \qquad (20)$$

$$2H_2O_2 + \text{p-hydroxybenzoic} + \text{4-aminoantipyrine} \xrightarrow{POD} \text{quinoneimine} + 4H_2O \qquad (21)$$

The colored complex absorbance intensity was monitored at 510 nm, using a JascoV-350 UV–Vis spectrophotometer for the physical adsorption parts, and Thermo Fisher Evolution 200 UV–Vis spectrophotometer. The absorbance intensity was recorded after 20 min, when 2 mL of POD mixture

was added to 0.5 mL of D-glucose (90 $g.L^{-1}$), and afterwards 0.5 mL of enzyme solution (1 $mg.mL^{-1}$ with active 0.0858 $U.mg^{-1}$) was added to the mixture. A calibration curve provided by the supplier was used, which allowed to quantify the number of enzyme unit per 0.5 mL of enzyme solution (Figure 14).

Where the unit of glucose oxidase is the amount of enzyme that oxidizes 1.0 µmole of β-D-glucose to D-gluconolactone and H_2O_2 per minute at pH 5.1 at 35 °C [19].

To verify the reusability of the samples, enzymatic activity assays were performed for multiple cycles as described previously, where one cycle assigned to conducting the activity assay for 20 min, followed by rinsing samples twice with phosphate buffer solution. The samples were then restored at 4 ºC for at least 24 h until the next cycle.

However, for the adsorption of GOx on 1 cm² carbon sample in 3 mL of enzyme solution, two pH values were used (pH 7 or 5.5). The activity of immobilized enzyme on 1 cm² was compared to the total activity of free enzyme (0.2574 U) presented in 3 mL, and "Relative enzyme activity %" after the immobilization was calculated as per the equation (Eq. 22).

$$\text{Relative enzyme activity (\%)} = \frac{\text{Activity of immobilized GOx } (\frac{\text{mU}}{\text{cm}^2})}{\text{Activity of free Gox (mU/0.5 mL) x 6}} \qquad (22)$$

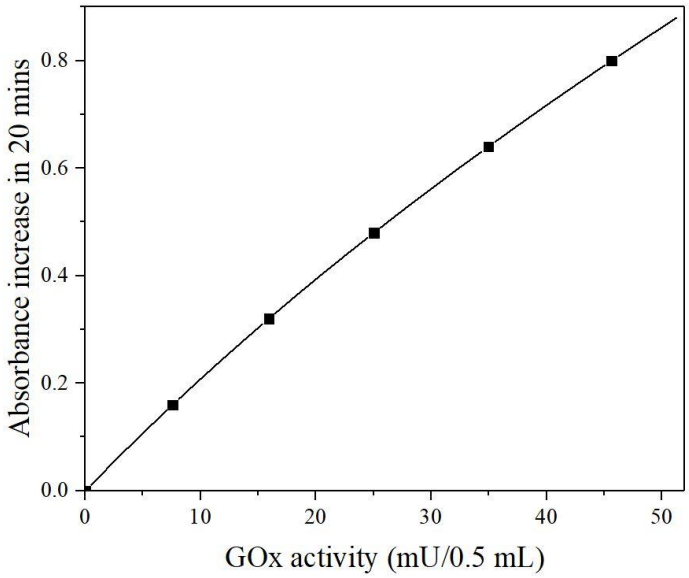


Figure 14 Standard curve relating glucose oxidase activity (mU/assay i.e per 0.5 mL) to absorbance at 510 nm

II- B- 8- Cyclic voltammetry (CV)

Cyclic voltammetry is a widely used technique for studying bio/electrochemical and reactions and response. It includes applying a linear potential on working electrode cycling between two limits (initial and final) against a reference electrode, using a certain rate of scans (Volts per second), and the response current obtained in the cell is then measured.

"CH Instruments" electrochemical workstation was used for CV measurements (Figure 15). The cell contains a standard three-electrode configuration. The software CH1650D was used to collect curves and OriginLab® software was used in plotting.

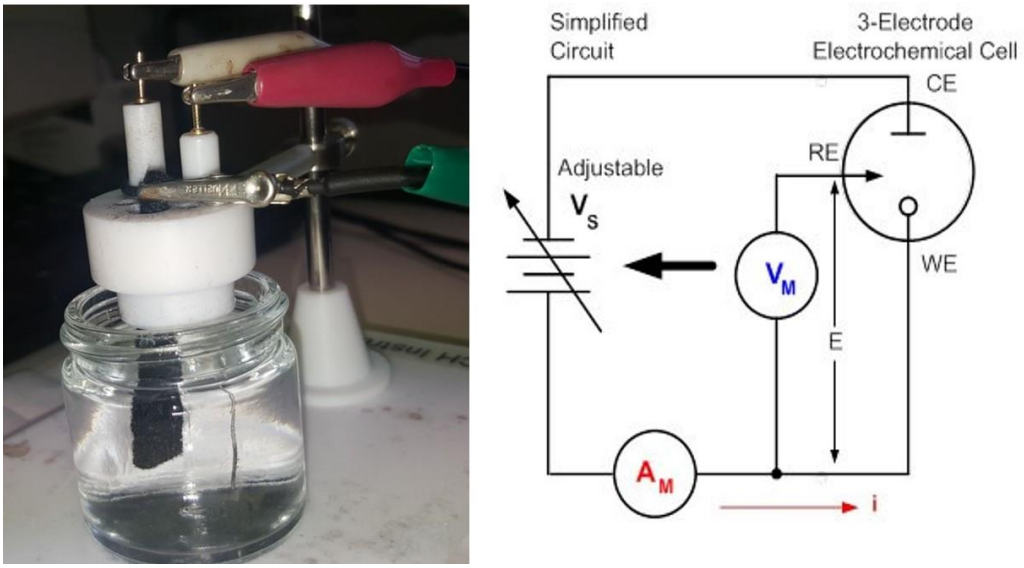


Figure 15 Cyclic voltammetry set up

The different carbon-based samples were used as working electrode (WE), with 1 cm^2 immersed surface area, the reference electrode (RE) used was Ag/AgCl, and the counter electrode (CE) used was platinum wire. The measurements were performed in phosphate buffered saline (PBS) solution (0.01 M) at pH 7 and room temperature. The scan range used was -0.4 – 0.6 V. Ferrocenecarboxylic acid (FCA) was used as a mediator (30 mM) to facilitate electron transfer. D-glucose was added to cell's medium as a substrate of GOx. The scan rate used was 0.01 $V.s^{-1}$, unless it is mentioned otherwise.

II- B- 9- Lineweaver-Burk fitting plots

This plot is a double reciprocal plot, which represents the graphics of enzyme kinetics equation that was proposed by Hans Lineweaver and Dean Burk. It can be described as the graphical analysis method of Michaelis-Menten equation (Eq. 23).

$$\frac{1}{V} = \frac{k_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (23)$$

From this equation, the enzyme kinetics like the apparent maximum velocity and apparent Michaelis-Menten constant can be determined.

In this work, the response of our obtained samples that are bio-functionalized with GOx was evaluated in solutions, which contain different concentrations of substrate, which is D-glucose in CV setup in the presence of FCA as a mediator. Following this step by applying the mentioned (Eq. 23), to determine the apparent I_{max} and K_m [240], which will help to understand the changes in affinity of enzymes towards the substrate with different immobilization methods.

II- B- 10- Bio-Fenton process for treatment of Remazol Blue RR dye solution

The immobilized GOx on different carbon-base samples were placed in a beaker (Figure 16). For each 5 mL of wastewater, 1 cm^2 of bio-functionalized carbon was used. The mixture contained $FeSO_4.7H_2O$ (1.5 $g.L^{-1}$), D-glucose (0.05 M), Remazol Blue RR (0.05 $g.L^{-1}$). The pH of the mixture was adjusted using acetic acid to be 4.5, the mixture's temperature was fixed on 30 ºC. The treatment was performed without stirring during 3 h for 1 cycle.

After treatment, the bio-functionalized carbon sample was removed from the medium, washed twice with buffer solution (pH 7), and stored at 4 ºC until further use. All experiments were performed three times.

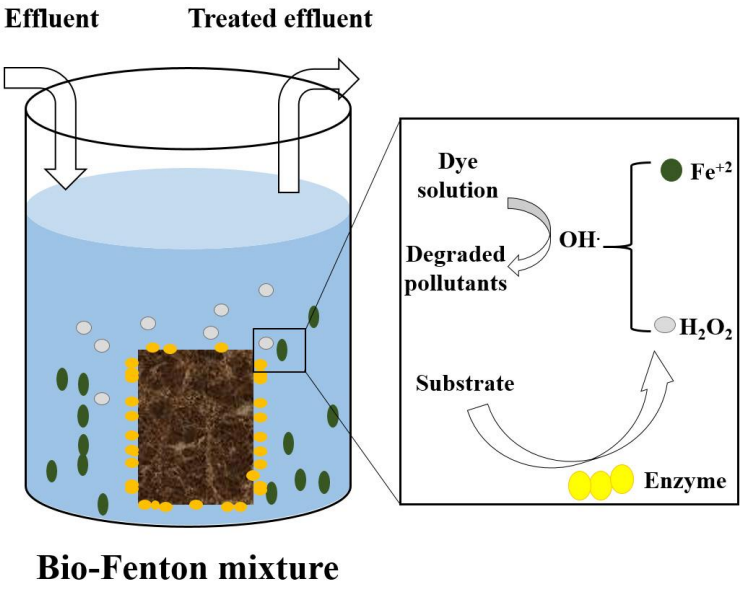


Figure 16 Bio-Fenton mixture

II- B- 11- Bio-Electro-Fenton process for treatment of Remazol Blue RR dye solution

As shown in (Figure 17), the prototype of the enzymatic BEF reactor used contained the following:

Anodic chamber: carbon-based bio-anode (obtained in **Chapter III**) was used. Each enzymatic bio-anode was 3 X 5 cm^2 all immersed in 100 mL of anodic solution. This solution contained 0.01 M PBS solution with 0.1 M D-glucose as the substrate of GOx, and 30 mM of Ferrocenecarboxylic acid (FCA) as a mediator. The pH of this chamber was adjusted to 5.5 in all experiments in favor of GOx enzymatic activity.

The reaction in the anodic compartment is illustrated in (Eq. 24)

$$\mathrm{D-Glucose} \xrightarrow{\mathrm{GOx}} \mathrm{Gluconic\ acid} + 2e^- + 2H^+ + O_2 \quad (24)$$

Cathodic chamber: bare carbon felt cathode that has been treated with CRP with 1% oxygen (CF 1%) was used as a cathode for all experiments. The dimension of the cathode was also 3 X 5 cm^2, and was changed for each experiment. The solution in cathodic chamber contained 0.01 M of PBS, 1.5 $g.L^{-1}$ iron sulphate hyptahydrate ($FeSO_4.7H_2O$) as a source of iron for Fenton reaction and 0.05 $g.L^{-1}$ Remazol Blue RR dye. The pH of this solution was adjusted to 3.5 to favor Fenton reaction illustrated in (Eq. 6 -7).

All experiments were performed twice at 21 ºC.

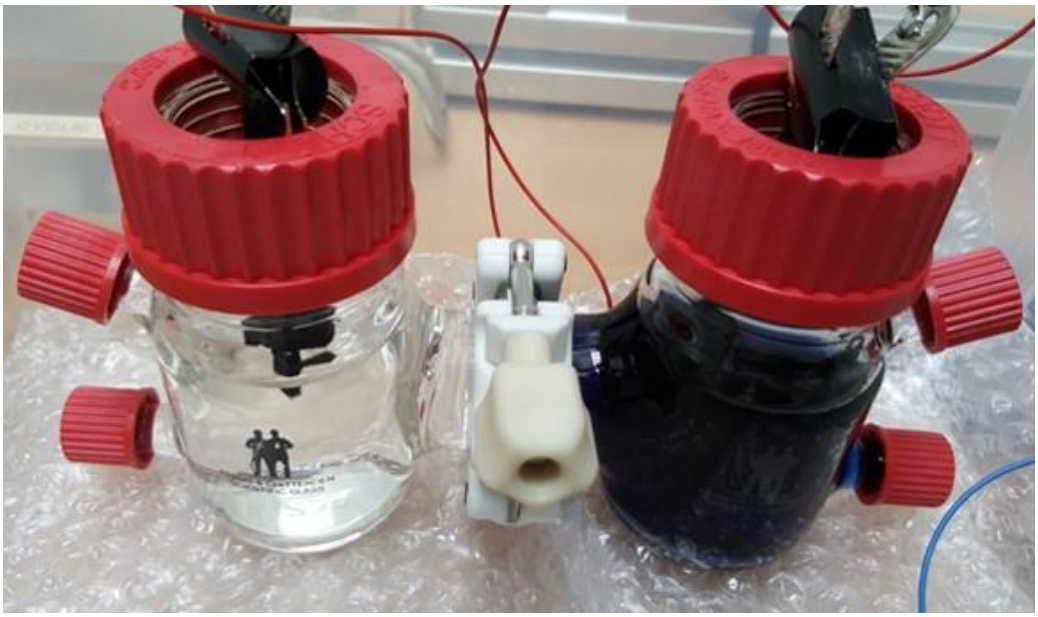


Figure 17 BEF prototype reactor used in this study

The electrical circuit was connected with wires that are attached to 2 cm of platinum wire (0.4 mm diameter) to hook the electrodes in place, and it was connected to the measuring unit.

The distance between the two electrodes was fixed to 10 cm for convenience, and the two chambers were separated by Nafion® (N 211) proton exchange membrane. Each experiment was performed during 12 h, with external resistance of 10 Ω.

II- B- 12- Discoloration determination of Remazol Blue RR wastewater

A solution of Remazol Blue RR was used as a modal pollutant in this study; a calibration curve was obtained from the UV-Vis absorption peaks of different concentrations of Remazol Blue RR (Figure 18). The color removal of the wastewater treated with BF or BEF process was assessed after treatment via Thermo Fisher Evolution 200 UV–Vis spectrophotometer to evaluate the efficiency of the treatment in discoloration of this dye. The absorbance peak at 605 nm was monitored before and after treatment, and then the calibration curve was used to estimate the concentrations of the colored

form of the dye left in the water post-treatment. The removal efficiency was calculated, where A_0 and A_1 are the initial and after treatment concentrations, respectively (Eq. 25).

$$\text{Color removal (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (25)$$

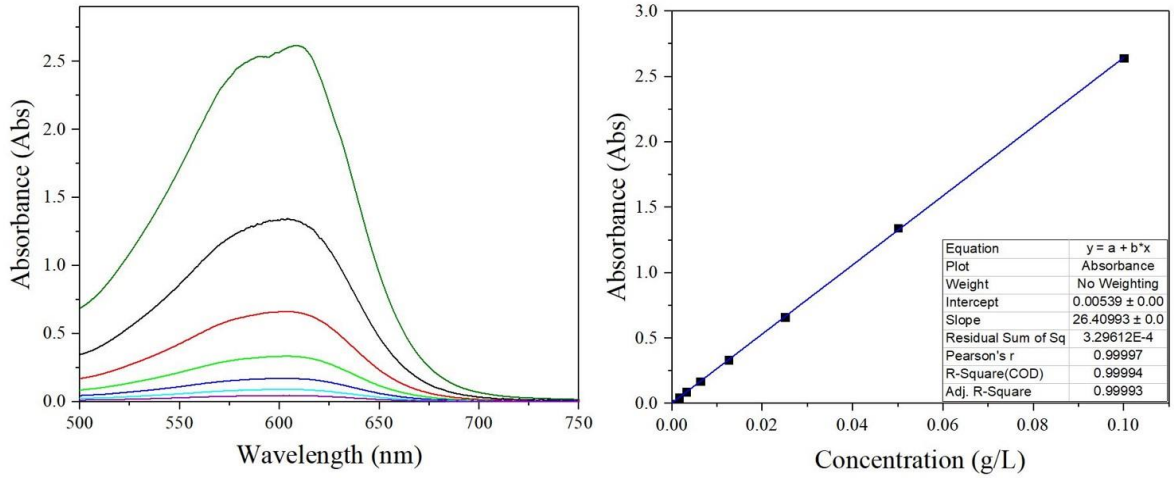


Figure 18 UV-Vis absorbance peaks of Remazol Blue RR in concentrations between 0.0015 and 0.1 g.L^{-1} at 605 nm, and Calibration curve concentration vs. absorbance - R^2 = 0.99994 (left to right)

II- B- 13- Chemical Oxygen Demand (COD) determination of Remazol Blue RR wastewater

Chemical oxygen demand (COD) was measured using vials provided by CHEMetrics, Inc. (USA). This test was carried out to measure the oxidizable organic matter quantity in a water sample. The reaction mixture contains the water sample and an acidic solution of potassium dichromate in the presence of a catalyst. This mixture undergoes digestion process at 150 ± 1 ºC for 2 h. Oxidizable organic compounds in water reduce the dichromate ion ($Cr_2O_7^{2-}$) to the chromic ion (Cr^{3+}). The decrease in ($Cr_2O_7^{2-}$) ion is measured colorimetrically at absorbance of 620 nm using UV-Vis spectrophotometer. Finally, COD values were estimated from the calibration curve provided by the manufacturer (Figure 19). The efficiency of COD removal (η %) as calculated using the following equation (Eq. 26) [125].

Where COD_0 and COD_1 are COD values for samples before and after the advanced oxidation, respectively.

$$\eta(\%) = \frac{COD_0 - COD_1}{COD_0} \times 100 \qquad (26)$$

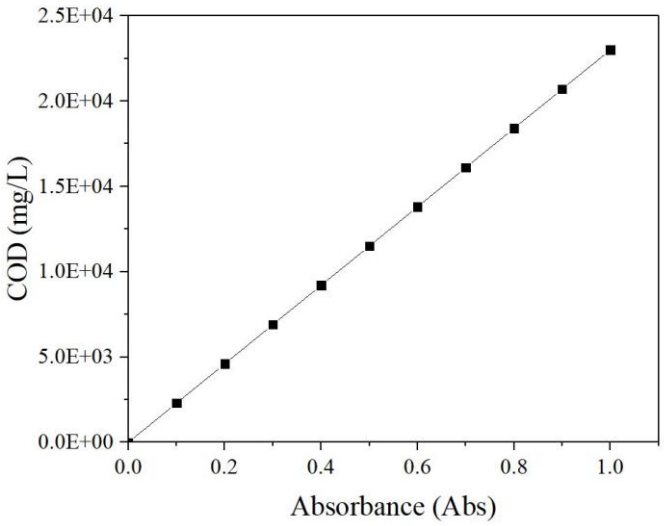


Figure 19 COD calibration curve as provided by the manufacturer

II- B- 14- Polarization curves

To examine the internal resistance of the cell, polarization curves were obtained by varying the external resistance between 1- 10000 Ω and measuring both cell voltage and the current passing through the electrical circuit using NI - USB 6229 BNC potentiometer and LabVIEW NXG in a synchronized manner. After obtaining the different values of both voltage and currents passing through the circuit, we calculated the current density and power density obtained, normalized to the surface area of the cathode used (15 cm^2).

Where, the power was calculated as follows (Eq. 27).

$$P = V \times I \qquad (27)$$

Where: P is the power generated from the cell (W), V: is the measured voltage (V), and I: is the current intensity measured (A).

Nomenclature of samples

Each type of samples used in **Chapter III** and **Chapter IV** was given a nomenclature according to the pretreatment and method of enzyme immobilization used to produce it (Table 2).

Table 2 Nomenclature of samples used in this thesis

Sample type	Without plasma treatment	With Cold Remote plasma CRP with O_2/ N_2 ratio=1 %	With Cold Remote plasma CRP with O_2/ N_2 ratio =2%
Bare carbon / adsorption	VCF	CF 1%	CF 2%
Coated carbon with PEDOT:PSS / adsorption	PPCF	PPCF 1%	PPCF 2%
Bare carbon - crosslinking	VCF - G	CF 1% - G	CF 2% - G
Coated carbon with PEDOT:PSS - crosslinking	PPCF - G	PPCF 1% - G	PPCF 2% - G

Chapter III

Eco-technologies for immobilization of glucose oxidase redox enzyme on carbon-based conductive textiles

III- A- Immobilization of glucose oxidase via physical adsorption on carbon felts treated with Cold Remote Plasma (CRP)

III- A- 1- Introduction

Textile materials based on carbon are chemically stable and corrosion resistant in water or solvent-based solutions. These properties, added to their good electrical conductivity, high porosity, and surface area make them a proper choice when a robust and relatively cheap material is required for bio/electrochemical applications [146,149,150]. These materials as well suited for electrodes and bioelectrodes applications that are used widely nowadays to fabricate biosensors for environmental or medical purposes, in addition to biofuel cells (BFC) and wastewater treatment setups.

However, the major drawback of their stability renders these materials inert, with hydrophobic tendencies, causing technical difficulties such as; poor affinity towards aqueous media, and poor adhesion with coating, polymers and resins during manufacturing [169].

Studies have been conducted in order to overcome these issues by modifying the surfaces of carbon materials. Variety of methods was used including: thermal, chemical or physical approaches to increase their surface energy, and to facilitate further treatments and finishing. These procedures were efficient in functionalization of carbon surfaces, improving mechanical properties, increasing adhesion forces in composites, and increasing power efficiency output of flow batteries [48–61].

However, most of these treatments depend on use of harsh chemicals and acids, high power consumption, or require long time for treatment. Hence, the use of milder and eco-friendly technologies would improve the environmental impact of such processes.

Plasma treatment is an effective dry eco-technology for surface modification. Unlike mentioned approaches, it does not require added chemicals and can be performed in a relatively short time.

Plasma eco-technology has been used for treatment of carbon textile materials using a variety of gases. Effects including increased wettability, improved adhesion of fiber-matrix interface in composite materials, and improved efficiency in flow batteries were achieved [58–61,167–173,241–

245]. Plasma treated carbon textiles possess improved surface energy better than their initial state; hence can be bio-functionalized with redox enzymes.

The combined properties of conductive carbon with customized surfaces via plasma, and immobilized redox enzymes give the opportunity to fabricate bioelectrodes for biosensors for medical purpose or pollution detection, self-powered implants, and electricity generation from bio-resources like bio-ethanol and sugars.

Bio-functionalization of carbon materials with plasma was reported. Materials including carbon nanotubes, nanoballs, and rods were functionalized with redox enzymes post plasma treatment. Gases such as CH_4, N_2 and O_2 were used in low temperature plasma, downstream oxygen plasma and atmospheric plasma jet [246–249]. As a result, high-performance bioelectrodes were obtained with improved enzymatic activity. These solid electrodes possess less specific surface area than carbon textile in form of nonwoven felts that are made from microfibers and possess higher surface area to immobilize more redox enzymes, and to allow diffusion of substrate by higher capillary uptake post-plasma, resulting in efficient bio-processes.

Nevertheless, efficiency of cold remote plasma (CRP) in modification of carbon-felt textiles for better bio-functionalization with redox enzymes has not been evaluated to the best of our knowledge. This plasma technology, away from discharge zone is well controlled, due to possibility of customizing type and percentage of the gas used, according to the desired acquired functions. CRP activates the surface of carbon fibers without resulting in hazardous sparkling. In fact, sparkling of conductive materials and textiles during treatment in vicinity of dielectric barrier discharge such as in air atmospheric plasma may cause ignition. In CRP, the discharge is around 0.9 m away from samples; free radicals produced are the only reactive species will be reaching carbon felt. CRP is thus suitable for electrically conductive materials, which are a main part of smart and functional textiles.

Additionally, the design of CRP instrument allows the treatment of materials without limitation on thickness, unlike some other types of setups that require certain thickness of the film or fabric. Hence, no limits for treatment of 3D structures like bulky felts in CRP, while both faces of textiles can be treated at once if desired, which saves both energy and time.

In this work, the efficiency of CRP (nitrogen + oxygen) to modify surface properties of commercial nonwoven Virgin Carbon Felt (VCF) was investigated. The treated felts were then subjected to immobilization of glucose oxidase redox enzymes EC (1.1.3.4) via adsorption, and activity of immobilized GOx was then evaluated. Surface modifications were characterized using different techniques, including SEM, FTIR, XPS, capillary uptake and water contact angle measurements, as described in method section. CV scans and colorimetric assays were carried out to evaluate efficiency of GOx immobilization and re-usability. The content of this part has been published in **Paper II**.

III- A- 2- Enzyme immobilization via physical adsorption

Physical adsorption was used for immobilization of glucose oxidase enzyme on different carbon-based samples. The samples were 1 cm^2 each, and were individually placed in 3 mL of phosphate buffer solution containing GOx (0.0858 $U.mg^{-1}$, 1 $mg.mL^{-1}$) for 24 h at 4 ºC.

This immobilization process was carried out at different pH values of 7 or 5.5 for untreated and CRP treated carbon-based samples. It is worth mentioning that the samples with CRP treatment were placed in the enzymatic solution directly after plasma. The obtained felts were then rinsed twice with phosphate buffer solution (pH 7) and stored at 4 ºC till use. The rinsing process was sufficient to remove any unfixed enzyme; and this was confirmed using the GOx colorimetric assay on the rinsing buffer solution: no pink coloration was observed**.**

III- A- 3- Results

SEM micrographs

SEM micrographs presented in (Figure 20) show the morphological changes after CRP treatment of the VCF felts. The micrographs show that fibers of VCF possess rough surfaces with grooves and striations appearing in a parallel manner along the fiber length. After CRP, regardless of the gas mixture used (N_2 + 1 % or 2 % O_2), there was a slight modification of the fiber surface with increased

number of deeper grooves. These observations are in agreement with another study related to the use of CRP plasma on a woven carbon cloth [237].

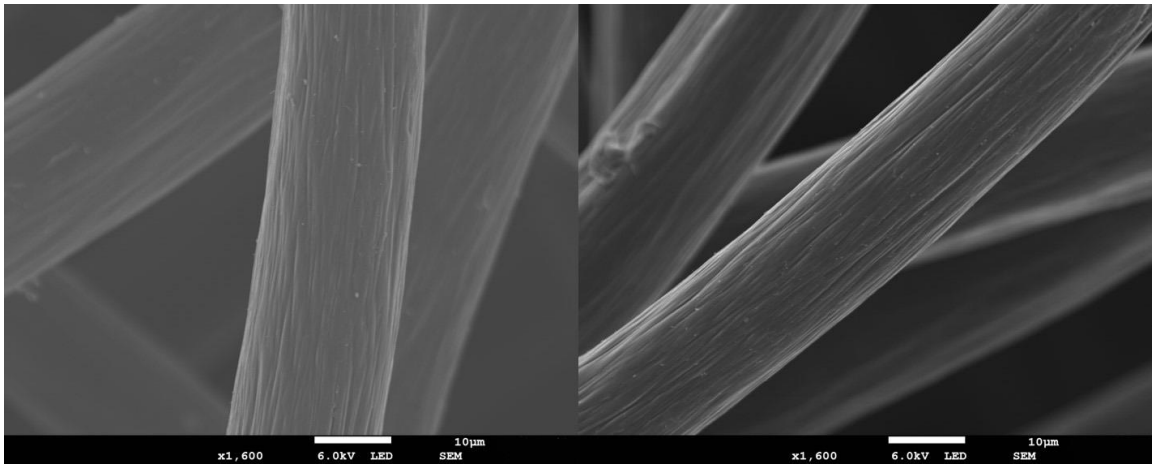


Figure 20 SEM micrographs of carbon felt before and after CRP treatment (left to right)

FTIR spectra

The FTIR spectra of bare carbon felts before and after CRP treatment with both (N_2 + 1 and 2 % O_2) gas mixtures are illustrated (Figure 21). New integrated functional groups appeared after the CRP treatment, and were similar regardless of the gas mixture used. These integrated groups were mostly oxygenated in addition to amino groups. The weak broad peak appeared at 3345 cm^{-1} was attributed to N-H stretching vibration of secondary amine [250]. The peak around 1650 cm^{-1} was attributed to C=O stretching vibrations of amide groups [251], in addition to the peak between 1550-1640 cm^{-1} which was assigned to N-H stretching vibration, indicating the presence of amide groups [250]. The double peak appeared at 2850 and 2920 cm^{-1} was assigned to stretching vibration of =C-H of an aldehyde group and the peak around 1733 cm^{-1} was attributed to stretching vibration of C=O, also indicating the presence of aldehyde groups. The strong peak between 1030 -1155 cm^{-1} was assigned to stretching vibration of C-O of an ether group.

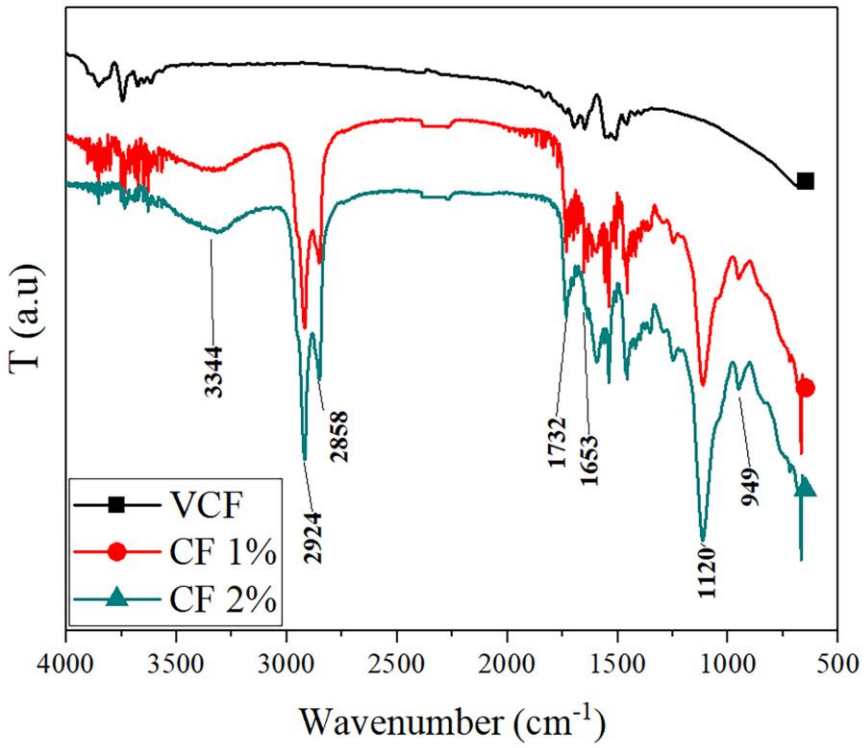


Figure 21 FTIR spectra of bare carbon felt before and after CRP

XPS analysis

The typical survey spectra by XPS of the bare felts: VCF, CF 1% and CF 2% were conducted. The main photopeaks C 1s, N 1s and O 1s are presented in relative atomic contents (%) (Table 3). From the spectra it was shown that C 1s, O 1s were predominant, while N 1s was also present in all samples.

Table 3 Relative atomic content as calculated from XPS spectra (%)

	C 1s	**O 1s**	**N 1s**	**C-C**	**C-O**	**C=O**
VCF	90.42	5.36	4.22	90	10	____
CF 1%	76	18.37	4.52	56.2	29.8	14
CF 2%	75	19.65	4.44	56.2	30.9	12.9

From the previous values, it can be noticed that after CRP, oxygen percentage (18%) was increased more than three times its initial value for the CF 1% samples. However, further increase in content of oxygen in the gas mixture (up to 2%) didn't result in a significant increase in oxygen content of the CF 2% sample. Meanwhile the nitrogen percentage did not show a significant increase after

plasma treatment for both gas mixtures used. The relative percentage of C-C was reduced notably (from 90% to 56%), due to the increase of oxygen percentage on the surface of the treated carbon felts via CRP. To further understand the values presented in the table, fitting and deconvolution of the C 1s photopeaks was conducted (Figure 22), the spectrum of CF 1% shows a peak at 284.8 eV that is related to graphitic carbon (C-C), the second peak at 286.2 eV is related to (C-O), and the third peak at 288.6 eV is related to carbonyl group (C=O). On the other hand, for the VCF, the spectrum showed a sharp peak for the C 1s that is related to graphitic carbon (C-C), with 10 % of (C-O) content only. CRP treatment increased significantly both (C=O) and (C-O) functional groups. FTIR spectra showed intense double peak between 2920 and 2850 cm^{-1} as previously mentioned, due to stretching vibration of aldehyde group. Thus, the increase in (C=O) is due to aldehyde groups and possibly amides groups that were also detected by FTIR. The (C-O) increase would be due to the formation of ether.

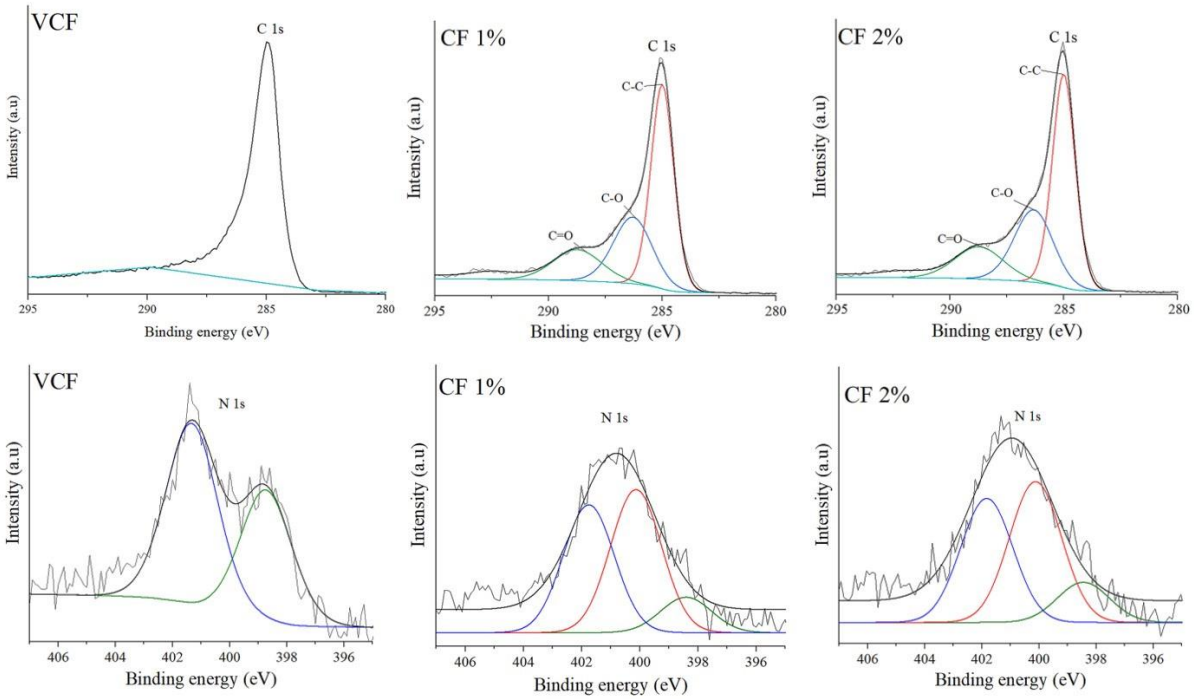


Figure 22 High resolution C 1s and N 1s XPS spectra for bare carbon samples before and after CRP

Photopeak deconvolution of N 1s spectra was performed (Figure 22). On VCF sample, N 1s spectrum shows two main peaks most probably related to pyridinium at 401.5 eV, and to pyridine at 398.4 eV. The ratio of pyridine compared to pyridinium is almost 1: 1.4 for VCF.

For CF 1% sample, the intensity of the peak at 398.4 eV (pyridine) decreased abruptly, and a new intense peak at 400.7 eV probably due to pyridone amines and amides appeared [252]. However, for CF 2% sample, the areal ratio of pyridone is slightly lower.

Water contact angle and capillary measurements

Water contact angle of the bare VCF was measured by sessile drop method using distilled water, at room temperature θº was 116 ± 1º (Figure 12). These hydrophobic tendencies affect their affinity towards aqueous enzymatic solutions that are used for immobilization by physical adsorption. Hence, the reduction of water contact angle is expected to result in higher affinity towards those mediums in order to facilitate the immobilization of enzymes. As a conventional method to increase the wettability of carbon materials, treatment with 10 % H_2O_2 solution at 90 ºC for 3 h has been described in literature [55], and when applied to the sample used in this study, the water contact angle obtained was 61º ± 3. However, this method has various disadvantages since it requires consumption of both energy and time, due to constant heating for several hours. This makes plasma treatment a better option to achieve the same results within shorter time and without the use of heated chemical solutions.

To compare the effect of CRP on VCF felts, capillary uptake and water contact angle of the samples were measured immediately after CRP treatment, and 4 weeks later, in order to study the ageing effect.

The variation of capillary uptake values and water contact angle without and after CRP treatment is presented (Table 4). Plasma treatment was efficient in reduction of water contact angle of VCF from 116° to around 57-59°, and increased the values of capillary uptake from almost 0 % to 650 -700 %, in a short time and without added chemicals. Better wettability of carbon felts was achieved using gas mixture containing 2 % oxygen. Nevertheless, after 4 weeks, a small decrease in wettability was observed, both in terms of increase in water contact angle, and decrease in capillary uptake. The decrease in wettability was higher for the CF 2% compared to CF 1%, with capillary uptake decreasing to around 570 %.

Table 4 Water contact angle and capillary uptake for bare carbon samples

Sample	Θ° directly after CRP	Θ° after 4 weeks	Capillary uptake after CRP (%)	Capillary uptake after 4 weeks (%)
CF 1%	59 ± 2	60 ± 2	663 ± 13	621 ± 8
CF 2%	57 ± 1	58 ± 3	713 ± 43	573 ± 28

The samples were subjected to ageing, and that resulted in oxidized species in both inside and the outer faces of the plasma treated carbon felts. Nonetheless, compared to other types of plasma treatment used to activate carbon fiber surface (active screen plasma [248]), the ageing effect is very slow with CRP (N_2 + O_2).

Characterization after enzyme immobilization

SEM and colorimetric assays

The SEM micrographs (Figure 23) illustrate the felt samples bio-functionalized with GOx enzymes. On the CF 2% samples, enzymes seemed adsorbed directly onto the fiber surface, as if they were on the upper edges of the parallel grooves along the fiber surface. However, the physical presence of the enzyme does not prove its activity, since denaturation may occur after immobilization, which results in enzymes losing their 3D structure and the active site not being accessible for substrate-enzyme interaction.

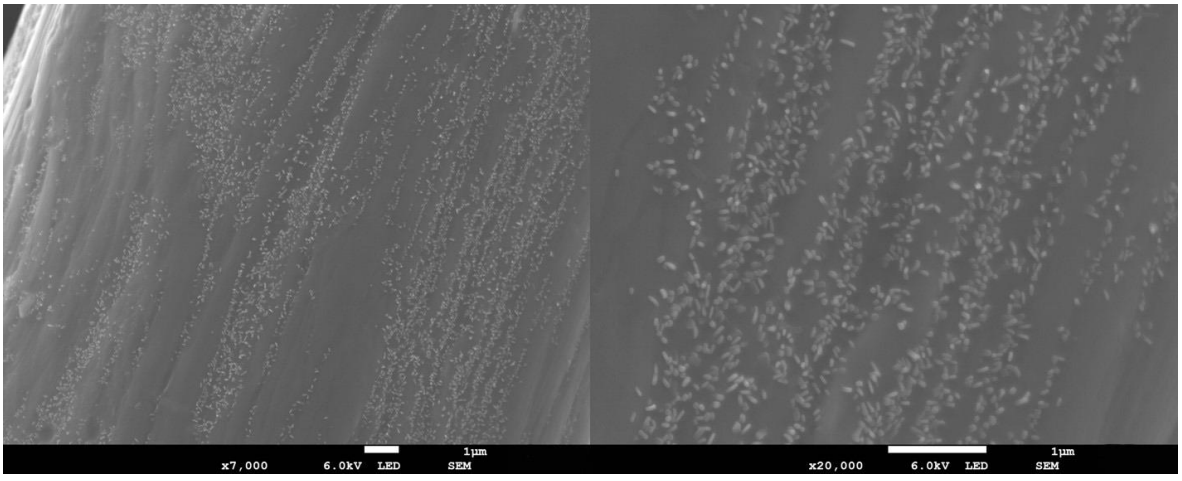


Figure 23 SEM micrographs of immobilized enzyme on bare carbon felts after CRP with magnification of X7000 and X20000 (left to right)

The enzymatic activity of the immobilized GOx was measured for all bare carbon felt samples, with or without CRP treatment using the glucose oxidase activity kit as described earlier. This activity test was conducted at 25 ºC and pH 7, according to the protocol provided by the producer.

As shown in (Figure 24) on bare untreated carbon (VCF), 35 % of the total free enzyme activity only was maintained after immobilization at pH 7 or pH 5.5. While CRP treatment allowed to maintain enzymatic activity as high as 55 to 60 % in the first cycle. Plasma treated samples both CF 1% and CF 2% gave higher values of relative enzymatic activity in most cases compared to VCF samples.

When enzymatic activities are compared for enzymes adsorbed at pH 7 and pH 5.5, we find from (Figure 24) that pH 5.5 value maintained better activities after immobilization process, for all carbon felt samples VCF, CF 1 % and CF 2 %.

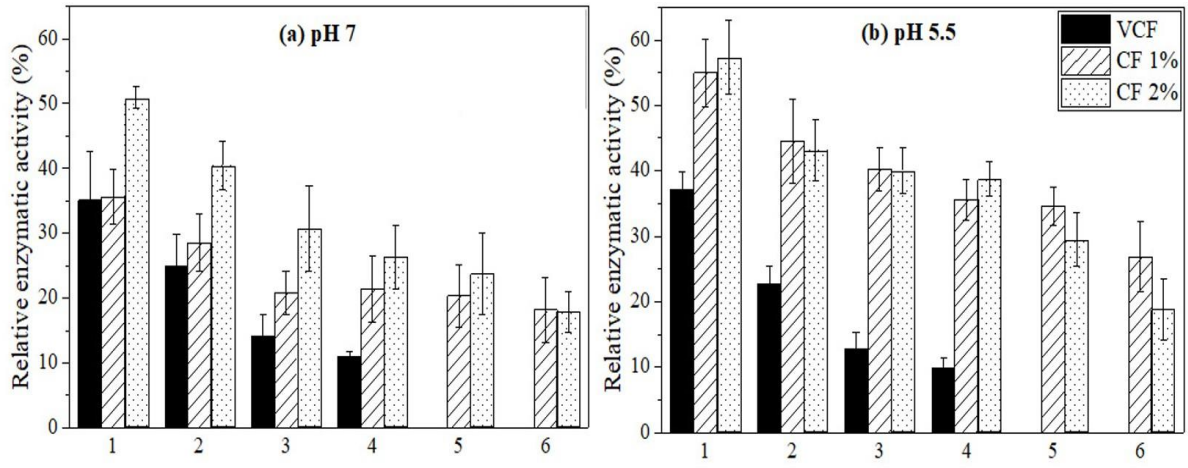


Figure 24 Relative enzymatic activity for immobilized GOx on VCF, CF 1% and CF 2%

Moreover, the reproducibility and stability of the samples were better at pH 5.5 than pH 7. It is worth mentioning that, enzymatic activity of free enzyme in 3 mL in the buffer solution used for adsorption on 1 cm² of bare carbon felt, was around 0.08 $U.mL^{-1}$. Hence, enzyme immobilization via physical adsorption, maintained a part of enzymatic activity after immobilization, since not all free enzymes would be immobilized successfully, and all immobilized enzyme do not necessarily maintain their bio-catalytic activity after the immobilization process.

Cyclic voltammetry scans of CF 1% samples in absence and presence of three concentrations of D-glucose (8.5, 17 and 25.5 mM) are shown (Figure 25). An increase of the bio-catalytic current occurred because of the increase of D-glucose added to the medium, and that indicates that GOx maintained its enzymatic activity after immobilization via adsorption and the obtained felts are bioactive, in accordance with the colorimetric assays.

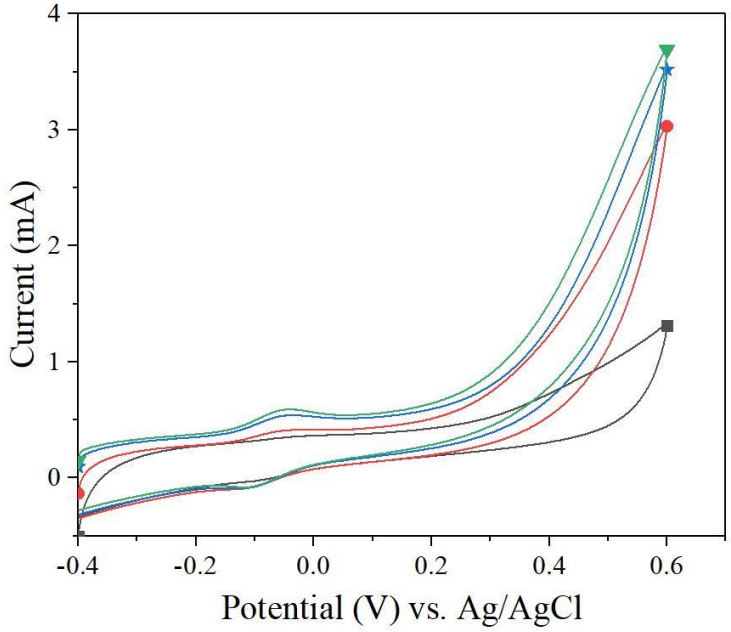


Figure 25 Cyclic voltammograms of CF 1% in 0.01M PBS vs. Ag/AgCl electrode in absence and presence of different D-glucose concentrations (0 - 8.5- 17- 25.5 mM respectively from bottom to top)

It's worth mentioning that, above 30 mM concentration of glucose, no further increase in the bio-catalytic currents, and a plateau was reached at around 3.5 mA and potential of around 0.5 V. This was applicable in case of CF 2% samples as well.

To evaluate the parameters of enzyme kinetics, the equation of Michaelis–Menten (Eq. 23) was used [240], in addition, Lineweaver-Burk method for fitting was used to estimate the apparent Michaelis constant (k_m) along with the maximum current response (I_{max}) for different samples (Figure 26). The I_{max} current reached the values 1.22, 2.92 and 3.8 mA, meanwhile, the apparent k_m was estimated to be 2.7, 3.8 and 5.2 mM for VCF, CF 1% and CF 2%, respectively.

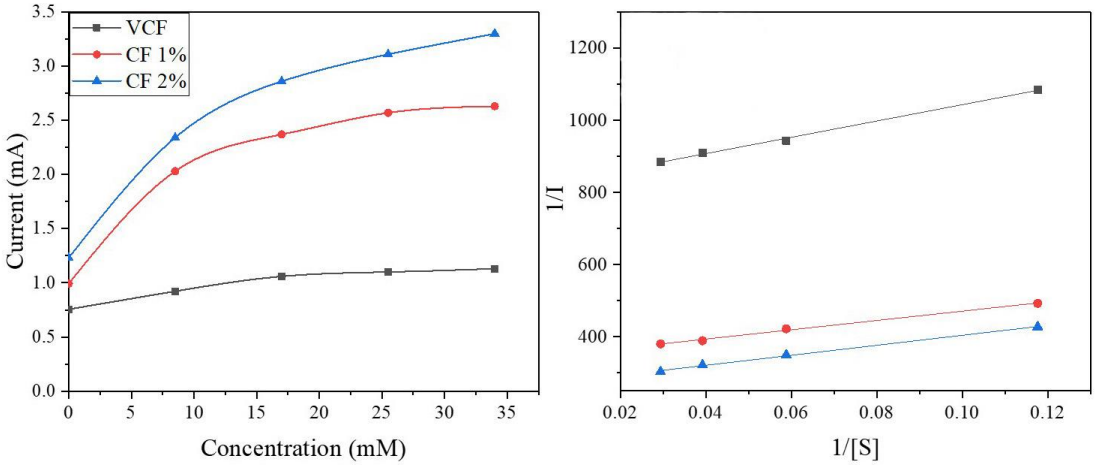


Figure 26 Catalytic current vs. D-glucose concentration at E = 0.5V, and Lineweaver–Burk fitting of the activity of GOx immobilized on VCF, CF 1%, CF 2% (R^2 = 0.998, 0.996, 0.998, respectively)

III- A- 3- Discussion

The purpose of this chapter was to investigate the efficiency of cold remote ($N_2 + O_2$) plasma (CRP) to activate bare Virgin Carbon Felts (VCF), for optimized enzyme GOx immobilization via physical adsorption. CRP treatments used showed to be an efficient approach to activate the surface of conductive carbon fiber. This treatment leads to both physical and chemical modifications of the surface of carbon fiber. Our results presented in this chapter agree with literature, when using reactive plasma of gas mixtures like oxygen and nitrogen, the main expected dominant effects are physical etching and surface functionalization [238].

In CRP, the carbon felt samples are treated far from the discharge, in a reactive zone that is free of ions, UV radiations and electrons. This approach was efficient in treatment of the conductive felts used in this study without the direct exposure to dielectric discharges, thus the etching effect is limited. In accordance, SEM micrographs showed slight topographical changes of the felts after CRP treatment.

Both FTIR and XPS analysis confirmed the integration of new functional groups mostly oxygenated (such as C-O and C=O) as well as amino groups, after treatment with both plasma gas mixtures. The percentage of oxygen atoms on the surface of carbon fibers was increased more than three folds, with the introduction of ether, aldehyde and amide groups. However, no further significant increase

in oxygen or nitrogen atom content was observed when 2% of oxygen was used instead of 1% in the plasma gas mixture.

A significant ratio of nitrogen atom was already present on the surface of untreated carbon fiber (4%), contrarily to a previous study [237]. This may be explained by the origin of the carbon felts used in this study, which has been obtained by oxidation of nitrogen containing polyacrylonitrile polymer (PAN). After CRP, only a very small variation in nitrogen atom content occurred. FTIR spectra showed peaks due to secondary amines and amides groups after CRP, while XPS spectra revealed that on VCF, almost 50% of nitrogen atoms were in the form of pyridine groups, which were converted gradually by plasma into pyridone. The possible functional groups on the virgin carbon fiber obtained from (PAN) and the possible functional groups that were integrated due to CRP (N_2 + O_2) treatments used are shown (Figure 27).

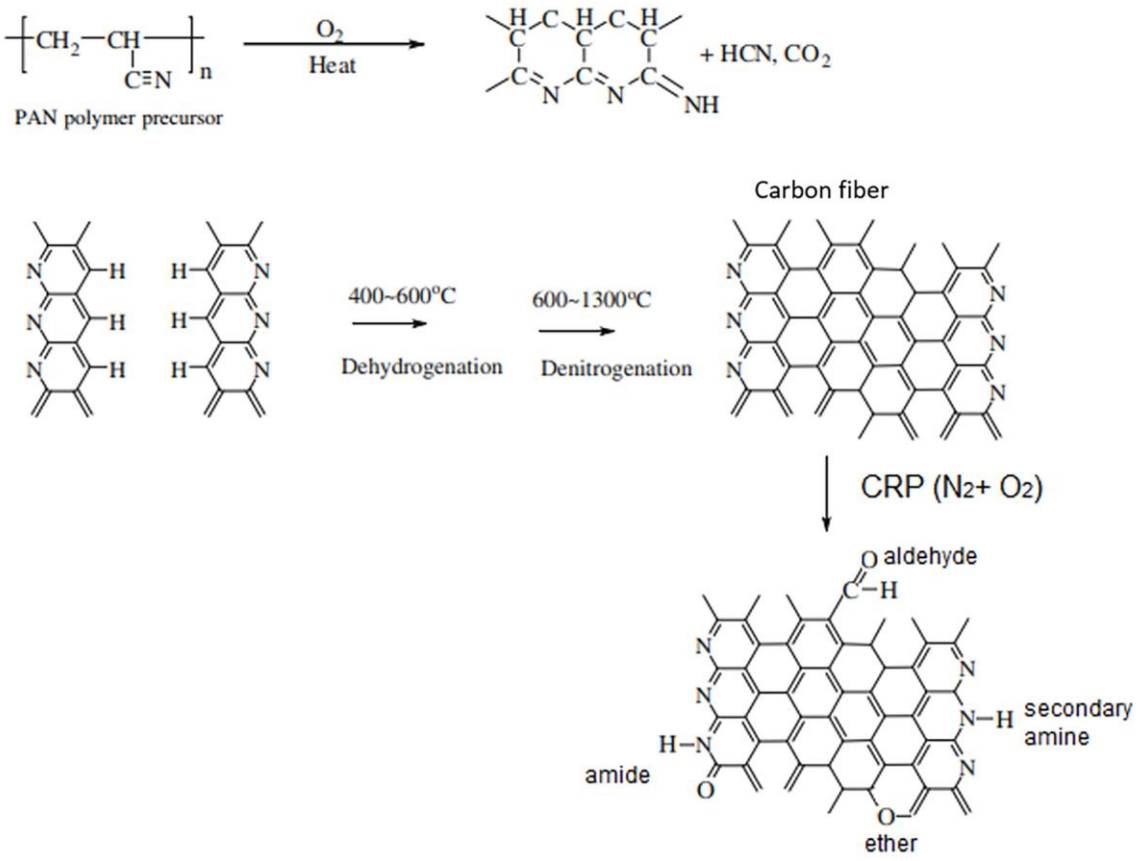


Figure 27 Possible chemical structure of carbon fiber, and possible chemical modification after CRP plasma treatment - adapted from [253]

New functional oxygenated groups would be integrated on fiber surfaces on both the external surface and inside of the felt, which result in an increase in wettability in terms of decreased water contact angle, and increased capillary uptake. Hydrophobic recovery due to ageing was very slow.

When pH 7 was used during the immobilization process, the enzymatic activity of GOx deviated, compared pH 5.5 which showed to be more stable and slightly higher. This might be due to optimal pH value for GOx (EC 1.1.3.4). For glucose oxidase, the pH range is broad and it is reported to be active in the range pH 4 - 7, but optimal enzymatic activity can be obtained in mediums of pH 5.5 [19]. Generally, the samples treated with CRP gave better enzymatic activity, shelf life and re-usability when compared to the untreated VCF samples. This might be due to better bonding between the surface and the immobilized enzymes as a result to the integration of new groups such as carbonyl and amino groups on the surface of samples after CRP treatment for both gas mixtures. These new groups help to create hydrogen bonding with the adsorbed amino acids of the enzymes. This leads to less leaching of the enzymes into the reaction solution, and consequently an extended reusability even when immobilized in a neutral buffer solution (pH 7). Furthermore, the conformation of GOx at the fiber surface allowed its substrate to access the active site, which resulted in maintaining its enzymatic activity and extending its reusability. The maximum currents (I_{max}) estimated from the CV scans show that CRP treated felts gave higher values compared to VCF (for one cycle scan), in accordance with the previous colorimetric assay for GOx. Since I_{max} value is attributed to the quantity of active units of enzyme, thus CRP treatment improved adsorption of the enzyme on surfaces of the treated felts, when compared with VCF. However, the apparent K_m values indicated that a slight decrease in the affinity of GOx towards its substrate occurred after the immobilization on the CRP treated felts. This might be due to the increased bonding between the integrated functions on carbon fibers and the enzyme. Consequently, only a slight increase in the substrate's concentration is required to reach the maximum enzymatic activity. Nevertheless, this small reduction in affinity was compensated by higher quantity of active units adsorbed on CRP treated samples, which resulted in higher I_{max} currents and activity overall.

It can be indicated from the previous results, that increasing the percentage of oxygen used in the gas mixture up to 2% did not have a proportional impact on the treated samples when compared to

1%. However, the more rapid loss in enzymatic activity during the sixth cycle, for adsorption at pH 5.5, using CF 2%, may be due to more rapid ageing effect of CF 2% compared to CF 1%. Therefore, further research on different percentages of gas mixtures can be beneficial in determining the optimal ratio for better wettability of carbon-based textile felts.

III- B- Immobilization of GOx on plasma treated dip-coated carbon felts with PEDOT:PSS polymer dispersion

III- B- 1- Introduction

To overcome the stability of carbon fibers, several approaches have been studied in order to change surface energy. One approach is coating of carbon fibers with conductive biocompatible biodegradable PEDOT:PSS polymer blend to increase surface energy, improve electrical sensitivity and biocompatibility [254]. Furthermore, this coating can improve electrode's capacitance by many folds [255]. Nevertheless, lack of functionality of PEDOT usually requires further treatment to improve its desirable characteristics such as electrical conductivity and/or surface energy [256]. It was reported in literature that bundles of carbon fiber were electrochemically modified with PEDOT in order to be used as a selective sorbent [257]. Additionally, chemical approaches were followed to treat PEDOT:PSS layers filled with carbon black or carbon nanotubes using dopants such as; dimethyl sulfoxide, ethylene glycol, and 1-butyl-3-methylimidazolium tetrafluoroborate [211]. Solvents like methanol and dimethyl sulfoxide glycol were also used for the same purpose [215–217]. However, despite their efficiency, these methods depend on using hazardous solvents and chemicals, which raise questions regarding the safety or working environment and end application. To avoid these complications, plasma treatment has been used in literature to treat carbon with PEDOT:PSS coatings, in order to increase surface energy for better adhesion and cohesion forces in composites, or to improve electrical conductivity [258].

Several types of plasma and gases have been reported, for instance microwave plasma, mild oxygen, atmospheric, hydrogen plasma, non-thermal plasma and light oxygen plasma were studied [258–262]. These treatments improved stability of PEDOT:PSS as a buffer layer for anode application [263], functionalized PEDOT:PSS with entrapped multi-walled nanotubes (MWNT) to improve the electro-catalytic behavior of the composite [264]. Finally, flexible 3D electrodes made from MWNT with PEDOT:PSS were successfully functionalized by plasma [265], in addition to synthesizing and modifying thin coatings [266–268].

Hence in this part of the work, cold remote plasma treatment was simultaneously carried out of both carbon felts and PEDOT:PSS coating in the same time, to evaluate the influence of this treatment on surface and electrical conductivity of felts dip-coated with PEDOT:PSS, for improved enzymatic activity of immobilized GOx. The carbon nonwovens were dip-coated with PEDOT:PSS, then the coated felts were subjected to CRP using the same conditions as the previous part. Finally, GOx was immobilized on obtained felts via physical adsorption using the same protocol mentioned in section (**III- A- 2**).

The interest of this method is preventing big impact of mass transfer limitation which was noticed in a primary study using GOx entrapped within the PEDOT:PSS coating, since the interactions between the enzyme and the substrate is slowed down [269]. Surface characterization techniques including FTIR, SEM, XPS, and wettability measurements were used to assess changes after CRP. In addition, electrical and electrochemical assessments like bulk resistivity and cyclic voltammetry (CV) were carried out to estimate conductivity behavior of the studied samples. Finally, enzymatic activity assays were performed like previously to check the success of GOx immobilization and reusability of the treated samples. The content of this part is presented in **Paper III**.

III- B- 2- Results

SEM micrographs

SEM micrographs in (Figure 28) illustrate the changes occurring before and after dip-coating the carbon felts with PEDOT:PSS dispersion. The pores of bare carbon felt can be seen, which are inherited in the nonwoven bulk. The individual carbon fibers appear with rough surfaces and grooves along the surface (Figure 29). This rough structure creates high specific surface area for GOx immobilization, and equally allows inflow of substrate and outflow of products of the reaction catalyzed by GOx. In the case of PPCF samples, the polymer blend seems to be distributed as dispersed spots over and within carbon felt.

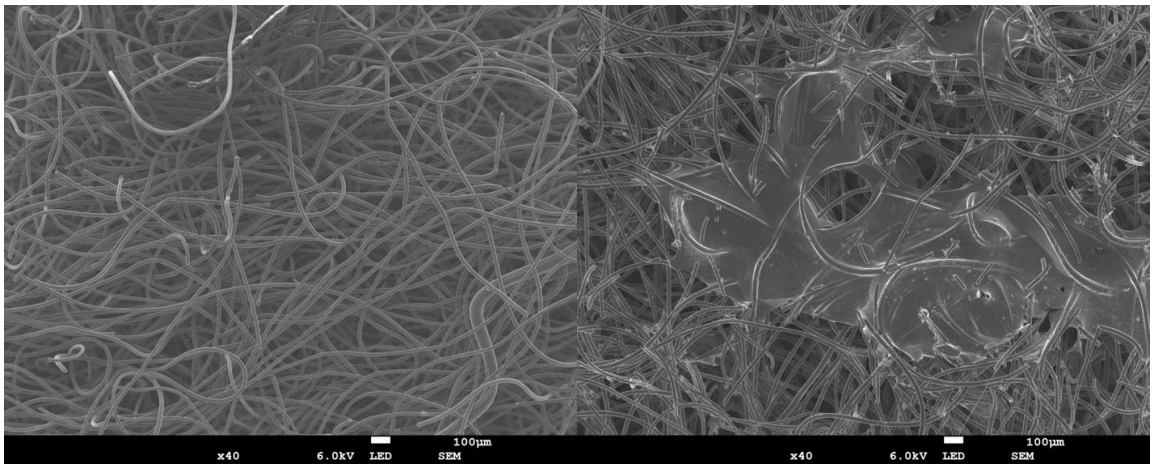


Figure 28 SEM micrographs of bare and PEDOT:PSS dip-coated carbon felts with magmagnification of X40 (left to right)

PEDOT: PSS film patches also appeared in pores and between the fibers. However, after CRP treatment (whatever gas mixture) the coating film appeared more uniformly distributed in areas where it was deposited, covering almost entirely and more homogenously, the individual carbon fibers surface as shown (Figure 29).

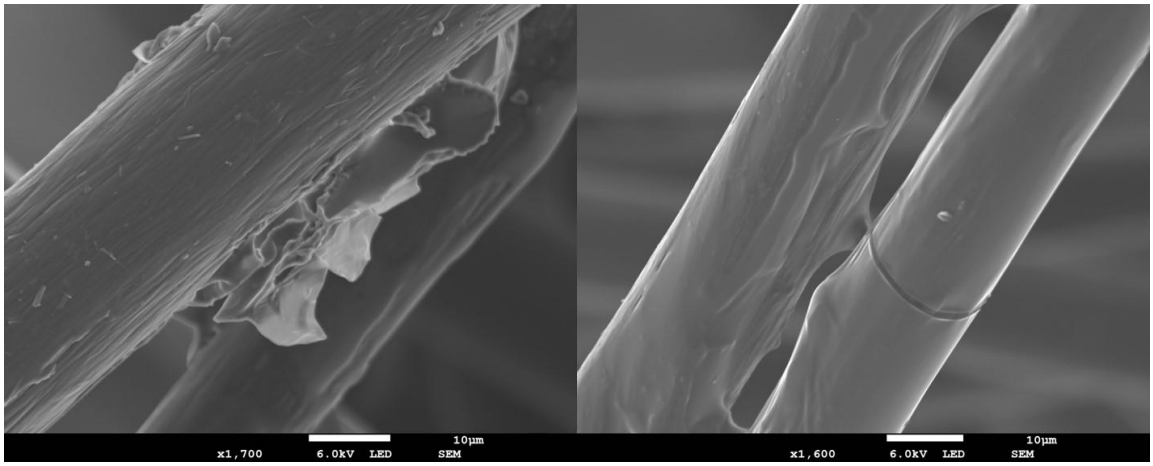


Figure 29 PEDOT:PSS deposition on carbon fibers before and after CRP 2% oxygen with magnification of X1700 and X1600 (left to right)

However, the samples subjected to plasma treatment showed to have some fractures in the coating layer after plasma (Figure 30), these fractures appeared to happen after forming the uniform coating on the carbon fibers.

The aim of using the diluted mixture of PEDOT:PSS was to maintain the open pores in the original untreated felts, and to prevent a thick cloaking polymeric film from forming over the surface of the samples, and as appeared from the micrographs that goal was achieved.

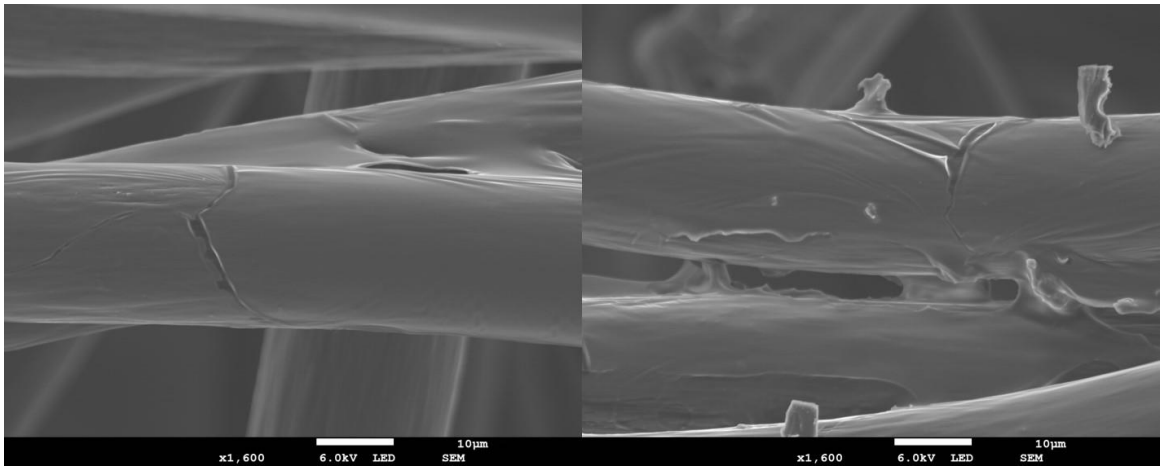


Figure 30 Fractures on PEDOT:PSS coating after CRP with magnification of X1600

Nevertheless, further porosity assessment was performed to estimate the pore size changes before and after dip-coating. Mean bubble point, which indicates the "bigger pore size", and both mean and median pore sizes were measured for both VCF and PPCF samples. The results presented in (Table 5) show that there was a small reduction in the biggest pore size, as well as the mean pore size. However, overall the dip-coating process maintained most of the initial porosity of the bulk, which is very important in order to maintain the high specific surface area of samples as previously mentioned.

Table 5 Changes in pore size after dip-coating with PEDOT:PSS diluted dispersion

Pore size (µm)	VCF	PPCF
Biggest pore size	99.6	94 ± 6
Mean pore size	50 ± 1	42.6 ± 6
Median pore size	39 ± 1	39.4 ± 2

FTIR spectra

FTIR spectra of VCF with or without PEDOT:PSS coating, and after CRP treatment using two gas mixtures are illustrated (Figure 31). The spectra after PEDOT:PSS coating confirmed integration of

carbonyl groups, and mostly oxygenated groups appeared for both PPCF 1% and PPCF 2% samples after plasma treatment, in addition to amino groups. Different peaks have appeared after CRP such as; a broad weak peak at 3350 cm^{-1} that was assigned to N-H stretch vibration, due to the aromatic secondary amines [270]. A double peak around 2850 and 2920 cm^{-1} was attributed to =C-H stretching vibration of an aldehyde group. A single peak appeared around 1733 cm^{-1} was assigned to C=O stretching vibration of carbonyl group, at 1250 cm^{-1} was attributed to =C-N vibration of secondary aromatic amines, while C=O stretch vibration around 1650 cm^{-1} was assigned to secondary amide group [271,272]. The peak between 1030 -1155 cm^{-1} was assigned to C-O group of an ether, while the weak peaks at 1415 cm^{-1} were assigned to symmetric stretching vibration of carboxyl group [194]. The peak at 940 cm^{-1} was attributed to deformation vibration of N-H group. The ring vibrations in aromatic amines appeared as interactions between C=C and C=N stretch vibrations between 1450 and 1615 cm^{-1}.

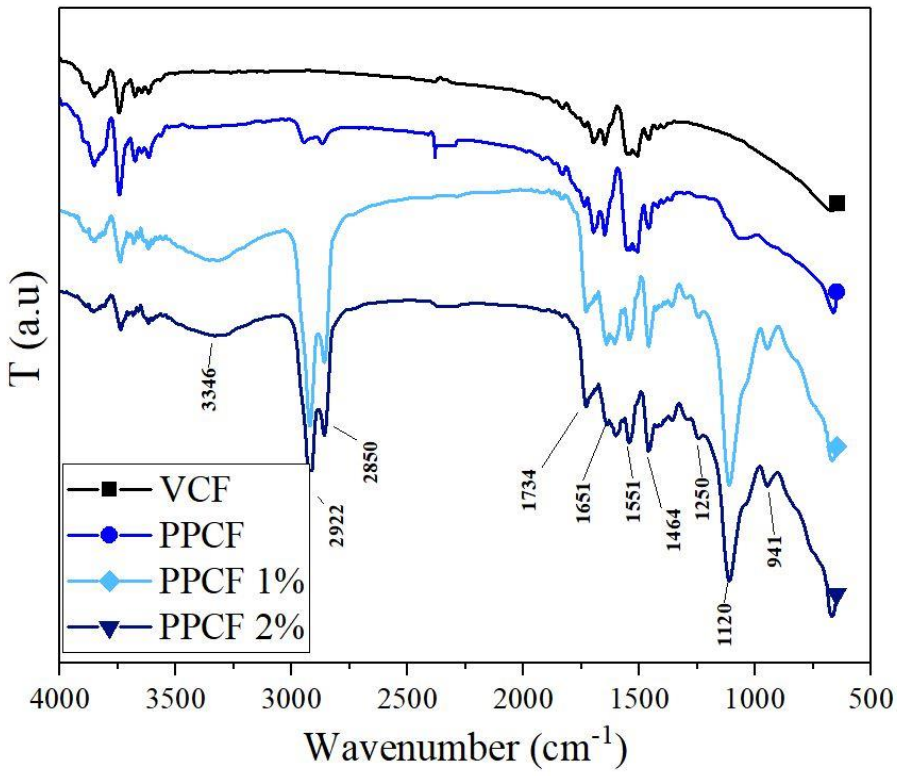


Figure 31 FTIR spectra of dip-coated samples with PEDOT:PSS compared to VCF

Water contact angle and capillary measurements

As mentioned before, the water contact angle of VCF was 116 ± 1º at room temperature and there was almost no capillary uptake for these felts. After the dip-coating process with PEDOT:PSS, water contact angle was reduced to 61 ± 5º at room temperature, and water capillary uptake was increased to 315% of the dry weight of felt sample.

After CRP treatment immediately, the water contact angles and capillary uptake values were measured for all samples. In addition, after 4 weeks from treatment these values were re-measured in order to estimate the ageing effect. Results are shown in (Table 6).

Table 6 Water contact angle and capillary uptake values after CRP for the fresh and aged samples

Sample	Θ° directly after CRP	Θ° after 4 weeks	Capillary uptake after CRP (%)	Capillary uptake after 4 weeks (%)
PPCF 1%	57 ± 4	64 ± 4	415 ± 8	410 ± 11
PPCF 2%	59 ± 8	70 ± 5	450 ± 14	427 ± 25

CRP treatment resulted in even further reduction of water contact angle for the dip-coated samples, and increased capillary uptake from almost 315 % to 415 – 450 % for the freshly treated PPCF 1% and PPCF 2%, respectively.

After storage for four weeks in clean closed dark containers, water contact angle increased specially for the PPCF 2%, from 59° to 70°, while the capillary uptake was almost maintained. This may be explained by the ageing effect that was also observed with other plasma treatments [238]. Nonetheless, in case of PPCF 2%, the ageing effect was more pronounced after storage.

The ageing phenomenon showed to be different between the internal and external fibers of PPCF treated with CRP. The samples maintained almost all the initial capillary uptake value, hence the oxidized species on internal fiber surfaces are not readily subjected to ageing as much as those external felt surface.

XPS

The typical XPS survey spectra of PPCF, PPCF 1% and PPCF 2% samples were collected. The main resonance peaks were C 1s, N 1s and O 1s in the untreated VCF felt as previously mentioned, and after PEDOT:PSS coating the C 1s, O 1s were predominant. The relative atomic contents of all samples are shown in (Table 7).

Table 7 Relative atomic content in percentage as calculated from XPS spectra

Sample	C 1s	C-C	C-O	C=O	O 1s	N 1s	S 2p
VCF	90.42	90	10	___	5.36	4.22	_____
PPCF	76.8	61.3	38.7	0	20.8	1.2	1.2
PPCF 1%	67.4	28.1	65.3	6.6	30.8	1.3	0.5
PPCF 2%	68.6	31.4	58.4	10.2	31.2	0	0.3

In the case of PPCF felt, compared to the untreated VCF, the coating alone has increased the percentage of oxygen atoms (from 5 to 20%), decreases the percentage of nitrogen atoms (from almost 4 to 1%) and sulfur atoms were also detected.

The high C-C content in VCF (90%) was reduced to 60% after the PEDOT:PSS coating in PPCF and to 30% after the CRP treatment (PCCF 1% and PPCF 2%). The relative carbon content on the surface is decreased by the increase of oxygen, this is due to oxygen presence in PEDOT:PSS and oxidation after CRP treatment.

In PPCF samples, the increase in oxygen atoms after PEDOT:PSS dip-coating is due to the increase in (C-O) groups only (38% compared to 10% in VCF), and no (C=O) group is detected. This might be due to (PEDOT) part of the coating that bears ether groups. However, after CRP treatment the (C-O) content increased from 30% to 65% for PPCF 1%, and to 59% for the PPCF 2%.

For better understanding, the different chemical group relative contents were compared to that of bare carbon felts treated with CRP using both gas mixtures, CF 1% and CF 2% presented earlier [273]. The 65% (C-O) content of PPCF 1% seemed to be a value close to the sum of (C-O) in PEDOT:PSS (38%) and (C-O) on CRP treated bare CF 1% (29%).

The (C=O) content is measured for PPCF 1% and PPCF 2%, their values are lower than those of bare samples CF 1% and CF 2%. A higher level is detected in PPCF 2% (10%) than in PPCF 1% (6%) compared to 0% in PPCF samples.

Analysis of N 1s high-resolution spectra show that a significant ratio of nitrogen atoms (4%) was already present in the untreated bare VCF, which may be explained by the fact that the carbon fiber felt used in this study has been synthesized by oxidation of nitrogen-containing polyacrylonitrile (PAN) polymer. After CRP, deconvolution of the N 1s photopeaks (Figure 32) and (Table 8) revealed that on VCF, almost 50% of nitrogen atoms were in form of pyridine (*BE* = 398.4 eV) and the other 50% in form of pyridinium (*BE* = 401.5 eV) [274]. After dip-coating with PEDOT:PSS (PPCF), the ratio of pyridine / pyridinium was almost maintained, although total nitrogen content was reduced to 1%. After CRP of PPCF with 1% oxygen, the nitrogen content on the surface was maintained to almost 1% in PPCF 1% , but a new peak at (*BE* = 400.7 eV) most probably due to pyridone amides appeared. This last peak was already observed in the case of VCF treated with 1% oxygen. Nitrogen was not detected in the case of PPCF 2%.

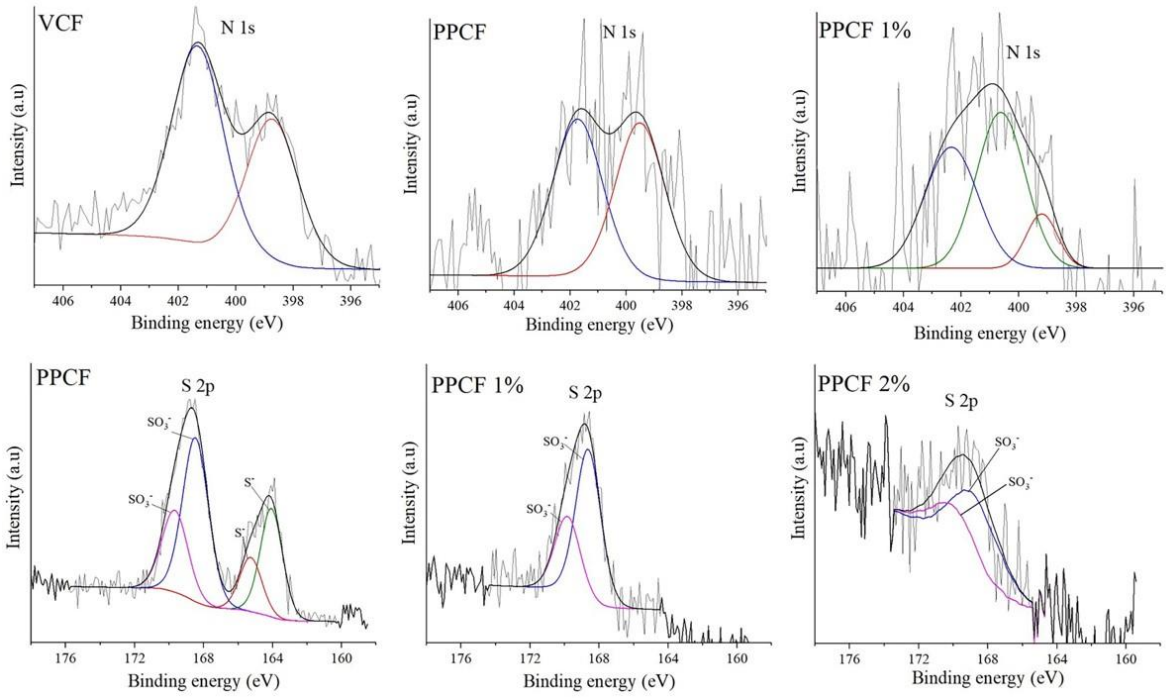
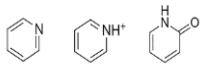


Figure 32 High-resolution XPS spectra of N 1s and S 2p for different carbon-based samples

There was a decrease in (S) content on PEDOT:PSS dip-coated felts after CRP, as the percentage of oxygen in plasma increases (Table 8) .The deconvolution of high-resolution spectra of S 2p show four peaks (Figure 32). Two peaks of higher binding energies (168.5 eV and 169.7 eV), corresponding to the sulfur atoms in PSS polymer and related to sulfonate groups (SO_3^-), and two peaks of lower energies (164.5 eV and 167.2 eV) related to sulfur (S^-) atom linked to carbon in PEDOT part.

Table 8 Relative atomic content and ratios as calculated from XPS spectra

Sample	S 2p (%)	S^- ($S\ 2p_{3/2}$) 164.1eV	S^- ($S\ 2p_{1/2}$) 165.7eV	SO_3^- ($S\ 2p_{3/2}$) 168.5eV	SO_3^- ($S\ 2p_{1/2}$) 169.7eV	S^- : SO_3^- ratio	N	 Pyridine Pyridinium Pyridone
PPCF	1.22	24	12	42	21	36:63	1.23	Pyridine/ Pyridinium 50/50
PPCF 1%	0.52	0	0	62	31	0:100	1.29	Pyridinium/ Pyridone 50/50
PPCF 2%	0.26	0	0	66	33	0:100	0	_____

The ratio of PEDOT/PSS can be estimated, for PPCF it was 1: 1.75; and after CRP (1% or 2%) peaks due to sulfur in PEDOT were not detected, only PSS seemed to be present.

CRP leads to oxidation of the positively charged PEDOT. As the charge of PEDOT becomes more negative, repulsion occurs between negative PSS and PEDOT. With 2% plasma, no S^- group, i.e. no PEDOT is detected on the surface.

Electrical resistivity and cyclic voltammetry

The measurements of the bulk electrical resistivity of each dry sample showed that PEDOT:PSS coatings for carbon felts maintained their initial conductivity of the bulk and very slightly improved it. Furthermore, after CRP treatment using 1% oxygen, the electrical resistivity of PPCF 1% sample was vaguely lower than the original PPCF before the treatment. However, when the percentage of

oxygen in the plasma gas mixture increased to 2%, the electrical resistivity increased remarkably and deviated among the samples (Figure 33).

In accordance, cyclic voltammetry scans showed better electrochemical behavior for samples coated with PEDOT:PSS than the bare VCF. Furthermore, the capacitive currents of PPCF 1% samples were higher than other samples for the same conditions and scan rate.

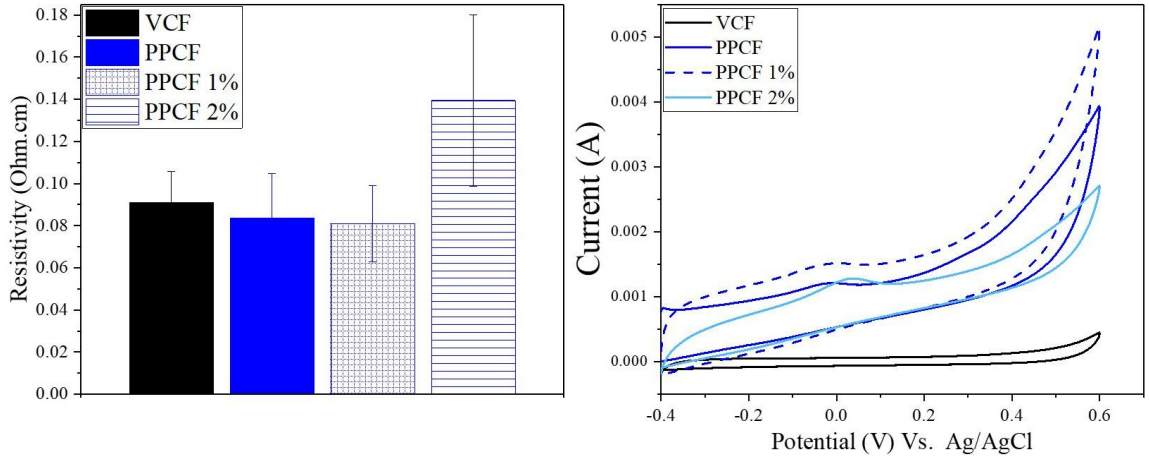


Figure 33 Electrical resistivity of the VCF, PPCF, PPCF 1% and PPCF 2% using 4-probe method, and cyclic voltammograms in 0.01M PBS solution at 0.01V.s^{-1} scan rate, pH 7 and room temperature (left to right)

SEM and colorimetric assays

The SEM micrographs of the felt samples bio-functionalized with GOx enzymes after CRP are shown in (Figure 34). It seemed from the micrographs that the regions of the samples with coating, the enzymes are mostly adsorbed onto the polymer spots, and noticeably smaller quantity onto the uncoated grooved regions of the bare carbon fiber surface.

The enzymatic activity for both free and immobilized enzymes was measured. (Figure 35) illustrates the activity expressed in percentage, compared to the activity of free enzyme present in 3 mL of aqueous solution (0.2574 U).

In accordance with the results obtained for the bare carbon samples, the CRP treated felts gave higher enzymatic activity values in most samples. When enzymatic activities are compared for enzymes adsorbed at pH 5.5 and pH 7, the pH 5.5 resulted in better results after immobilization process.

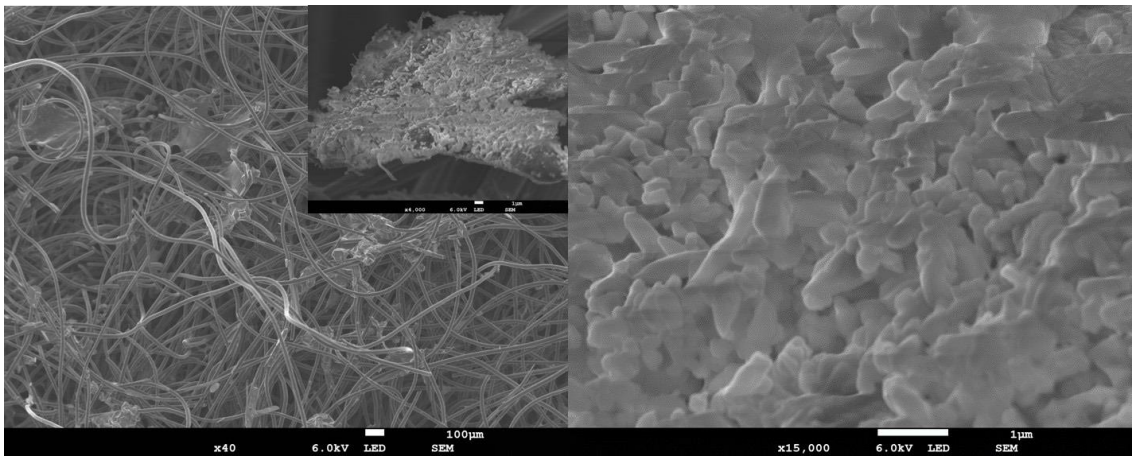


Figure 34 SEM micrographs of GOx immobilized on PPCF 2% in different magnifications (left to right X40, X4K inset, X15K)

As can be seen from the histograms, the standard deviation values were high for some samples immobilized at pH 7, and the stability and reproducibility of the samples of pH 5.5 were better. Based on the results, it is clear that highest activity was obtained for immobilization using PPCF 2% at pH 5.5, and up to 60% of the activity of free enzyme was maintained for the first cycle. In addition, PPCF 1% also resulted in a very close value for the first cycle but maintained a better stability in following cycles than PPCF 2%. Not all free enzymes necessarily maintain their bio-catalytic activity after immobilization, 37% only of the total free enzyme activity was maintained after immobilization on VCF as previously mentioned.

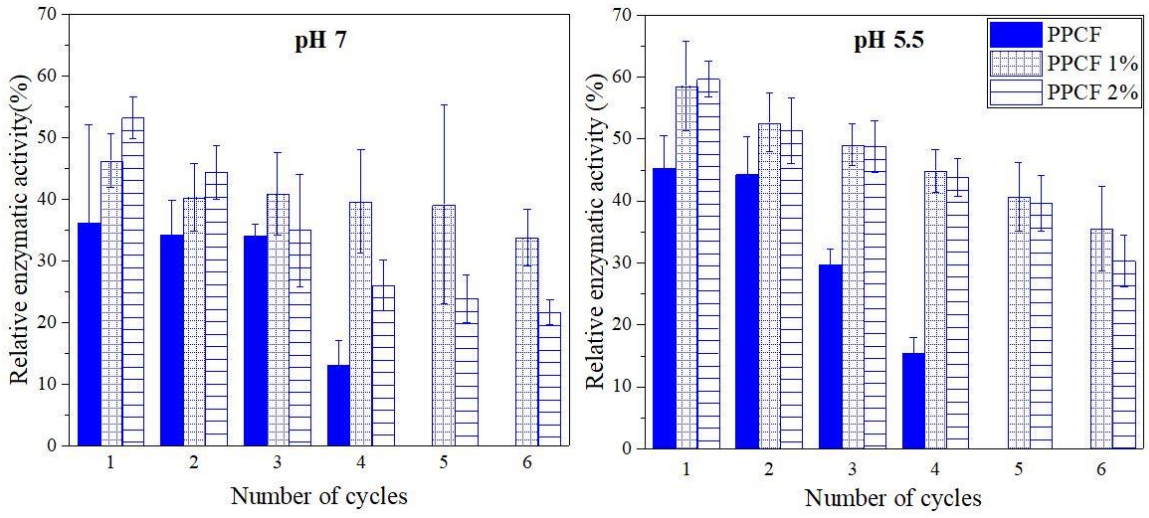


Figure 35 Relative enzymatic activity PPCF, PPCF 1% and PPCF 2%, respectively

To evaluate the reusability of these felts, six cycles of the activity were performed as mentioned earlier. CRP treated bio-functionalized samples maintained better enzymatic activity after higher number of cycles. The samples with no plasma treatment (PPCF) showed no enzymatic activity after the fourth cycle; while CRP treated samples maintained a high percentage of their activity after 6 cycles. PPCF 1% felt with enzyme immobilized at pH 5.5, maintained up to 36 % of activity in the sixth cycle, compared to 15 % of the activity on the fourth cycle for the PPCF samples, with same conditions.

Furthermore, CV scans were performed to check the redox behavior of the bio-functionalized samples in the PBS solution in presence of FCA and D-glucose as the mediator and substrate of the enzyme, respectively.

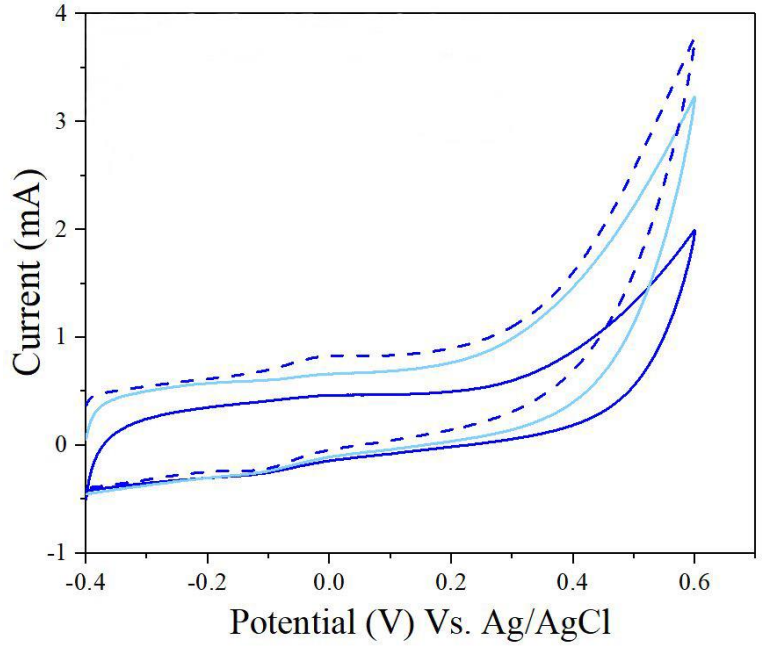


Figure 36 Cyclic voltammograms of PPCF 1% with immobilized GOx in 0.01M PBS and in presence of FCA as a mediator at scan rate of 0.01V.S^{-1} at pH 7 and room temperature using different concentration of D-glucose (0 - 8.5 - 17 mM respectively from bottom to top)

To assess the response of GOx immobilized on the felt when the substrate is added, CV scans of PPCF 1% with and without D-glucose were performed at a scan rate of 0.01 V.s^{-1} and in the presence of FCA in PBS buffer (Figure 36). The results show the increased oxidation current with the increased added D-glucose, which indicates that GOx are maintaining a part of their activity after the immobilization process. However, it is worth mentioning that no significant increase in the

oxidation current was observed when D-glucose concentration was higher than 17 mM for most dip-coated samples.

Cyclic voltammetry response or catalytic currents of different samples in absence and presence of different concentrations of D-glucose (8.5, 17 and 25.5 mM) are shown (Figure 37). An increase of the bio-catalytic current occurred because of the increase of D-glucose added to medium, suggesting that GOx maintained its enzymatic activity after immobilization via adsorption and the obtained felts with coatings are bioactive, in accordance with the colorimetric assays.

It's worth mentioning that, above 17 mM concentration of glucose, no significant increase in the bio-catalytic currents, and a plateau was reached at around 2.7 mA and potential of around 0.5 V for PPCF 1%. To estimate the parameters of GOx kinetics, Lineweaver-Burk was also used to determine the apparent I_{max} and K_m from the plots at 0.5 V, the apparent I max values were 1.48, 2.71, 2.45 , while the apparent Km were 5.62, 1.86, 2.29 for PPCF, PPCF 1% and PPCF 2%, respectively.

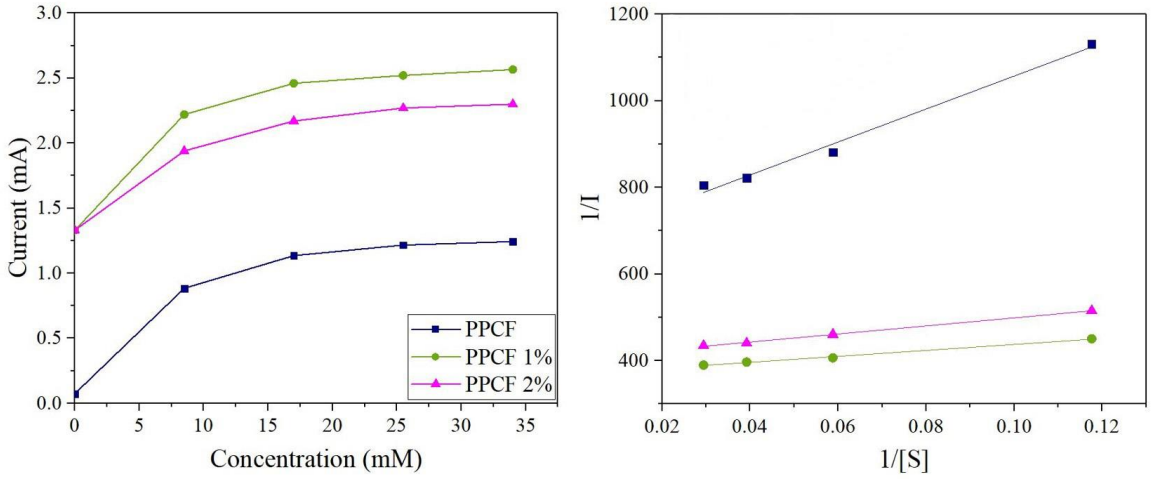


Figure 37 Catalytic current vs. D-glucose concentration at E = 0.5V, and Lineweaver–Burk fitting of the activity of GOx immobilized on PPCF, PPCF 1%, PPCF 2% (R^2 = 0.9897, 0.9955, 0.9986 respectively)

III- B- 3- Discussion

The purpose of this study was to investigate the influence of CRP (N_2 + O_2) on properties and conductivity of carbon nonwovens dip-coated with PEDOT:PSS aqueous dispersion, for optimized immobilization of GOx enzyme.

On the virgin untreated VCF fibers, the PEDOT:PSS coating appeared to be relatively thick, and deposited as patches due to the use of diluted dispersion, which resulted in uneven coverage after drying. Nonetheless, following CRP treatment, this film became thinner after better spreading on the surface of the fibers, and more even in the spots were the polymer is deposited. It seemed that the CRP treatment of the PPCF would activate both the uncoated part of the carbon fiber along with the PEDOT:PSS coating. The increase in oxygen content of the uncoated regions of the carbon fiber increases its surface energy [273], and allows better spreading of the PEDOT:PSS on the surface of the individual carbon fiber within the felts. This better coverage was probably due to the interactions between the hydrophilic negatively charged PSS, and the hydrophilic positively charged pyridinium regions of the carbon felts through electrostatic and hydrogen bonds. Indeed, FTIR spectra showed the peaks between 1450 and 1615 cm^{-1} that were assigned to ring vibrations in aromatic amines (pyridine and pyridinium). These peaks were present even after plasma treatment, in addition to the peak around 940 cm^{-1} that was attributed to the out of plane N-H deformation vibration of the pyridinium that was still present in the samples after the CRP [271]. That coverage prevented the efficient detection of nitrogen groups of the carbon surface as it was observed in the corresponding XPS spectra. The thinner coating layer formation explains the significant decrease in intensity in both PEDOT sulfur and PSS sulfur peaks in the PCCF 1% fiber surface XPS analysis. The PEDOT sulfur peaks were reduced to even a greater extend in PPCF 2% due to two main factors, an over-oxidation of PEDOT sulfur, in addition to phase segregation with the tendency of PSS to be on the outer layer, since the ionic bonds between the PEDOT and PSS were broken due to charge changes after oxidation in PPCF 2%.

The electrical resistivity in case of 1% plasma was maintained and slightly improved. This might be due to the reduction in positive charge of the PEDOT, which would lead to breaking of the ionic bonds between the PEDOT oligomers and the PSS chains. The negative charges remaining on the PSS chains repulse each other, resulting in an improvement of their alignment [275]. In this stage, the conductive PEDOT oligomers accumulate between the aligned chains of PSS forming local grains of improved conductivity [275] (Figure 38). However, with increased plasma treatment (2% oxygen), most of the electrostatic bonding between the hydrophilized negatively charged PEDOT

and the hydrophilic negatively charged PSS are broken. In addition, oligomers of PEDOT were formed due to chain scission and formation of –C=O and –COOH groups as was shown in XPS and FTIR spectra, and conductivity is decreased because of oxygen interposition [276]. Furthermore, the polymer spreading could have negatively influenced the electrical conductivity because of higher local strain which causes fracture of the PEDOT:PSS film [215] (Figure 30), in addition to the previously mentioned changing in the PSS and PEDOT phase segregation at this point, which result in increased PSS rich insulating regions (Figure 38).

The increase of the water contact angle and water capillary uptake in the plasma treated samples can be attributed to the oxidation of the bare and coated fibers and the integration of the new functional groups on these surfaces. Furthermore, the increased hydrophilicity of the PEDOT:PSS coating within the felts increased the water uptake values and maintained it even after ageing for 4 weeks.

The enzymatic activity values when pH 5.5 was used during the adsorption were higher than of those at pH 7 in general. This is maybe due to the fact that pH 5.5 is considered the optimum value of the enzyme glucose oxidase GOx (EC 1.1.3.4) which can still be active over a range between pH 4 and 7 [277].

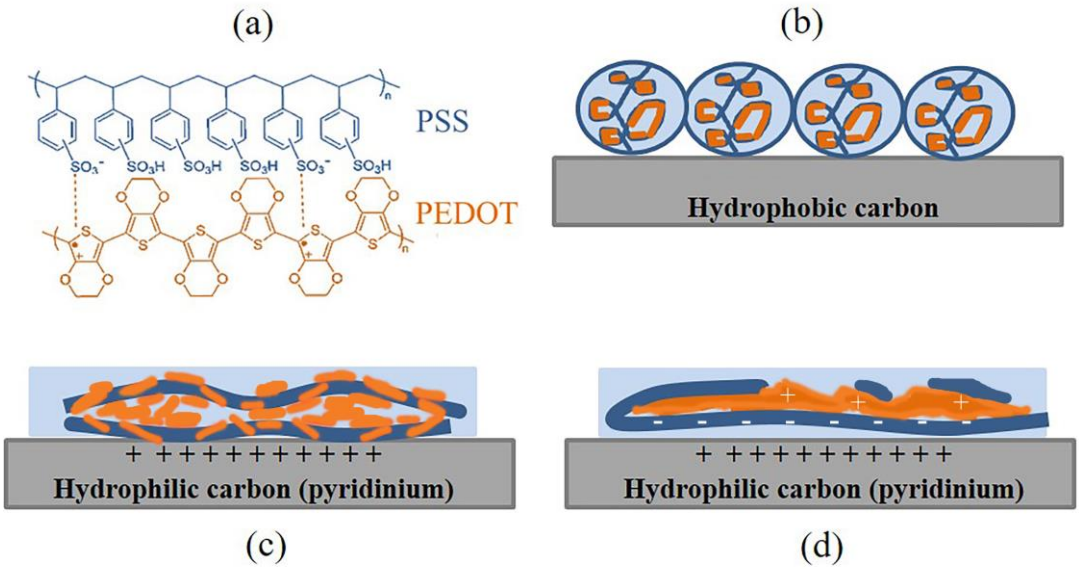


Figure 38 The structure of PEDOT:PSS (a), colloidal gel particles of PEDOT:PSS on hydrophobic carbon (b), ionic bonds between PSS and hydrophilic regions of carbon, and ionic bonds between PSS and PEDOT start to break (c), and PEDOT oligomers form conducting grains (d)

The reusability and shelf life were improved after the CRP treatment for all samples. This might be due to amino groups on the surface of the treated fibers as previously shown in FTIR and XPS spectra, in addition to the oxygenated groups like carbonyl. These groups can improve the bonding between the enzymes and the surface of the felts especially with hydrogen bonding which reduces the leaching of the enzymes and maintain the activity for higher number of cycles. Furthermore, the GOx enzyme has an Isoelectric Point (IP) of 4.2 [277], it exists in the form of an anion in the range of pH above this value, which is the range used in our study. Thus, it can form electrostatic bonding with the positively charged regions on the surface after CRP treatment (pyridinium and PEDOT).

The apparent I_{max} values determined from CV scans show that CRP treated felts resulted in higher currents (for one cycle scan), in accordance with chemical colorimetric assay for the enzyme. Since I_{max} is attributed to quantity of the active units of enzyme, thus plasma improved the adsorption of GOx on felts surface, in comparison with PPCF. The apparent K_m values indicate an increase in enzyme's affinity towards its D-glucose occurred after immobilization on CRP treated samples, which suggest a better confrontation between the enzyme and the treated surface, which maintained the accessibility of the active sites and helped GOx to reach its maximum capacity at slightly lower concentrations of glucose.

However, the increase of the percentage of oxygen in the gas mixture used for CRP up till 2% did not show to give better characteristics for our samples, on the contrary, it had a negative influence on the electrical conductivity of the coated felts (due to the modification of PEDOT:PSS). Hence, the 1% oxygen showed to be sufficient in increasing the wettability in general of the coated carbon felts with PEDOT:PSS, and slightly improved the electrical conductivity and the enzyme immobilization process of the treated samples. Nevertheless, more research should be held to optimize the ratios of the gas mixture used in CRP treatment, and to study their influence on the structure and uniformity of polymeric coatings used in functional and smart textiles.

III- C- Immobilization of glucose oxidase via crosslinking with a naturally occurring crosslinking agent (Genipin)

III- C- 1- Introduction

The use of genipin is expanding in many fields and industries, and the focus on reducing its cost is a main field of research, due to the big potential of this material as mentioned in **Chapter I**.

Application of genipin in crosslinking of redox enzymes has been reported with the focus on crosslinking hydrogels like chitosan and gelatin as matrix for enzymes.

However, very rarely the use of this crosslinker was reported in direct reaction and crosslinking of pure enzymes without the matrix.

It was reported that genipin was used to crosslink glucose oxidase with another enzyme catalase for the synthesis of gluconic acid [278]. This attempt was successful in reducing the distance between the enzymes to improve the efficiency of the production of gluconic acid.

However, the use of glucose oxidase directly with genipin for immobilization on conductive textile has not been reported to the best of our knowledge. Since genipin can react with primary amino acids as shown in (Figure 39), the obtained aggregates can be immobilized on a conductive textile carrier.

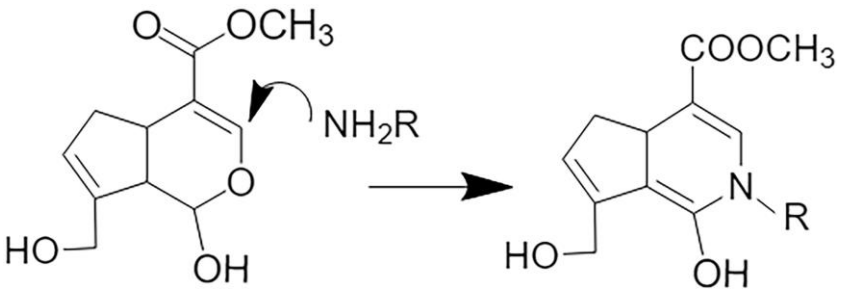


Figure 39 Genipin reaction with primary amines

Hence, in this chapter, an attempt of conducting this reaction to immobilize glucose oxidase was carried out, in order to produce a bio-functionalized carbon-based felt with low toxicity and fewer steps. Characterization of the obtained materials was carried out using UV-Vis spectroscopy and

FTIR. Furthermore, the impact of crosslinking on bioactivity and reusability of GOx was also assessed using the colorimetric chemical assays and cyclic voltammetry scans, as described in the previous chapters.

III- C- 2- Enzyme immobilization via crosslinking with genipin

Solution of 30 mM (or 60 mM) of genipin was prepared using Milli-Q water, and glucose oxidase was added to the mixture at room temperature (1 $mg.mL^{-1}$). The mixture was stirred on a magnetic stirrer for 30 min, and then 1 mL of the previous mixture was added to 1 cm^2 of carbon-based sample. The samples were kept for 24 h at 4 ºC, followed by rinsing twice with a buffer solution (pH 7) and stored at 4 ºC for further use.

The activity of GOx immobilized on the various carbon felts using genipin, was compared to that of GOx enzymes in the cross-linked aggregates of GOx/genipin, which were 0.62 ± 0.04 and 0.69 ± 0.007 $U.mL^{-1}$ for 30 mM and 60 mM of genipin respectively, in the free state without immobilization on carbon-based samples. The results of the activity are presented in percentage.

III- C- 3- Results

UV-Vis spectroscopy

The reactional mixture of GOx enzyme with genipin was studied before its immobilization on carbon felts, using UV-Vis spectrophotometer. After 1 h of preparing the mixture, it maintained its initial appearance as a pale yellow-colored solution.

The UV-Vis spectrum showed a significant peak between 200 - 300 nm (Figure 40), which is attributed to both GOx and genipin which have an absorbance peak around 280 nm [19], and 240 nm [220], respectively.

However, when the spectrum was collected for the same mixture after 8 h, a new absorbance peak appeared around 600 - 605 nm. This peak was intensified even more, after 24 h as illustrated (Figure 40).

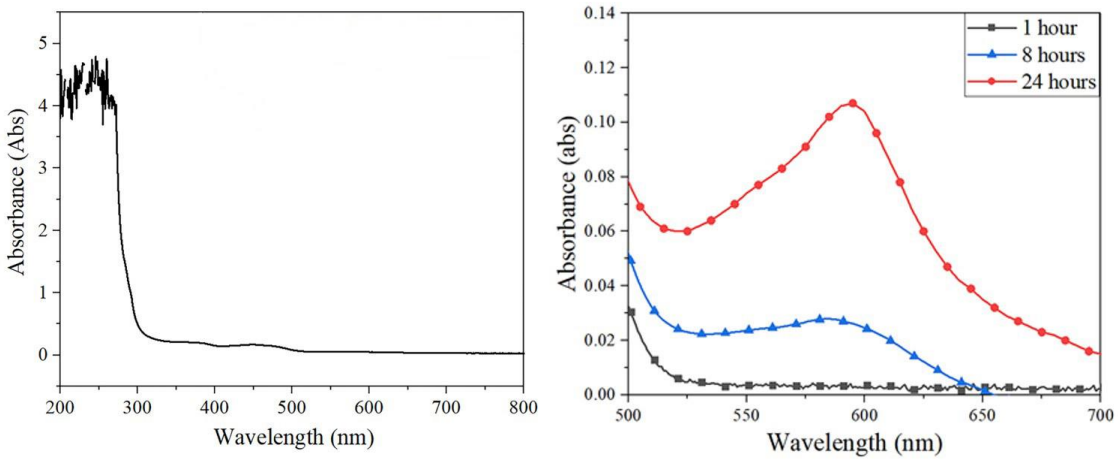


Figure 40 UV-Vis absorbance spectrum for GOx and genipin mixture after 1h, and UV-Vis absorbance of GOx crosslinked with genipin with time after 1 - 8 - 24h (left to right)

This peak appeared in the area of the blue absorbance of the spectrum, which was also detectable by naked eye that the enzyme/genipin mixture yielded a blue hint coloration regardless of the low concentration of GOx used (Figure 41).

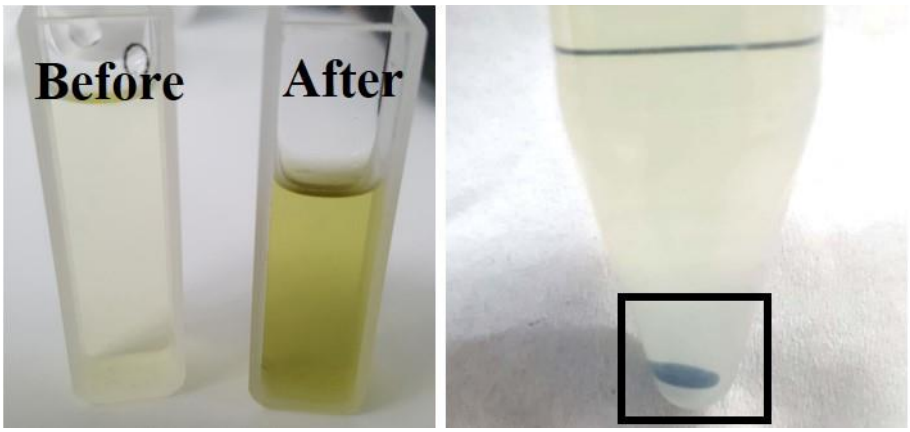


Figure 41 Blue pigment formation from GOx crosslinked with genipin 30 mM and 60 mM (left to right)

When the concentration of genipin increased in the mixture to 60 mM, the same phenomena occurred. However, precipitation of the genipin crystals occurred due to its high concentration in the solution, and some of these crystals were blue tinted as observed by the naked eye and under microscope, especially on the side where it is the most exposed to water (Figure 42).

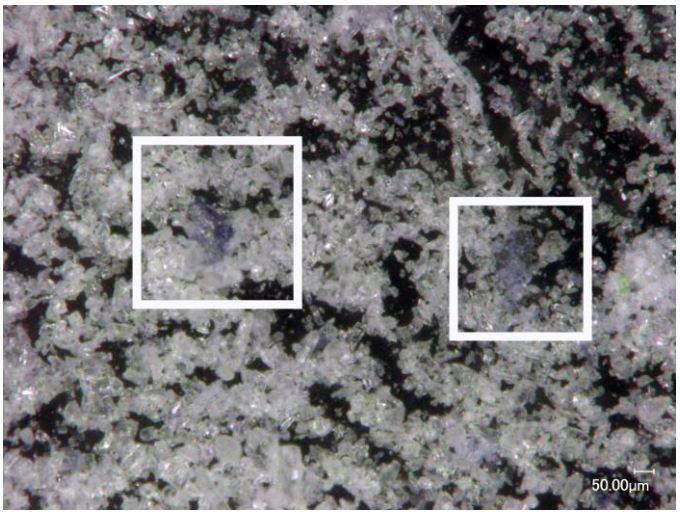


Figure 42 Microscope image of blue color formation due to GOx crosslinked with genipin

FTIR of GOx crosslinked with genipin

The FTIR spectra of genipin, GOx before and after crosslinking reaction are illustrated (Figure 43).

The FTIR spectrum of genipin shows an absorption peak at 1680 cm^{-1} which was attributed to C=O stretching vibration of carboxymethyl group, and 1622 cm^{-1} was assigned to C=C absorption peak of the olefin ring in genipin [279]. The peak in the region around 2800 – 3000 cm^{-1} was assigned to C-H stretching vibration. The double peak between 3000 - 3600 cm^{-1} in genipin spectrum is due to the overlapping of O-H and aromatic C-H vibration bands.

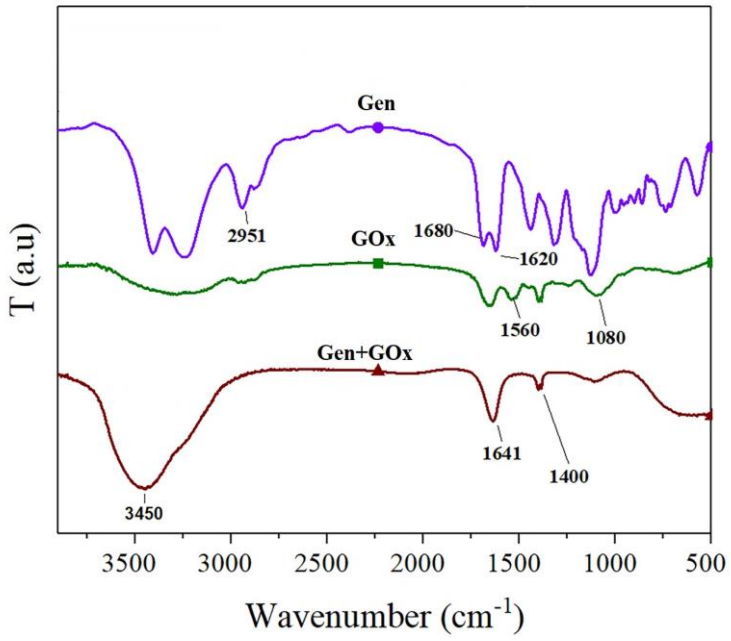


Figure 43 FTIR of genipin, GOx and their mixture after crosslinking for 24h (from top to bottom)

The spectrum of GOx shows the peak at 1560 cm^{-1} that was attributed to the primary amines that did not appear after crosslinking for 24 h. The spectrum of mixture after crosslinking shows that the peak at 1642 cm^{-1} was more pronounced, and was attributed to the formation of aromatic amine groups [250]. While the peak at 1400 cm^{-1} was assigned to C-N stretching vibration and the peak at 3450 cm^{-1} was assigned to stretching vibration of the O-H group.

SEM and colorimetric assays

SEM micrographs in (Figure 44) illustrate the deposition of the enzyme crosslinked with genipin on both bare and PEDOT:PSS coated samples that have been treated with CRP. The distribution and density of the crosslinked aggregates seemed dispersed and similar for samples before and after plasma treatment on the surfaces and within the different samples.

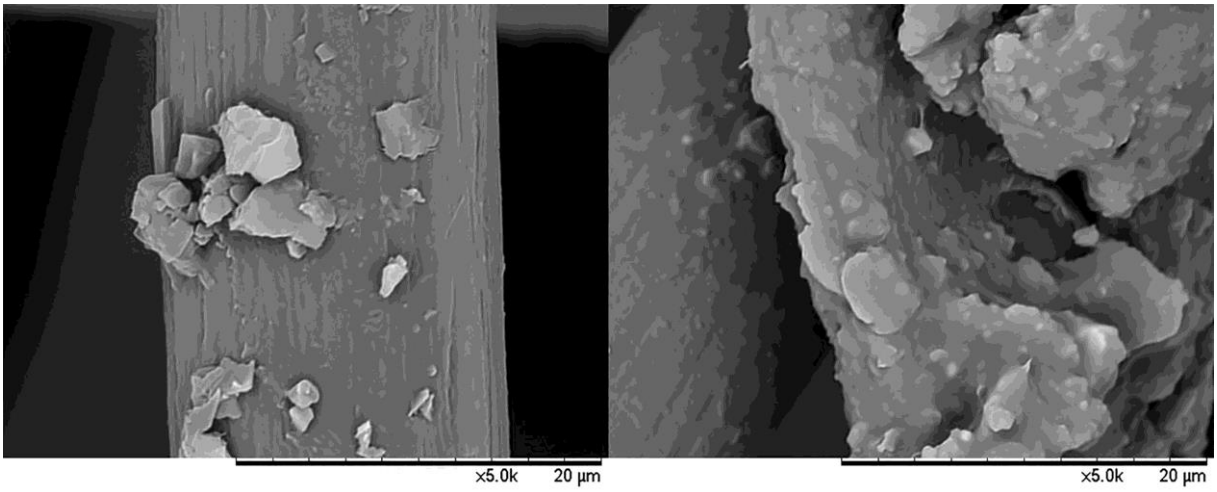


Figure 44 SEM micrographs of bare, and PEDOT:PSS coated CRP treated carbon samples with GOx crosslinked with genipin and X5000 magnification (left to right)

The relative enzymatic activity and reusability of the samples bio-functionalized with GOx crosslinked with both concentrations of genipin (30 mM and 60 mM) are illustrated (Figure 45) and (Figure 46). The activity of GOx immobilized on the various carbon felts using genipin, was compared to that of GOx enzymes in the cross-linked aggregates of GOx/genipin, in their free state without immobilization on carbon-based samples. The results of the activity were presented in percentage.

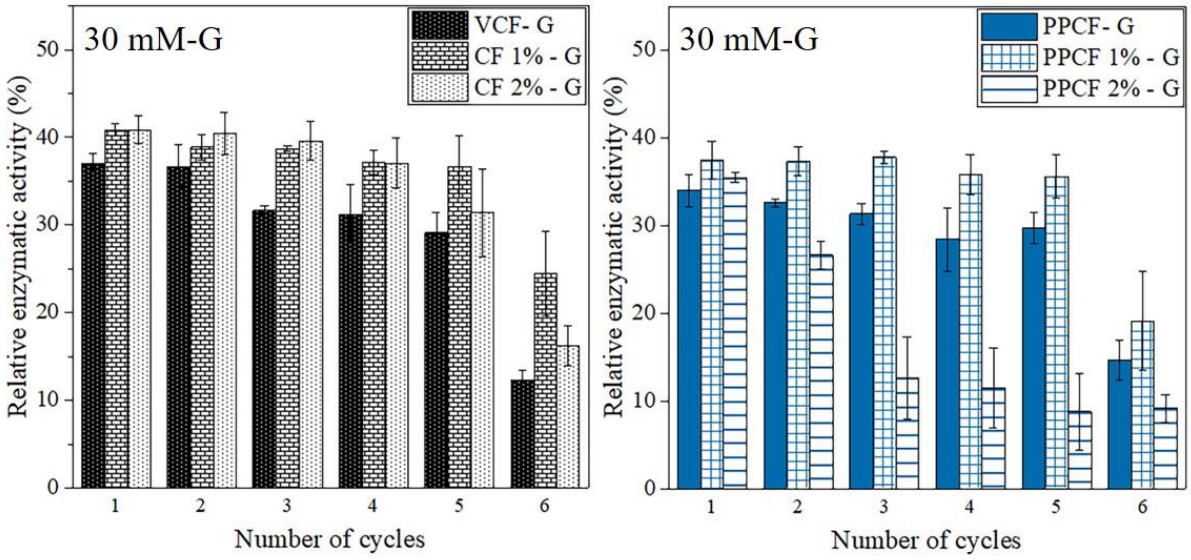


Figure 45 Relative enzymatic activity of GOx crosslinked with (30 mM) genipin after immobilization on bare carbon felts and PEDOT:PSS coated carbon felts (left to right)

It is noticeable from the histograms that around 40% of the enzyme activity was maintained after immobilization on different carbon felts using genipin. This percentage is slightly lower than results obtained by physical adsorption of enzymes. Nonetheless, genipin improved the stability in performance of the different samples; even in the case of felts without plasma (VCF –virgin carbon felt and PPCF PEDOT:PSS coated carbon felt) which showed better stability and reusability up to the sixth cycle, as determined by colorimetric assay using GOx activity kit.

However, the activity of PPCF 2% samples in general declined with the increased number of uses, in accordance with the previous remarks, that PPCF 2% was subjected to more pronounced ageing than other samples.

Furthermore, the higher concentration of genipin used to crosslink the enzyme (60 mM) did not result in higher enzymatic activity. Indeed, excessive crosslinking agent may result in loss of flexibility of the enzyme and thus reduces activity. However, the stability and reusability in performance for all samples used was improved. This might be due to the reduction of enzyme loss in the medium due to higher concentration of the crosslinker, which prevents leaching and improves stability [280].

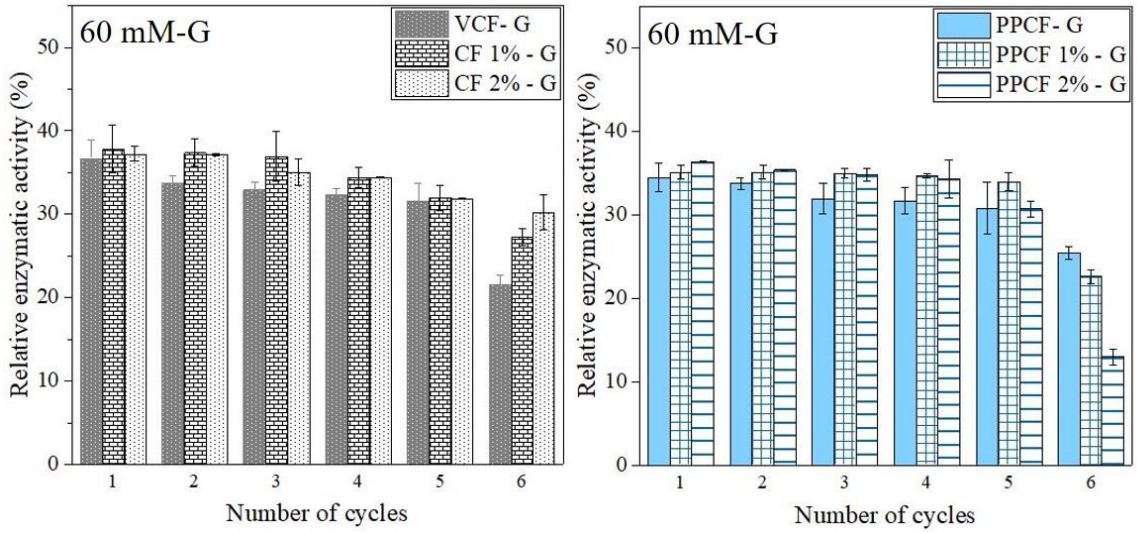


Figure 46 Relative enzymatic activity of GOx crosslinked with (60 mM) genipin immobilized on bare carbon samples and dip-coated samples with PEDOT:PSS (left to right)

In addition to the formation of aggregates of crosslinked GOx and genipin, the carbon-based felts as a support or a carrier made the reusability of these aggregates possible for several enzymatic activity cycles. The enzyme-genipin aggregates allow formations of hydrogen bonds, due to hydroxyl groups from genipin with different carbon-based samples, in addition to possible bonding between genipin and the secondary amines on the surface of carbon-based samples. However, a slightly better performance for carriers or felts treated with plasma 1% oxygen was noticed.

Concerning the GOx activity with higher concentration of genipin, the effect of the carrier or textile felts showed to be limited (Figure 46).

Cyclic voltammetry

The cyclic voltammetry scans for CF 1% - G and PPCF 1% - G in the absence and presence of different concentrations of D-glucose and using two concentrations of genipin (30 and 60 mM) are illustrated (Figure 47) and (Figure 48).

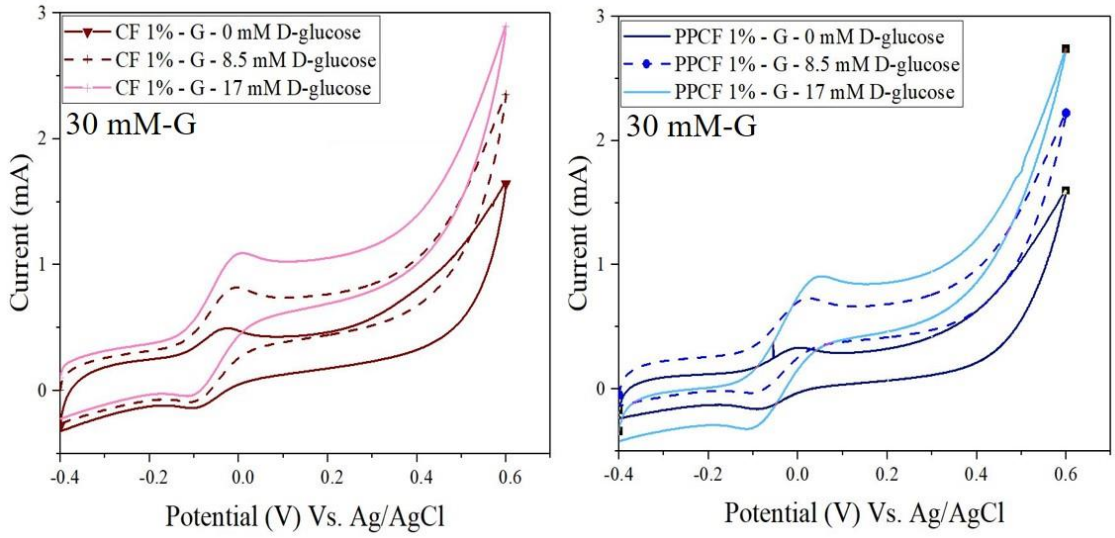


Figure 47 Cyclic voltammograms for GOx crosslinked with (30 mM) genipin immobilized on CF 1% - G and PPCF 1% - G in absence and presence of D-glucose (0, 8.5, 17 mM) at scan rate of 0.01 V.s^{-1} using FCA mediator

It was noticed that, an increase in the substrate concentration resulted in an increase in the biocatalytic activity of electrodes, suggesting that the immobilized enzymes maintained a part of their activity after crosslinking process. This effect was more pronounced when the concentration of genipin used was 30 mM. Moreover, a plateau was reached around 3 mA currents in most samples and regardless of the increased substrate concentration in accordance with similar observations in the previous chapters. When genipin concentration was 60 mM, the saturation with substrate was achieved at lower glucose concentrations.

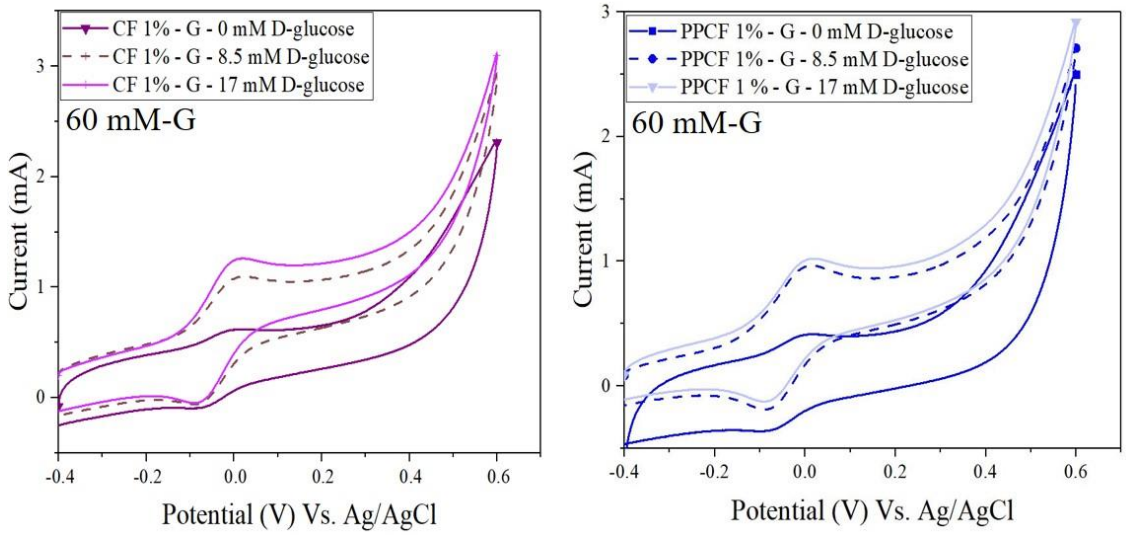


Figure 48 Cyclic voltammograms for GOx crosslinked with (60 mM) genipin immobilized on CF 1% - G and PPCF 1% - G in absence and presence of D-glucose (0, 8.5, 17 mM) at scan rate of 0.01 V.s^{-1} using FCA mediator

The apparent Michaelis - Menton constant K_m was estimated from the Lineweaver - Burk fittings (Figure 49). For the samples immobilized on CF 1%, K_m = 2.28 and 2.21 for 30 and 60 mM genipin respectively.

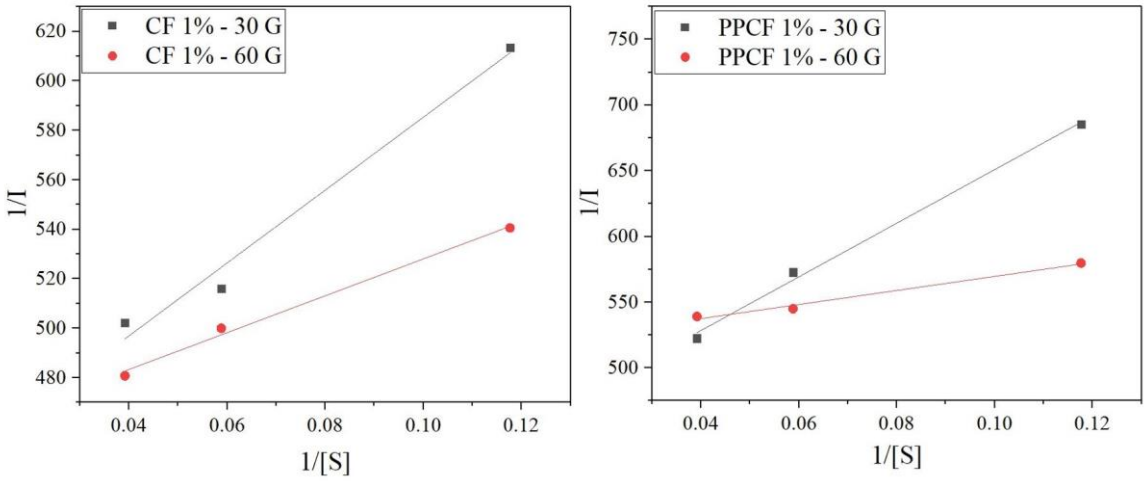


Figure 49 Lineweaver–Burk fitting of the activity of GOx crosslinked with genipin (30 or 60 mM) on CF 1% samples (R^2= 0.9834, 0.9939 respectively), and on PPCF 1% samples (R^2 =0.9960, 0.9883 respectively)

For samples immobilized on PPCF 1%, K_m = 4.56 and 1.1, for 30 and 60 mM genipin respectively. The apparent constant value of K_m = 4.56 for PPCF 1% - 30 mM indicates that there was a decrease in the affinity of GOx towards its substrate with maintained values for the current, since I_{max} is an indicator of the amount of the immobilized active units, while K_m indicates the affinity of these units towards the substrate regardless of their amount. This suggests that higher concentration of the D-glucose is required to achieve the same response, it can be seen from the scans that indeed this type of sample produced slightly lower currents and lower enzymatic activity as determined by the colorimetric assay.

III- C- 4- Discussion

The purpose of this study was to investigate the possibility of directly crosslinking GOx enzymes with a naturally occurring less toxic crosslinking agent genipin, and immobilize the obtain aggregates on carbon-based conductive textiles.

As observed by UV-Vis spectrophotometry, the blue pigment formation during the reaction between GOx enzyme and genipin was an evidence of the crosslinking phenomenon between genipin and amino acids with the increased absorbance at 600 nm (blue color) with time, up to 24h.

It was reported that only the primary amines can react with genipin to form blue pigments in presence of oxygen [224]. It is proposed that a spontaneous reaction between genipin and amino acids occurs which leads to the formation of an aromatic monomer. In a second step, intermolecular crosslinking may occur due to radical reaction [225]. Genipin is also reported to be capable of crosslinking proteins, a genipin molecule is able to crosslink two free groups of lysine amino acids residues on protein macromolecular chains [226], and GOx has 15 lysine residue per each subunit.

When the concentration of genipin was as high as 60 mM, the precipitated crystals were tinted blue in a more pronounced manner on the side where the crystals were in direct contact with water. This is because oxygen is vital to the blue color formation resulting from polymerization of genipin molecules after their reaction with primary amino acids of GOx, and it is present as dissolved oxygen in the solution [281].

FTIR spectra of genipin and GOx before and after crosslinking confirmed the formation of aromatic amines at 1642 cm^{-1} and C-N at 1400 cm^{-1} after the reaction, therefore, the crosslinking reaction occurred and a ring opening reaction took place for these amines (Figure 39). In addition, the peak of primary amines of GOx spectrum at 1560 cm^{-1} did not appear after crosslinking for 24 h, which can be due to the disappearance of the primary amine groups during the crosslinking process.

The mechanism of crosslinking between enzymes and genipin can be explained by opening the ring of genipin due to the reaction of primary amines of GOx with carbon number 3 in genipin, and/or the formation of amide by ester substitution with the amino groups of GOx [281]. Besides those, the reaction with secondary amines may occur in an intermediate stage before the formation of the aromatic amines.

The enzymatic activity measured by the colorimetric assays, indicates that the use of crosslinker improved the stability of activity of the obtained felts, especially for the high concentration 60 mM. When compared to the felts obtained previously by physical adsorption, all the samples were partially active in the sixth cycle including the samples without plasma treatment (VCF and PPCF). However, higher concentration of genipin did not affect significantly the activity of the obtained felts; it was slightly lower than the activity

obtained at lower concentration (30 mM), but it showed to be more stable when reused. This may be explained by similar quantity of enzyme used in both cases, but with higher crosslinker concentration, more enzymes are crosslinked along with side crosslinking between the genipin molecules themselves, and possible bonding between genipin and secondary amines existing on the surface of carbon samples, which stabilizes the aggregates immobilized on the carbon material [227]. The lower activity may be explained by slight deformation and less mobility of GOx due to crosslinking, which cause slight reduction in the activity. This lack of mobility of crosslinked enzyme may result in less accessibility to the enzyme active sites and cause saturation at lower concentrations as appeared in (Figure 48). The slightly lower enzymatic activity resulted from samples coated with PEDOT:PSS in general when compared to bare carbon may be caused by the coverage of fibers by the polymer, which limits the reaction with secondary amines on the surface of samples and caused less stability especially for PPCF 2% samples.

Chapter IV

Application of immobilized enzymes for Bio-Fenton and Bio-Electro-Fenton for sustainable treatment of Remazol Blue RR effluent

IV- A- Introduction

The huge amounts of textile effluents production on annual basis worldwide are becoming a growing concern globally. With the wet process consuming fresh waters to the disposal of effluent that are rich in dyes and additives. The range of dye loss in effluents ranges from 2 to 50 % depending on the type of dye and fibers used in the process. Reactive azo dyes are particularly dangerous, since they are heavily used in the industry and they can be hard to treat with degradation products that can cause cancers [282].

The modal pollutant chosen in this study was Remazol Blue RR by Dystar, which is a heavily used dye in textile industry worldwide. It is a formulation of two dyes; a disazo-divinylsulfone and formazan-vinylsulfone-copper complex reactive azo dye. It is mostly composed of 50 – 60 % dyestuff, 30 – 40 % inorganic salts and up to 5 % functional additives, according to the manufacturer. This reactive dye has a maximum absorbance wavelength at around 605 nm [283].

Several studies focused on the removal of this dye from wastewater, using approaches like adsorption, advanced oxidations or bio-treatments. A complete decolorization of Remazol Blue RR via *Clostridium* species was achieved after 24 - 72 h [284], while high color removal was also achieved by laccase enzyme in a membrane reactor [285], anaerobic treatment [286], and white-rot fungi [283]. Adsorption on natural powders from leaves also showed to be successful in color removal of this dye, using Neem tree leaf powder [287]. In addition, metal coagulation was used like alumina-coagulation to adsorb this dye from effluents [288]. Furthermore, photo-Fenton was used to treat textile wastewater that contain Remazol Blue RR [282]. Many of these studies focused mostly on color removal, taking into consideration that it is hard to degrade and produce amines that are dangerous cancerous materials.

Hence, in this chapter the different carbon felts with immobilized GOx enzyme whose fabrication were described in previous chapters, will be assessed in two sustainable methods of wastewater treatment using Bio-Fenton (BF) and Bio-Electro-Fenton (BEF) methods for degradation of Remazol Blue RR dye solution.

This is the first attempt to achieve BF with immobilized enzyme on a textile material. While for BEF method, the use of enzymatic bio-anode instead of microbial consortium was reported, since the immobilized enzymes can be removed directly from the reactor when desired. In addition, they can be used directly, and reused without the need of time for bacterial multiplication (ranging from few hours to days). Additionally, with the enzymes, less sludge will be formed in the anodic chamber compared to microbial consortium.

The first method BF depends on the bioactivity of the immobilized enzymes, while the other method BEF depends on the bioelectrical activity of the bio-anodes, which were described in the previous chapters.

The efficiency of these methods was evaluated for color and COD removal using UV-Vis spectroscopy methods (as described in **Chapter II**), to estimate the level of pollutant degradation post-treatment. Color removal does not reflect the level of degradation of the dye, COD removal is a more real indicator of dye degradation.

Furthermore, simultaneous energy production while performing degradation of dye is a wise approach to zero energy depollution. Thus, the general power output of the BEF reactor has also been assessed since this method was reported in literature using microbial consortiums and not directly immobilized enzymes on the anodes.

IV- B- Bio- Fenton process for treatment of Remazol Blue RR dye solution

Discoloration and COD removal

A solution of Remazol Blue RR (0.05 $g.L^{-1}$) was used as a modal pollutant in this study. The color removal of the wastewater treated with BF process was assessed after treatment via UV-Vis spectrophotometer to evaluate the efficiency of the treatment in discoloration of this dye. BF mixture contained $FeSO_4$ (1.5 $g.L^{-1}$), D-glucose (0.05 M) as well.

GOx enzyme immobilized on different carbon-based materials with different methods and pretreatments were used for BF process, and 1 cm^2 of the carbon-based felt for each 5 mL of the dye solution. CF 1% - G samples resulted in up to 93% discoloration of Remazol Blue for the first use as observed in the UV-Vis spectra of the solutions before and after treatment (Figure 50), the color was almost entirely removed and the peak at 605 nm disappeared post-treatment (3 h).

It can be seen from the histograms (Figure 51), that for CF 1% with GOx immobilized via adsorption, 83% of the dye discoloration occurred while for GOx immobilized directly on VCF almost 60% of discoloration occurred in the same conditions. When these mentioned samples were reused for a second time in the same process, discoloration values of Remazol Blue decreased to 82% for the CF1% - G, to 57% for the CF 1% and 34% for the VCF, in the same used conditions and for 3h.

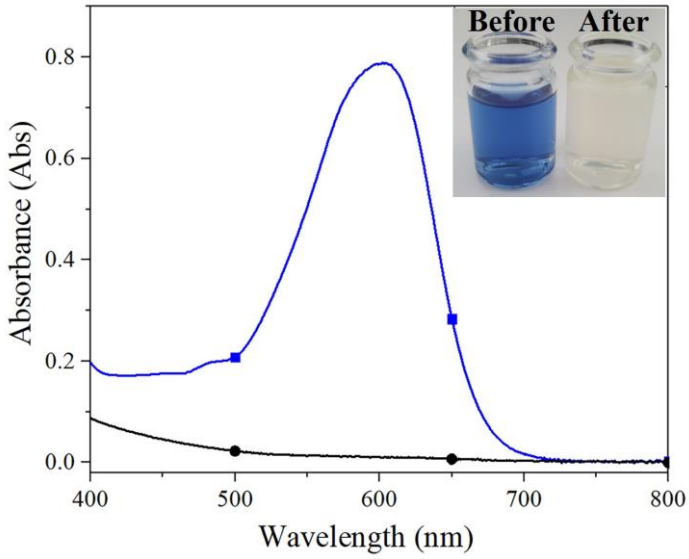


Figure 50 UV-Vis absorbance of Remazol Blue RR solution before and after BF treatment using CF 1% - G (Inset: the corresponding samples)

As far as the carbon felts dip-coated with PEDOT:PSS are concerned, they resulted in less efficient discoloration, with the highest efficiency reported for PPCF 1% - G samples (67% discoloration), while PPCF 1% and PPCF resulted in 49 and 33 % discoloration respectively, for the first use.

It is worth mentioning that, these samples were not efficient in the second use for the BF process and insignificant discoloration occurred for all samples dip-coated with PEDOT:PSS.

COD removal efficiency was up to 30% of the initial values before treatment when CF 1% - G was used for the first time, while it was reduced to 21 and 10% for CF 1% and VCF, respectively. Meanwhile, for samples dip-coated with PEDOT:PSS, the efficiency was 23 and 17% for PPCF 1% - G and PPCF 1%, unlike PPCF samples which did not result in any COD removal after treatment regardless of the partial color removal achieved. It is worth mentioning that the COD removal was insignificant for the second use for these samples, even when there was a partial discoloration.

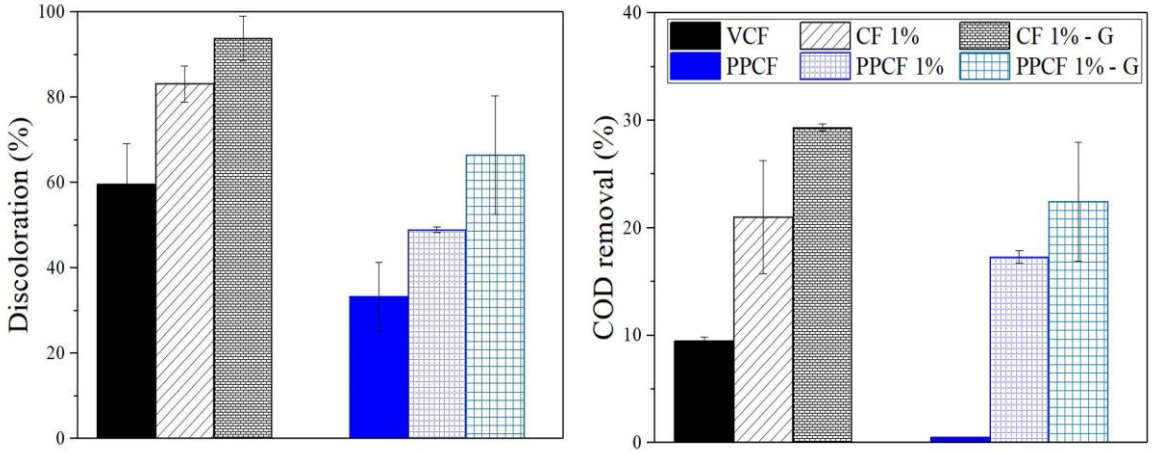


Figure 51 Discoloration efficiency of BF treatment for Remazol Blue RR solution, and COD removal efficiency using different carbon-based felts with immobilized GOx (left to right)

IV- C- Bio-electro-Fenton process for treatment of Remazol Blue RR dye solution

Discoloration and COD removal

A solution of Remazol Blue RR (0.05 $g.L^{-1}$) was used as a modal pollutant in this part of the study as well. The color removal of the wastewater treated with BEF process in the cathodic chamber of the cell (see section **I- B- 3**) was evaluated after treatment via UV-Vis spectrophotometer to estimate the efficiency of the treatment in discoloration of the dye effluent. Four types of carbon-based samples were used in this configuration in the anodic chambers as bioelectrodes, while keeping the same type of cathode as a bare plasma treated carbon felt CF 1% in all experiments and the iron ions were

in a free state within the cathodic solution (1.5 $g.L^{-1}$). Similar to what have been done in BF process, the color removal as well as the COD removal were assessed post-treatment (12 h), the detailed method used is explained in **Chapter II** and results are illustrated (Figure 52).

Color removal showed to be close for both anodes with immobilized GOx with genipin, with color removal efficiency reaching 31% and 34 % for CF 1% - G and PPCF 1% - G, respectively. For bio-anodes produced via physical adsorption of enzymes, discoloration was close to 20%.

Furthermore, the COD removal were in accordance with the color removal results, and the efficiency obtained reached up to 36% for genipin-based anodes, and 27% for anodes with enzyme adsorption. The color reduction is illustrated in (Figure 52), along with the absorption spectra before and after treatment at 605 nm wavelength.

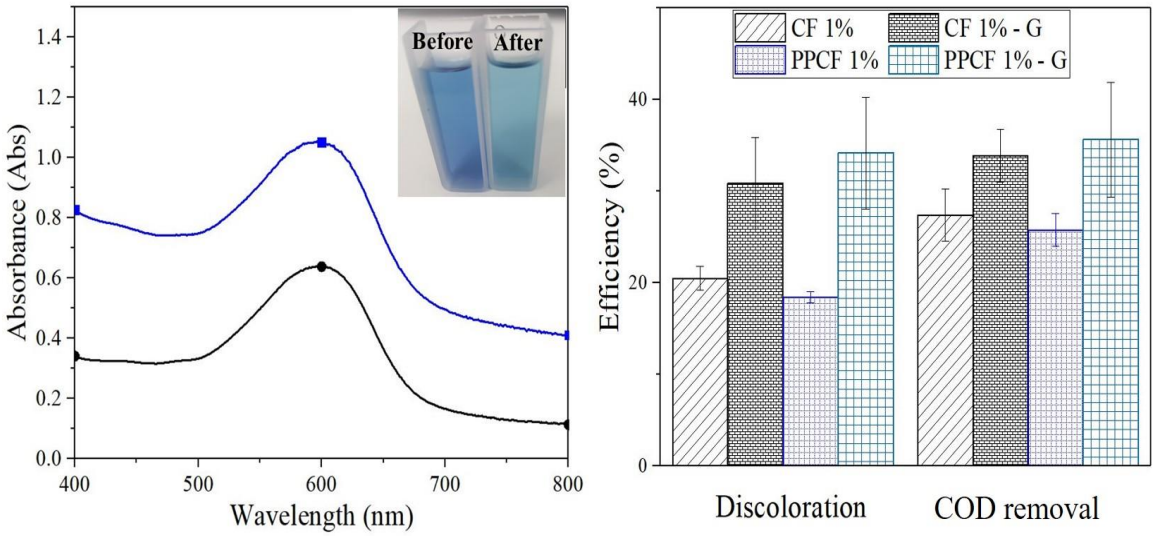


Figure 52 UV-Vis absorbance of Remazol Blue RR solution before and after BEF treatment using PPCF 1% - G (Inset: corresponding samples), and discoloration and COD removal efficiency for different samples using BEF setup (left to right)

Power and polarization curves

The power density and cell polarization of reactors with two different bio-anodes are illustrated (Figure 53), since the use of different bioelectrodes as anodes in BEF reactor affected the power generation output of the cell overall. As presented, the PPCF 1% - G showed enhancement in power generation more than the bare carbon CF 1% - G, with higher voltage and power generation.

With external resistance varied between 1 - 10000 Ω, a maximum power density of 0.5 $\mu W.cm^{-2}$ was obtained at a current density of 15 $\mu A.cm^{-2}$ in the case of PPCF 1% - G bio-anode, and the power was stable until reaching the current density of 30 $\mu A.cm^{-2}$. Meanwhile, when CF 1% - G was used as bio-anode the power density generated reached up to 0.16 $\mu W.cm^{-2}$ at a current density of around 10 $\mu A.cm^{-2}$.

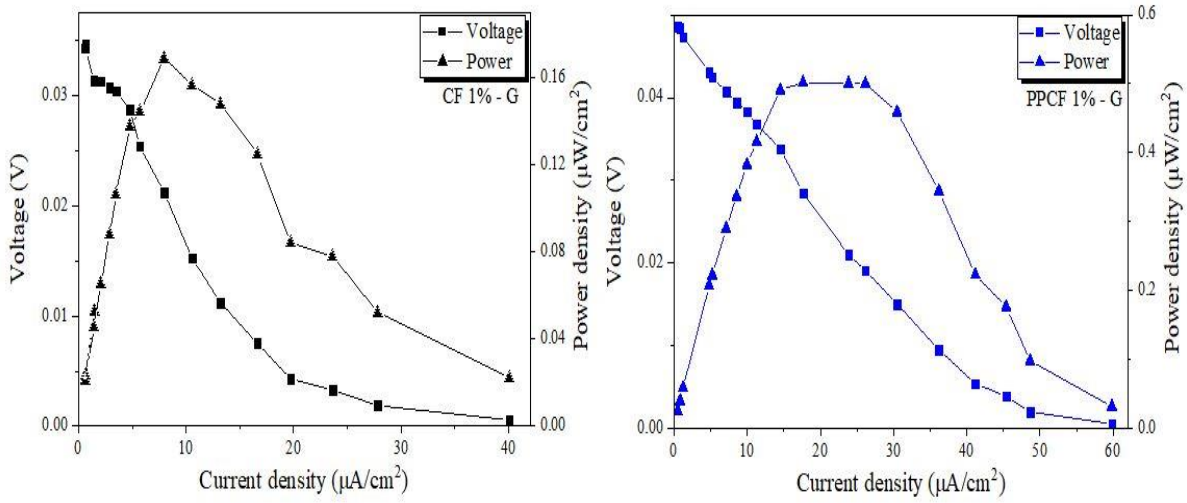


Figure 53 Polarization curves of BEF reactors using CF 1% - G and PPCF 1% - G bio-anodes (left to right)

These two cases resulted in better power output of the reactor in general than the bio-anodes obtained via physical adsorption as presented in (Table 9), while the control cell with VCF with GOx immobilized via adsorption resulted in only 0.037 $\mu W.cm^{-2}$ at a current density of 1 $\mu A.cm^{-2}$.

On the basis of the shape of the voltage against the current density curve, it can be concluded that losses in the reactor in all cases was due to ohmic over potentials, since the relationship between the voltage and the current at intermediate current densities tends to be almost linear.

Table 9 Power density and current density obtained by different BEF reactors

Bio-anode	Power density ($\mu W.cm^{-2}$)	Current density ($\mu A.cm^{-2}$)
VCF – GOx (Control)	0.037± 0.004	1 ± 0.2
CF 1% - GOx	0.09 ± 0.02	5 ± 2
CF 1% - G - GOx	0.16 ± 0.01	10 ± 2
PPCF 1% - GOx	0.11± 0.1	10.5 ± 1
PPCF 1% - G - GOx	0.5 ± 0.1	15 – 30 ± 1

IV- D- Discussion

The objective of this chapter was to investigate the efficiency of the produced bio-functionalized conductive textiles as biochemical /bioelectrochemical active materials for wastewater treatment using sustainable approaches as (BF) and (BEF). In the BEF, their efficiency as bio-anode was tested.

The reactive group of the Remazol Blue RR is vinyl sulphone $–SO_2CH=CH_2$. From the structure of this dye, it contains sulfonated aromatic amines, which are not fully biodegradable according to literature [286]. This might be due to the formation of sulfanilic acid as degradation product that may interfere and couple with other products and hinder the degradation process.

It was shown from the color removal results via BF that the plasma activated bare carbon samples performed better than the samples dip-coated with PEDOT:PSS, and the samples with GOx crosslinked with genipin overall resulted in better discoloration than samples obtained by physical adsorption, reaching to almost complete color removal after 3 h. This might be due to better attachment of the crosslinked aggregates to carbon support and to the enzyme itself. This reduced effect of leaching into the BF medium during treatment, and further resulted in better stability and reusability for the second cycle for the bare samples as well, by maintained enzymatic activity and consequently production of hydrogen peroxide that is crucial for Fenton reaction to occur. Meanwhile, in the case of carbon dip-coated with PEDOT:PSS, the obtained efficiency in discoloration was lower; this might be because with added coating, we add an organic material to the medium, which makes it even harder to degrade the sum of pollutants and led to less efficiency. However, the PPCF 1% - G showed better discoloration that confirmed the better stability of the immobilized GOx with genipin, in similar manner to the bare carbon felts. When comparing with the colorimetric assay of enzyme activity in all cases, the reusability was possible for higher number of cycles. This indicates that BF mixture contributed to denaturation of immobilized enzyme due to lower pH values and the traces of copper that is included in the dye formula.

Furthermore, COD removal via BF process showed to be better for bare carbon and for genipin crosslinked samples overall, in accordance with color removal results. This might be due to sulfur

species that are contained in the PEDOT:PSS and $FeSO_4$ which may contribute to the formation of sulfanilic acid that leads to slowing down the dye degradation process, besides the added organic material via coating and glucose. The interference of ferrous ions with the COD mixture may also occur, resulting in their oxidation by potassium dichromate. Thus, other analytical techniques might be more suitable for use in future studies to determine the degradation levels better than COD for BF treatment such as HPLC.

Concerning BEF process, the results obtained showed that the use of different bio-anodes resulted in different outcome from the reactor. This process was more efficient than BF in COD removal, but less in color removal since in BF the addition of glucose to the mixture contributes to increasing the overall COD of the solution that lead to decrease in the total efficiency of COD removal. Furthermore, both types of samples (bare and coated) with GOx crosslinked with genipin performed better than GOx immobilized via adsorption. That was in accordance with our previous findings.

In addition, the power output from the cell improved for all samples when compared to the control bio-anode obtained by physical adsorption of GOx on untreated VCF (Table 9). PPCF 1% - G samples resulted in the power density up to 0.5 $\mu W.cm^{-2}$ that was stable when the currents were 15 – 30 $\mu A.cm^{-2}$. This might be due to the effect of PEDOT:PSS coating that improves the capacitance of bioelectrodes while the crosslinking improved the stability GOx and reduced leaching, consequently the electron transfer process was better and resulted in higher current and power densities. This value of power density is low compared to conventional biofuel cells that use enzymes on both electrodes or noble metals as cathodes (ranging from 1.38 to 176 $\mu W.cm^{-2}$) [289].

However, the polarization curves showed a linear relation between the current and voltage at intermediate current densities. This indicates that the cell is experiencing ohmic overpotentials that are causing losses in power. These losses may be caused by the materials of electrodes used, along with the electronic components between the measuring instruments and the ionic resistance in the electrolyte (low concentrations).

It can be noticed that, bio-anodes that resulted in higher power and current densities (both genipin electrodes), were more efficient in both color and COD removal, which is related to generating more electrons that can be accepted at the cathode's surface and reduces the dissolved oxygen to hydrogen

peroxide, consequently formation of $OH^{\cdot}$ radical to contribute to Fenton reaction. The higher COD removal in BEF better than BF process might be due to pH level in the cathodic chamber that is more suitable for Fenton reaction, in addition to the fact that cathodic solutions do not contain glucose unlike BF mixture. However, the configuration in the prototype used for BEF can be improved, to control the ohmic losses that contributed to reducing the efficiency of the reactor. Finally, it should be mentioned that regardless of the high potential of Fenton reaction as an advanced oxidation process in the treatment of the effluent, its efficiency is still dependent on chemical structure and molecular weight of the pollutant used [282].

These results show that the GOx enzyme functionalized carbon felts samples showed to be efficient in both bioactivity (as bio-functionalized felts), or bioelectrical activity (as bioelectrodes) in real applications related to wastewater treatment. These sustainable applications show to be promising methods in treatment of persistent organic pollutants like Remazol Blue RR dye that is considered harder to treat than other similar dyes and is commonly used worldwide in textile industry. The Simultaneous energy production while performing degradation of pollutants in BEF systems is important approach towards zero-energy depollution of organic matter.

Chapter V

General conclusions, challenges and future perspectives

V- A- Summary, Conclusions and Contribution

Summary

The objectives of this thesis were to investigate the use of different eco-technologies as strategies for immobilization of redox enzymes on conductive carbon-based felts, to produce bio-functionalized textiles for a future use in sustainable applications. We have outlined the main approaches used in efforts towards modifications of textile materials that can perform in both bio-chemical and bioelectrochemical wastewater treatment applications.

After highlighting the main advances in literature regarding this topic in **Chapter I**, the materials used in this studies with the detailed methods and protocols used for obtaining the modified textile materials were described in **Chapter II**, along with the characterization methods and techniques to evaluate the treatments conducted.

Chapter III included the obtained results from the eco-technologies used in this thesis.

The first approach is Cold Remote Plasma (CRP) with gas mixture of N_2 and O_2, as a dry nondestructive method, was used to modify the surfaces of bare carbon nonwoven felts without added chemicals and within a short time. Immobilization of glucose oxidase (GOx) redox enzyme via physical adsorption followed CRP, which was efficient in modifying the surface of carbon felts by integrating new functional groups like C-O and C=O in addition to amines. These groups improved hydrophilicity of carbon and facilitated the immobilization process. Furthermore, the immobilized GOx on CRP treated felts maintained higher activity and stability up to 6 cycles, and showed to be active and responsive to the addition of substrate in cyclic voltammetry scans.

The second approach used was using dip-coating with biocompatible and biodegradable conductive polymer blend PEDOT:PSS followed by simultaneous CRP for both carbon and coating, to improve surface characteristics followed by immobilization of GOx via physical adsorption. The plasma improved coverage of fibers with coating, conductivity, and capillary uptake due to oxidation of both carbon and coating which lead to better capillary uptake and improved the mass transfer process. Consequently, this method improved the immobilization process, activity and reusability of the obtained felts.

The third approach was the use of naturally occurring crosslinking agent (genipin) to immobilize GOx. This crosslinker proved to be 5000 - 10000 times less toxic than conventional chemical crosslinkers used in literature.

The immobilization process took place over the carbon-based samples, which helped to stabilize the obtained aggregates and significantly improved the stability of enzymes due to bonding between the felts and the crosslinked GOx. The results confirmed the formation of blue pigment that indicates the formation of bonds between genipin and the primary amino groups within the enzyme.

The bio-functionalized textiles obtained in the previous chapter were assessed for possible applications in wastewater treatment. Since textile industry contributes remarkably to production of wastewater, sustainable methods were proposed in **Chapter IV** to use the obtained textile samples in the treatment process and the primary obtained results were presented.

The first approach was Bio-Fenton (BF), which was useful in evaluating the enzymatic bioactivity of the immobilized GOx. This approach was efficient in color removal and partial COD removal, which indicates the partial degradation of dyestuff of Remazol Blue RR. The bare carbon samples with crosslinked enzymes resulted in better degradation overall and stayed partially efficient in removing color in the second use.

Meanwhile, the second possible application Bio-Electro-Fenton (BEF) was useful in evaluating the bioelectrical activity of the obtained samples as bio-anodes. The bio-anodes were responsible of generating power to stimulate the degradation of the dyestuff in BEF reactor. All the obtained bio-anodes resulted in improving of power output of the cell when compared to the control. However, bio-anodes with crosslinked GOx resulted in better color removal, COD degradation and higher power density generated overall.

Conclusions

Hence, it can be concluded based on results and observations obtained in this work that:

1) Cold remote plasma is an efficient eco-technology for the treatment of conductive textile materials like carbon, without the use of added chemical, destructive effects, hazardous sparkling or long treatment time. It can be used with customized gas blends according to the functionality required,

with possibility of treating big batches of samples with different thickness on both sides simultaneously. Consequently, it facilitated bio-functionalization with enzymes in this study and improved stability and activity when compared to untreated samples.

2) PEDOT:PSS as a coating for conductive textile materials showed to improve conductivity, biocompatibility and surface properties of the samples. The oxidation of this coating via CRP further improved these desirable properties and enhanced the activity and reusability of immobilized enzymes. As a biodegradable eco-friendly conductive coating, PEDOT:PSS blend has a great potential in wide spectrum of applications.

3) The gas mixture of nitrogen with 1% oxygen was sufficient to integrate functional groups such as C-O and C=O as well as amino groups, which resulted in improved hydrophilicity and maintained enzymatic activity. However, the increase of oxygen percentage to 2% did not show a significant proportional effect on the treated samples.

4) Genipin as natural crosslinker with low toxicity showed to be a good candidate for directly crosslinking GOx enzymes without the use of polymer matrix. Its low toxicity and biocompatibility improved the stability of crosslinked GOx remarkably and prevented leaching phenomenon better than physical adsorption.

5) Within the theme of sustainable development, the obtained materials from this study were primarily evaluated for wastewater applications that consume less energy, less added chemicals, reduce wastes and help prevent health and environmental hazards. The primary results showed the feasibility and significant potential of BF and BEF using immobilized GOx on carbon-based textiles for degradation of persistent dyestuff like sulfonated reactive dyes. The color removal efficiency varied from around 40% and 90% for BEF and BF, respectively, while COD removal reached up to 30 and 36 %, respectively.

Furthermore, in BEF setup, a synchronized power generation was estimated to reach 0.5 $\mu W.cm^{-2}$ for bio-anodes with crosslinked GOx.

6) These obtained carbon-based textiles maybe used in variety of applications related to power generation and pollution control.

Main Contribution

Within the frame of this thesis, approaches and materials for resource-efficient processes were proposed and evaluated regarding the use of enzymes immobilized on conductive textiles. These new and fast growing types of materials are hot topic of research considering their great potential in smart and functional textiles, which are becoming a crucial part of applications for daily use on both domestic and industrial levels.

Here the points that have an added value and practical contribution in this field are presented:

- The use of cold remote plasma was assessed as an eco-friendly pretreatment of immobilization process on carbon felts with or without biocompatible conductive polymer coating.
- The possibility of direct crosslinking of GOx enzyme with genipin without hydrogels or other polymers like gelatin was evaluated.
- To the best of our knowledge, the first attempt of BF treatment of wastewater using immobilized enzymes on textile materials was reported, which is usually achieved by enzymes in free-state, or immobilized on metallic carriers or within hydrogels.
- Microbial BEF reactors gained a lot of attention in the past few years; here in this work an attempt of enzymatic BEF reactors for wastewater treatment was reported.

V- B- Challenges and future perspectives

The ability of enzymes to catalyze reactions in mild conditions, as well as their high specificity, make them well-appreciated materials in both industry and academia. Immobilized enzymes often show high stability and good activity that helps to better control the process and contributes to the

reduction of costs. Immobilization of enzymes is still facing some challenges in order to be efficiently used on large scales. Several phenomena may occur during immobilization that can cause total or partial loss of enzymatic activity or enzyme units. Enzyme leaching to media, inconvenient confrontation of enzymes on the support causing blocked active site, and denaturation due to harsh conditions for some immobilization methods like pH and temperature changes can lead to the loss of the folded 3D structure of the protein and the loss of the active site and activity are some of these phenomena. These lead usually to short shelf life and reduce stability and efficiency of the bio-functionalized materials with enzymes. Another challenge can be the high cost of some types of enzymes, which are not yet produced on industrial level, which hinders their use in large-scale applications.

Concerning BF and BEF process, the high cost of materials used to construct these systems, like metals and polymers used for the electrodes, and membranes like Nafion® are still challenging the scaling up of these methods. Furthermore, regardless of their efficiency in degradation of pollutants, power density generated from BEF is still low for industrial level. Thus, electrodes need to be larger with higher efficiency in electron transfer.

Further research in the field of redox enzyme immobilization seems to have endless ideas and possibilities. Advances in textile nanotechnology are promising to provide efficient substrates for immobilization. Nano fibers from electrospinning of conductive polymers, conductive coatings and fillers are few of the possibilities. Identification of effective immobilization techniques on appropriate supports could help improving human life and welfare, with more efficient disease detection biosensors, degradation of contamination from water bodies and generating electricity from wastewaters of other industries are few prospects in the field of redox enzymes immobilization.

In regards to wastewater treatment configuration, BF and BEF using cheaper membranes will significantly reduce expenses, such as GORE-TEX® and PVA modified membranes. Cheaper electrode materials like graphite and carbon with coatings can improve the performance overall. Furthermore, wastewater with added iron scrap waste, from steel and mining industry can be used for Fenton's reaction. Similarly, the use of sugary wastewater from food industry can be valuable as substitute to pure substrates in anodic chambers. All these possibilities can reduce the costs

remarkably and improve the performance of these reactors for better degradation of persistent pollutants.

As for the processes reported in this work, further research on certain points would be beneficial in better understanding the phenomena observed and optimizing the conditions for improved results. For instance, the influence of different gas mixtures for CRP treatment of carbon felts with or without the conductive coating (PEDOT:PSS) would permit to optimize the experimental conditions to improve surface energy and electrical conductivity. In addition, the influence of different parameters and concentrations on the crosslinking process of GOx with genipin could be further studied to improve the enzymatic activity and stability of obtained felts. Improving the prototype setup for BEF process and using robust electrical wiring, would help decreasing the ohmic losses that were observed, thereby improving the power output and dye degradation. Finally, degradation of different modal pollutants via BF and BEF processes should be studied such as other types of dyes, since the modal pollutant used in this study is known to be hard to treat and contains traces of metals that may have affected the enzymatic activity in BF process.

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