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PhD THESIS

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GRAPHENE-BASED NANOMATERIALS FOR BIOMEDICAL APPLICATIONS

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Contents

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Summary of the PhD thesis

General and specific objectives

The progress of medical diagnosis has been significant in the wake of biosensors, which offer fast, precise and non-invasive detection methods for different biomarkers. This is especially important in terms of personalized medicine as these instruments have the capacity to allow real-time monitoring thereby enabling prompt diagnoses and effective treatment planning. Important areas that can benefit significantly from improving biosensor technologyare represented in the medical field by managing bone-related conditions and assisting in stem-cell therapies for bone repair.

Osteoporosis, osteoarthritis, and bone fractures among others are significant global health problems affecting millions worldwide. Therefore, it is crucial that early diagnosis and continuous monitoring be carried out for successful management and treatment of these conditions. Traditional diagnostic techniques are invasive, time-consuming and may not provide immediate clinical decision making due to lack of real time data. This gap can be filled by biosensors, especially electrochemical ones.

Electrochemical biosensors could help detect specific osteogenic biomarkers indicative of bone health or bone disease, thus providing early promising solutions for diverse bone-health issues. Biomolecules like proteins, peptides or nucleic acids are associated with resorption, formation, or repair processes in bones. Having the ability to track these biomarkers continuously gives specialists better understanding about each individual patient,which leads to more accurate diagnoses. Moreover, biosensors can also aid clinicians to check how diseases progress over time and to evaluate the efficacy of the treatment.

Biosensors play an important role in regenerative medicine as well, especially when itcomes to stem cell treatments for bone repair purposes. Stem cell based therapies show greatpotential in promoting bone regeneration and healing, however, their success largely dependson monitoring closely and in real-time stem cells activities at specific sites within injured tissues alongside local environmental conditions. Therefore, highly important information could be obtained through detecting the presence, as well as the concentration

levels, of osteogenic biomarkers responsible for the differentiation of stem cells into osteogenic cells,enabling the optimization of stem cell therapies and ensuring their efficacy.

Deoxyribonucleic acid (DNA) is emerging as an intriguing biological material for biosensing because of its enhanced biocompatibility, thermal stability, and ease of functionalization. It is well recognized that DNA and its assembly structure can be used to detect a variety of targets, including proteins, enzymes, ions, nucleic acids, and small biomolecules. Dynamic networks based on DNA hybridization can be used to amplify biosensor signals thanks to the contunous development of DNA nanotechnology. Moreover, DNA may organize other functional elements and construct complex three-dimensional (3D) nanostructures. When compared to commonly used bioprobes, DNA exhibits superior addressability, adjustable stiffness, and more sustained biological activity, making it an excellent option for intelligent biosensing. DNA probes, such as aptamer, have reportedly been found to have improved thermal stability, tunable biological affinity, and increased resistance to nuclease enzyme assault by manual screening and modification. In order to get precise control over the spatial position of alterations, DNA may also be utilized to construct programmable supermolecular structures. This might greatly enhance the performance of the biosensors and even serve as inspiration for the development of other innovative detection platforms.

Considering that established molecular technologies used in DNA and biomarker detection, i.e., polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), are unsuitable for point-of-care (POC) tests because they require extensive laboratory work, expensive reagents and qualified personnel, it is emphasized the necessity for improving the biosensor technology in order to provide real-time biomarker detection ina noninvasive, cost-effective and patient-friendly manner.

In this context, taking into account the arguments discussed above, the main objective of my PhD thesis is to contribute to the field of personalized medicine by developing a novel electrochemical biosensor able to accurately detect osteogenic biomarkers in real-time in order to meet the need for rapid, simple and continuous in-situ monitoring required in the development of POC devices. At the same time, I aim that this work will provide the means to design a robust and versatile detection platform, that can be easily modified to accurately

detect and quantify a wide range of biomarkers beyond osteogenic ones, with high industrialization prospects.

The general objective of the present thesis is sustained by several specific goals, as follows:

- Comparison of glassy carbon electrodes (GCE) and screen printed carbon electrodes (SPCEs) so as to determine which type of electrode provides the best performance for biosensing applications;
- Exploring the functionalization of reduced graphene oxide (RGO) with diazonium salt to make possible the covalent attachment of probe onto the electrodes with the aim to increase the sensitivity of the detection platform;
- Investigations of RGO functionalization with gold nanoparticles (AuNPs) for the increase of electron transfer rate and consequently for the increase of biosensor sensitivity for a non-covalent immobilization of probe;
- Testing the most promising RGO-based platform that can detect specific osteogenic biomarkers, i.e., alkaline phosphatase (ALP) and Runt-related transcription factor 2 (RUNX2);
- Thorough characterization and structural comparison of each detection platform proposed and developed in these studies.

This PhD thesis is divided in two parts: the first part presents the state-of-the-art in the field of biosensors, addressing the materials and methods used for their fabrication, while the second part is focused on original contributions aimed to address the current need for better biomarker detection techniques. The six chapters comprising this thesis are summarized as follow:

Chapter 1 presents general aspects about biosensors, discussing their history, classification, main advantages and drawbacks, as well as their applications. The literature study is rounded by theoretical information about graphene and graphene-related materials, focusing on their properties, strengths and limitations in biosensing applications, a summary of the synthesis methods, as well as the reduction methods of graphene oxide (GO) and its importance, i.e., enhancing the surface area and the electrical conductivity of the material.

Moreover, graphene functionalization methods are discussed, especially functionalization with diazonium salt and gold nanoparticles, which was performed to increase even more the surface area of the electrode and to deliver anchors for the covalent attachment of probes.

In chapter 2 are presented the first original results obtained in this work. In this thesis I focused on comparing GCEs with SPCEs in order to determine which is the most suitable working electrode (WE) to use further in the fabrication of the detection platform. To this purpose, both electrodes were coated with GO by the same method and tested for their DNA detection ability. It was found that while both surfaces have a good adsorption capacity for DNA, GO/SPCEs shows a higher sensitivity towards detection of DNA hybridization, detecting the target up to 10 nM, compared to GO/GCE which detected the target up to 25 nM. As such, these findings play an important role in POC settings since SPCEs are affordable, portable as well as easy to use, making them suitable for large-scale production and real-time diagnostics.

In chapter 3 is presented a new direction, where SPCEs are modified with RGO functionalized with diazonium salt in an attempt to increase the sensitivity of the detection platform. The novelty of this study consists in providing a controlled and stable RGO-based electrodes functionalization by means of diazonium chemistry so as to anchor carboxylated aryl groups (Ar-COOH) on the RGO surface for the covalent attachment of amino-modified ssDNA probes via carbodiimide coupling, enhancing ssDNA probe immobilization compared to other methods. Morphological, structural, and electrochemical characterizationconfirm that through diazonium chemistry a more stable detection platform can be obtained,compared to previous studies. Moreover, the saturation level of amino-modified ssDNA probe was determined to be 10 µM and it was shown that the DNA hybridization process was successfully detected from 200 nM to 1 nM, by monitoring the changes in the electrochemical signal. The results are confirmed by assessing the electron transfer kinetics at the electrode surface through cyclic voltammetry (CV) scan dependence studies, as well as by modifications of charge transfer resistance (Rct) reported by electrochemical impedacne spectroscopy (EIS).

Chapter 4 explores the functionalization of RGO with gold nanoparticles and the

subsequent modification of SPCEs to provide a way for the non-covalent attachment of probe onto the working electrode. The fabrication of this platform involves a two-step electrochemical procedure, the first step concerning the GO reduction, while the second step involves the electrochemical reduction and simultaneous electro-grafting of AuNPs on the RGO surface. The conclusion of this study was that AuNPs introduction into the system contribute to an increase in surface conductivity, and therefore to a more sensitive oligonucleotide detection.

Chapter 5 presents a novel approach for osteogenic biomarkers detection, specifically of ALP and RUNX2, based on the results obtained in the previous studies. The proof-of-concept relies on SPCEs modified with RGO as the simplest, most rapid and cost-effective protocol to fabricate the detection platform. For the first time an electrochemical biosensor for RUNX2 detection is proposed and the results obtained from numerous characterization techniques show that the RGO-based platform can detect both RUNX2 and ALP biomarkers up to 1 nM, with better results in terms of sensitivity obtained for RUNX2.

In chapter 6 are presented the general conclusions of the original contributions obtained during the PhD program, which are described in detail in the second part of this thesis.

The PhD thesis ends with a list of bibliographic references and with a list of publications and conferences where the results were disseminated during the course of my PhD program.

State-of-the-art and motivation

The development of electrochemical biosensors has been actively advancing over the last years due to a large demand for high-performance detection devices. When reviewing the literature on suitable materials for biosensing, graphene and its derivatives have garnered significant attention due to their outstanding electrical, mechanical, and chemical properties [1-3]. In this PhD thesis it is presented the investigation, optimization, and characterization of several electrochemical detection platforms fabricated with graphene-based materials for the detection of DNA hybridization, with the final aim to detect osteogenic biomarkers.

Detecting osteogenic biomarkers is highly important in the fields of regenerative

medicine, orthopedics, and osteology, as they are indicators of bone formation and bone health, offering crucial information about the biological mechanisms related to bone metabolism, growth, and repair [4, 5]. Therefore, early and accurate detection of these biomarkers can aid the early diagnosis of skeletal disorders, such as osteoarthritis, osteoporosis, and other metabolic disorders. Moreover, monitoring osteogenic biomarkers in real-time is helpful in predicting therapeutic outcomes, especially for applications aimed to treat bone defects using bone grafts, orthopedic implants, and regenerative medicine based on stem cells, seeking to improve bone regeneration and healing [6]. The identification of osteogenic biomarkers is essential for supporting the development of novel therapeutic approaches in the management of bone health and disease since it makes it easier to evaluate the quality of the bone tissue for each patient and the efficacy of treatment plans.

Graphene is a two-dimensional (2D) carbon based nanomaterial with many remarkable properties that make it suitable for use in biosensing. As a result of its excellent electrical conductivity, substantial surface area, and good chemical stability [7], graphene has been explored in a variety of biosensing applications where the interaction with biomolecules is highly important [8-10]. To date, numerous biosensors based on graphene have been proposed for DNA detection [11-13]. In this thesis, several graphene derivatives were investigated, namely GO, RGO, RGO functionalized with aryl carboxylic groups by diazonium chemistry (RGO-Ar-COOH), and RGO functionalized with gold nanoparticles (RGO-AuNPs), to determine for each of them specific advantages regarding stability, ease of functionalization, electrical conductivity, and capacity to bind biomolecules. It has been shown that the modification of electrodes with GO or RGO can significantly improve DNA biosensor performance, as the oxygenated functionalities present on the material surface aid the immobilization of DNA probes [14, 15], while the reduction of GO improves the conductivity and the overall electrochemical properties of the detection platform [16, 17].

Additionally, the properties of a detection platform can be enhanced even more by incorporating AuNPs into the system, which was proven to increase the sensitivity of electrochemical biosensors for DNA detection [18, 19]. Gold nanoparticles have superior electrical properties and the ability to enhance the surface area and the conductivity of the

electrodes used to fabricate biosensors, as demonstrated by several studies [20-22]. For instance, Liu et al. [23] fabricated a DNA biosensor based on RGO and AuNPs for the detection of Mycobacterium tuberculosis, showing that the nanoparticles contributed to improving the immobilization capacity of the probe, while also enhancing the charge transfer, leading to a highly sensitive and stable biosensing device.

Moreover, diazonium chemistry has been employed as a robust and reproducible method for the covalent immobilization of DNA probes on graphene-based electrodes. This technique involves electrochemical reduction of diazonium salts to graft aryl groups onto the electrode surface [24, 25], creating reactive sites for attaching amino-modified DNA probes. In particular, this methodology enhances stability and hybridization efficiency of DNA biosensors making them applicable in sensitive and selective DNA detection [26, 27]. Considering that diazonium salts can be unstable and even explosive [28], not many scientists have ventured into exploiting their properties in biosensing applications. Nevertheless, several studies were conducted to investigate how diazonium chemistry can be safely used in the design of biosensors [29-31]. Wang et al. [32] proposed an immunosensor for alpha- fetoprotein detection, a diagnostic biomarker for liver cancer, using an electrode modified with graphene and diazonium chemistry to immobilize the bioreceptor. They conclude that the use of graphene improved the sensitivity and stability of their system, while the functionalization with aminophenyl groups facilitated the immobilization of probes.

Although significant progress has been made in the field of electrochemical biosensors, there are still several challenges that need to be overcome before DNA biosensors can be mass-produced and commercialized. Among these limitations, the high reproducibility, high specificity and sensitivity need to be addressed. Moreover, other features that require additional investigations are the long-term stability as well as the robustness of these biosensors in real-world situations. Therefore, it is expected that research will be focused on optimizing the fabrication protocol of these platforms, on enhancing the biosensors performance by increasing the selectivity, sensitivity, and specificity, as well as on adapting the proof-of-concept devices into integrated systems for point-of-care applications.

This thesis aims to build upon the advancements mentioned above by exploring novel biosensing platforms and addressing the current challenges in the field. To this end, I first tested and compared two types of electrodes, i.e., GCEs and SPCEs, which were chosen for their superior properties in terms of versatility, ease of functionalization, high conductivity, and chemical stability. Two platforms were fabricated started from these electrodes, that were modified with reduced graphene, extensively characterized by transmission electron microscopy (TEM), Raman spectroscopy, Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD), cyclic voltammetry, and electrochemical impedance spectroscopy. The platform designed with SPCEs showed an increased sensitivity for DNA detection, and considering the additional advantages, like being easier to characterize, being robust and having the capacity to develop miniaturized biosensors, SPCEs were chosen to continue this research.

Another goal was to explore the covalent immobilization of the bioreceptor onto SPCEs in order to determine if a higher biosensor sensitivity can be obtained. Therefore, the electrodes were modified with RGO functionalized by diazonium chemistry, to provide aryl functional groups on the electrode surface, to serve as anchors for the attachment of DNA probes modified with amino groups, through carbodiimide coupling, as a covalent approach. The electrochemical platform was characterized by several techniques, including scanning electron microscopy (SEM), Raman spectroscopy, CV, and EIS, showing that an increased electron transfer was obtained at the interface of the electrode after DNA hybridization, detecting the target in a range of 200 nM – 1 nM, and that the proposed platform can be developed as a superior biosensing device.

Moreover, another RGO functionalization method was investigated so as to verify if a high sensitivity can be achieved by immobilizing the bioreceptor via the non-covalent way. In this study, the RGO-modified SPCEs were functionalized with gold nanoparticles by the electrochemical reduction of chloroauric acid and each step of the modification was characterized by SEM, X-ray Photoelectron Spectroscopy (XPS), CV, EIS, and chronoculometry studies. The results showed that functionalizing RGO with electro-grafted AuNPs led to a higher surface conductivity, and therefore, an increased sensitivity and specificity in DNA target detection. This electrochemical platform was shown to

differentiate between complementary and non-complementary target and also to detect the target analyte up to 1 nM.

Finally, based on all previous original studies, a simple, cost effective and efficient detection platform is proposed as a solution to detect and quantify the osteogenic biomarkers RUNX2 and ALP. SPCEs were modified with RGO and the bioreceptors corresponding to each biomarker were immobilized on the surface of the electrode using a physical adsorption approach. The modified electrodes were extensively characterized by XPS, contact angle measurements, SEM, Raman spectroscopy, and by electrochemical methods, i.e., CV, and EIS, all these methods confirming the electrodes modification with electrochemically reduced GO, as well as the immobilization of the bioreceptors. The electrochemical tools demonstrate in this study that these pilot platforms detect both osteogenic biomarkers with a concentration of 1 nM, showing a higher sensitivity in the case of RUNX2, having the potential to be mass-produced at industrial scale.

General conclusions and original contributions

The main original contributions presented in my PhD thesis consist of:

1. *Comparing GCE and SPCE for the detection of oligonucleotides*

To the best of my knowledge, there is no other published study on the comparison of GCEs and SPCEs modified with GO to be employed as an electrochemical detection plaform for DNA hybridization. This study finds both platforms effective in DNA adsorption and hybridization. However, the DNA hybridization detection sensitivity is higher in GO/SPCEs, with a detection limit of 10 nM, compared to 25 nM for GO/GCEs. This higher sensitivity, along with the low cost, portability, affordability, and ease of miniaturization of SPCEs, makes them more suitable for POC applications. Characterized using techniques such as TEM, Raman spectroscopy, FTIR, XRD, CV, and EIS, this study demonstrates that SPCEs could be pivotal in developing efficient biosensors for real-time diagnostics and personalized medicine.

2. *Using diazonium chemistry for the functionalization of RGO to provide anchors for the covalent attachment of DNA probes.*

To date, there is no other study that has used in situ generated diazonium salt to modify RGO/SPCE with a carboxyphenyl layer to provide anchors for the covalent attachment of the bioreceptor with the aim to design an electrochemical detection platform for DNA hybridization. This innovative approach significantly improves the sensitivity of electrochemical biosensors for DNA detection. The functionalization of the graphene-modified electrode surface via diazonium chemistry was performed by means of electrochemistry (CV) and enhances stability and hybridization efficiency by covalently immobilizing amino-modified ssDNA probes, facilitated by carbodiimide chemistry. The carboxyphenyl-grafted layers was shown to enable controlled and stable probe immobilization, resulting in a superior performance for DNA hybridization detection. This method achieved a detection limit as low as 1 nM, demonstrating a marked increase in sensitivity. These findings were validated through various characterization techniques, including SEM, Raman spectroscopy, CV, and EIS, underscoring the potential of this approach in creating highly sensitive and reliable biosensors.

3. *Modification of RGO/SPCEs with gold nanoparticles for the fabrication of a DNA electrochemical biosensor*

In this study it was presented for the first time the modification of RGO/SPCEs with electrografted AuNPs to provide the means for the physical adsorption of probes in designing an electrochemical biosensor for DNA hynridization detection. Incorporating AuNPs into RGO-modified SPCEs by a simple electrochemical technique, significantly enhances their electrochemical properties and sensitivity, as evidenced by SEM, XPS, CV, EIS, and chronocoulometry analyses. This biosensor demonstrates exceptional sensitivity, detecting DNA oligonucleotides down to 1 nM concentration, even with probe immobilization achieved through physical adsorption. The findings underscore the potential of graphene-based materials combined with metal nanoparticles like AuNPs to produce robust biosensors with superior performance, marking a significant advancement in biosensor technology for applications in biomedical diagnostics.

4. *Designing an electrochemical detection platform for RUNX2 and ALP based on RGO-modified SPCEs*

The novelty of this study lies in pioneering the electrochemical detection of osteogenic biomarkers, specifically ALP and RUNX2, using a graphene-based platform on SPCEs. While previous research has proposed biosensors for ALP detection, none have utilized RGO-modified SPCEs, making this approach distinctively innovative. Furthermore, no prior studies were found on the electrochemical detection of RUNX2, highlighting the unique contribution of this research. Characterization techniques such as SEM, Raman spectroscopy, XPS, and contact angle measurements confirmed the successful modification of SPCEs with RGO, while electrochemical methods including CV and EIS validated the effective immobilization of bioreceptors onto RGO through physical adsorption. The study demonstrated a remarkable detection limit of 1 nM for both biomarkers, with superior sensitivity observed for RUNX2 over ALP. These findings underscore the potential of RGO-modified SPCEs as a reliable and scalable biosensing platform, poised to advance biomedical diagnostics and facilitate cost-effective mass production in industry.

From all of the experimental studies, several final conclusions can be drawn. Primarily, graphene-based materials have shown a high potential to increase the sensitivity of electrochemical DNA biosensors. Secondly, they can be further improved by functionalization RGO using diazonium chemistry or metal nanoparticles such as AuNPs for enhanced target detection. Thirdly, SPCEs offers practical advantages over other electrodes like GCEs, particularly in point-of-care applications due to their affordability, ease of use, as well as scalability for mass production.

Additionally, the experimental work performed during the course of this PhD program aims to address some limitations of current diagnostic technologies, namely the need for real-time monitoring, non-invasiveness and cost-effectiveness that can only be done through integrating advanced materials and techniques into biosensor designs. These developments have the capacity to improve current therapies for bone-related diseases and to provide patients access to custom-made solutions for faster and easier recovery.

Finally, these studies provide extensive research on biosensor technology which is highly important for the development of next-generation detection tools, giving insight into how they can be improved for superior results. In clinical settings it is expected that the implementation of these biosensors will improve the ability of healthcare providers to deliver personalized care effectively, contributing to the future of medicine.

Publications list and dissemination of results

This PhD thesis is a compendium of the following scientific research articles, published during the course of my PhD program, with a cumulative impact factor of 13.9:

1. **Chiticaru EA**, Toader GA, Ioniță M. Towards point-of-care medical applications using electrochemical biosensors. U.P.B. Sci. Bull., Series B, Vol. 86, Iss. 1, 2024. ISSN 1454-2331. IF=0.

2. **Chiticaru EA**, Pilan L, Ioniţă M. Electrochemical detection platform based on RGO functionalized with diazonium salt for DNA hybridization. Biosensors. 2022 Jan 13;12(1):39. IF=5.4.

3. **Chiticaru EA**, Damian CM, Pilan L, Ioniță M. Label-Free DNA Biosensor Based on Reduced Graphene Oxide and Gold Nanoparticles. Biosensors. 2023 Aug 8;13(8):797. IF=5.4.

4. **Chiticaru EA**, Ioniță M. A Novel Approach Using Reduced Graphene Oxide for the Detection of ALP and RUNX2 Osteogenic Biomarkers. Current Issues in Molecular Biology. 2024 May;46(5):4489-505. IF=3.1.

Additional studies, as review articles and book chapter, were also published during the course of this PhD program:

1. Tite T, **Chiticaru EA**, Burns JS, Ioniţă M. Impact of nano-morphology, lattice defects and conductivity on the performance of graphene based electrochemical biosensors. Journal of nanobiotechnology. 2019 Dec; 17:1-22; IF=10.2. (review article).

2. **Chiticaru EA**, Ionita M. Graphene toxicity and future perspectives in healthcare and biomedicine. FlatChem. 2022 Sep 1; 35:100417; IF=6.2. (review article).

3. **Chiticaru EA**, Muraru S, Ioniţă M. From unidimensional carbonaceous materials to multidimensional structures through molecular modeling. In Carbon Related Materials:

Commemoration for Nobel Laureate Professor Suzuki Special Symposium at IUMRS-ICAM2017 2021 (pp. 1-21). Springer Singapore (book chapter).

The results obtained during this program were disseminated at the following national and international conferences:

1. 6th International Congress on Biomaterials & Biosensing (BIOMATSEN); Oludeniz, Turkey; 17-23 October 2021; poster presentation: “Label-free DNA biosensor based on reduced graphene oxide functionalized by diazonium chemistry”, authors: **Elena A. Chiticaru**, Luisa Pilan, Mariana Ioniţă.

2. 12th International Congress on Advances in Applied Physics & Materials Science (APMAS); Oludeniz, Turkey; 13-19 October 2022; poster presentation: “Impedimetric biosensor based on reduced graphene oxide functionalized with gold nanoparticles for DNA detection”, authors: **Elena A. Chiticaru**, Mariana Ioniţă.

3. RealMe conference (Transnational Multiplier Event); Bucharest, Romania; 18-19 October 2023; poster presentation: “Electrochemical label free biosensor based on reduced graphene and gold nanoparticles for DNA hybridization detection”, authors: **Elena A. Chiticaru**, Celina M. Damian, Luisa Pilan, Mariana Ioniţă.

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