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# Biosensing based on pencil graphite electrodes

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ABSTRACT

Pencil leads have been increasingly used as electrode material in electrochemical applications. Commonly denominated as pencil graphite electrodes (PGE), they represent a viable alternative to other standard electrodes due to their comparable electrical properties but mainly for their low cost and availability, enabling disposable applications. In order to achieve the best analytical performance literature evidences the type of lead (hardness level) and electrode surface pre-treatment are critical to the envisaged application. The present review describes the use of PGE in biosensing analysis, more specifically those sensors comprising immobilized enzymes but also briefly referring nucleic acids and other biological entities. It lays an emphasis in the immobilization process of the biological entities while focusing in the analytical performance of each biosensor, mainly sensitivity, linear range and limit of detection as comparative criteria. This review also addresses the main characteristics and properties of PGEs as transducer material in the electrochemical field.

## 1. Introduction

Writing and drawing with ever sharp utensils based on carbonaceous threads of Ø ≤ 2 mm encased in mechanical devices, have been a common alternative to standard pencils since the beginning of last century. Also in the continuous search for easily polarizable electrodes electrochemists were attracted by their electrical conductance properties, tiny dimensions, low cost and ubiquity. Commonly referred as pencil graphite electrodes (PGE), first reports dated back to the 50's/60's. At the time, a pencil lead served as anode against the classic dropping mercury electrode into a microcoulometric arrangement for the numbering of electron charges involved in irreversible electrolysis processes [1]. In the 80's, surface covering with a thin layer of polyvinyl chloride plasticized with dioctylphthalate enabled successful use in titrations performed at null current [2]. Independent results obtained from voltammetric studies with well-known redox probes $[Fe(CN)_6]^{3-/4-}$ and $[Ru(NH_3)_6]^{2+/3+}$, highlighted the higher stability and analytical reproducibility regarding the much more expensive glassy carbon (GC), pyrolytic graphite and carbon fiber electrodes [3,4]. Sustained development of electrochemical applications and patent request referring different usages began in the nineties mainly by Asiatic research groups [5–12]. Meanwhile, the first biosensor implemented with a PGE was described in 1990 by Pishko group [13] and transduced the interaction between substrate and the glucose oxidase enzyme wrapped in polycation redox polymer formed from osmium complexes with poly(vinylpyridine) and immobilized on its surface. The opposite charges between the reduced form of the enzyme and the polymer enabled a simplified electron transfer feature, direct "wiring" to the active catalytic centre, and obviated use of additional redox mediators.

Pencil mines are produced by extrusion of a mixture composed of graphite powder and clay (used as binder material) in water, afterwards heated up to 1000 °C in order to gain rigidity. Immersion in a wax bath enables pores filling and confers smooth appearance to the end product [14]. An approximate composition of around 68% (w/w) graphite, 26% (w/w) clay and 5% (w/w) wax was recently stated as example of medium hardness Staedtler HB [15]. The thinnest leads tend to easily break due to the brittle nature of clay and require additional use of a polymer to provide resilient bonding after thermal treatment [16,17]. Besides differing in gauge, pencil mines come in diverse hardness varieties depending on the graphite/clay proportion in the extruded mixture, which can also differ between manufacturers. The ones labelled as H have lower graphite/clay ratios and give harder writing sensation, while B mines are produced with higher carbonaceous content to enable softer and darker writing. Thus, PGEs can be implemented from mines with different graphite/clay ratios corresponding to the scale from 10H to 10B (22 Types) (Japanese Mitsubishi Pencil Co. (Uni)). From pure theoretical point of view, since electrical properties of electrodes such as conductivity largely depend on the carbonaceous content, generic use of softest pencils would seem obvious [18]. In fact, softer leads were advantageously used to draw miniaturized electrodes over cellulosic flat surfaces. For that purpose, pencils like 6B [15,19] or 8B [18] were the best due to the enhanced

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electrochemical performance obtained. In other applications, variation in hardness (clay content) as well as in a particular pencil brand have shown marked influence on the achieved performance [16,17,20]. For instance, 5H pencils provided higher oxidation peaks in the determination of analgesic drug acetaminophen when compared with 2B mines [21]. In the determination of caffeic acid, better signal-to-noise ratio was observed for HB mines when compared to the 2B, 2H and 5H counterparts [22]. To unveil the influence of hardness, PGEs from three different manufacturers, labelled from 4B to 4H were evaluated by cyclic voltammetry (CV) [17]. The anodic peak currents clearly raised from softest to medium type leads (HB) and stabilized for those with increased hardness while potential peak separation ($\Delta E_p$) consistently decreased. The authors of the study also observed that similarly labelled pencil mines could have significant differences concerning to reproducibility and redox peak ratios according the manufacturer. Kariuki [20] studied the surface structure and electrochemical properties of PGEs. Higher oxygen/carbon ratio, as well as higher silica and aluminium content with increasing in hardness was observed by means of X-ray photoelectron spectra (XPS). Resorting to the redox probes $Fe(CN)_6^{3-/4-}$ and $Ru(NH_3)_6^{3+/2+}$, a lower peak separation $\Delta E_p$ was more evident for HB mines due to faster heterogeneous electron transfer rate. The surface reactivity of chemical species at HB mine exhibited improved kinetics regarding to H and B varieties though without a clear tendency between these last. As it happens with other carbon electrodes, namely glassy carbon, pyrolytic graphite, spectroscopic graphite and carbon fibres, the prevalent structure of the carbonaceous component was carbon to carbon bonds in the $sp^2$ configuration. Differences in electrochemical properties were thus ascribed to variations in size and orientation of graphitic inter- and intraplanar microcrystallites [23,24]. On other hand, the electrochemical response of a PGE of suited hardness might change dramatically after pre-treatment. To accomplish determination of phenols, best results were obtained after anodization treatment and responses improved for soft mines up to the 6B. Phenol adsorption at unacceptable levels was observed for softer varieties with consequent fouling and signals loss. Without pre-treatment however, the HB variety presented the best results with responses worsening with increasing in softness [25]. Also importantly, the choice between PGEs for biosensing applications revealed specific chemical interactions between the analyte and the clay/graphite composite regardless to any pre-treatment given to the electrode prior the analysis (under detailed discussion below). In this context, best results for hydrogen peroxide ($H_2O_2$) biosensing [26] or in the detection of DNA hybridization [27] were consistently achieved with 6H labelled PGE.

The use of modified or non-modified PGEs for the determination of organic and inorganic compounds were reviewed by other authors [28–30]. However, the results discussed above highlight several features of PGEs which also affect the immobilization of different biological entities during implementation of biosensors as well their final analytical performance. A systematic literature review is carried-out concerning use of this electrodic material for biosensors comprising enzymes, nucleic acids and whole organisms, in order to guide the researchers in future works. As far as we know, this is the first review regarding the application of this type of material in transducing schemes with biological entities. Here we give insight of (i) pencil lead characteristics and features; (ii) immobilization processes of the biological entities while (iii) addressing the achieved analytical performance as comparative criteria between the described biosensors.

### 1.1. Differences between PGE and other carbon electrodes

In order to highlight the surface composition of different PGEs we have analysed 3 types of pencil leads (4B, HB and 4H) by energy-dispersive X-ray spectroscopy and scanning electron microscopy. The results obtained are illustrated in Fig. 1 (unpublished). The spectra reveal silicon, aluminium and oxygen as main components of clay besides the carbon microcrystallites in chunks of juxtaposed thin platelets with approximately 2–10 μm length (Fig. 1a).

Traditional hand-polishing (0.05 μm alumina) revealed entangled filament structure (Fig. 1.b) with darker but less defined regions in the less disordered 4B lead. In the study conducted by Kariuki [20], Raman spectra showed that HOPG electrode had the lowest ratio between the two 1370 $cm^{-1}$/1583 $cm^{-1}$ bands ($D/E_{2g}$ ratio) reflecting ordered basal plane surface structure. The disorder rises for PGE followed by GC and is in direct relation with the clay content. In turn, redox peaks obtained with different probes, $Ru(NH_3)_6^{3+/2+}$, $Fe^{2+/3+}$, dopamine and $Fe(CN)_6^{3-/4-}$, showed this last as the more appropriate to test for surface characteristics namely disorder and oxygen to carbon ratio. The highly disordered edge plane in GC electrode presented faster electron transfer kinetics translated in bit smaller peak separation for each redox probe, $\Delta E_p$, when compared to PGEs labelled as HB. Basal plane HOPG electrode showed higher values of $\Delta E_p$ and also lower reproducibility. On the contrary, specific analysis regarding guanine oxidation signal [31,32] or the determination of the carcinogen 7,12-dimethylbenz[*a*] anthracene [33] revealed better electrochemical results of PGEs over GC. While the stated conclusions can be looked as guiding criteria to select a particular electrode for an intended application, other studies demonstrated equivalent performance between both electrodes [5,16,34]. An important aspect to be taken into account considers the differences on carbon electrode surface structures leading to apparent high double layer specific capacitances in the implemented electrochemical method which may affect the quality of the electrode response. A first cause is related to the presence of other constituents besides graphite [33]. As can be seen from Fig. 2 (data not published), PGEs generally show potential limits *vs* Ag/AgCl between −1.2 V and + 1 V in 0.1 M KCl, being pristine B mines characterized by larger capacitance but also poor signal-to-noise ratio. Other factors influencing the performance of the PGEs are related to surface roughness as well as to porosity of the material. In the presence of void space, unsuspected compounds in solution might be adsorbed thus imparting uncontrollable background currents [24]. A particular feature of PGEs is that the surface contacting the sample is a mix of insulating clay regions and conductive graphite regions unlike GCE and carbon fiber electrodes. This aspect determines an akin of “microelectrode array” behavior with corresponding better signal-to-noise ratios as noticed in few studies regarding adsorptive stripping analysis involving nucleic acids [31,32,35]. A second aspect relates with the higher relative porosity which in the case of the same biopolymer can improve orientation, coverage and redox activity. In the absence of a general consensus about their comparative performance with the more traditional carbon electrodes, PGEs have the enormous advantage of being inexpensive and commercially available in different diameters and hardness suited to encompass a larger number of applications as disposable electrodes. Moreover, if specific pre-treatment to improve the general outcome of the electrode is intended, experience shows that achievement of adequate performance with GC can be rather fastidious [24]. On contrary, pencil lead electrodes can be cut each time offering renewable and reproducible individual surfaces for simpler and faster polishing procedures [32]. They are also better materials for miniaturization and portability purposes, though the considerable dimensions for in vivo applications when compared to carbon fibres electrodes [36–38].

During biosensors construction process, different pre-treatments of electrodes are usually referred, namely mechanical (polishing process), chemical (using an organic solvent like ethanol or acetone), electrochemical (potentiostatic/potentiodynamic) or a combination of the three. There is not a preferred protocol since it depends on factors such as the diameter of leads used, target analyte or immobilized compound. Nevertheless, whatever the procedure used it decisively influences the final performance in specific applications. If creation of functional groups, mediation of the heterogeneous electronic transfer to electroactive species or adsorption enhancement of these last is envisaged, electrochemical pre-treatments should be adopted [24,39]. Most works describing nucleic acid sensors with PGEs employed high fixed positive

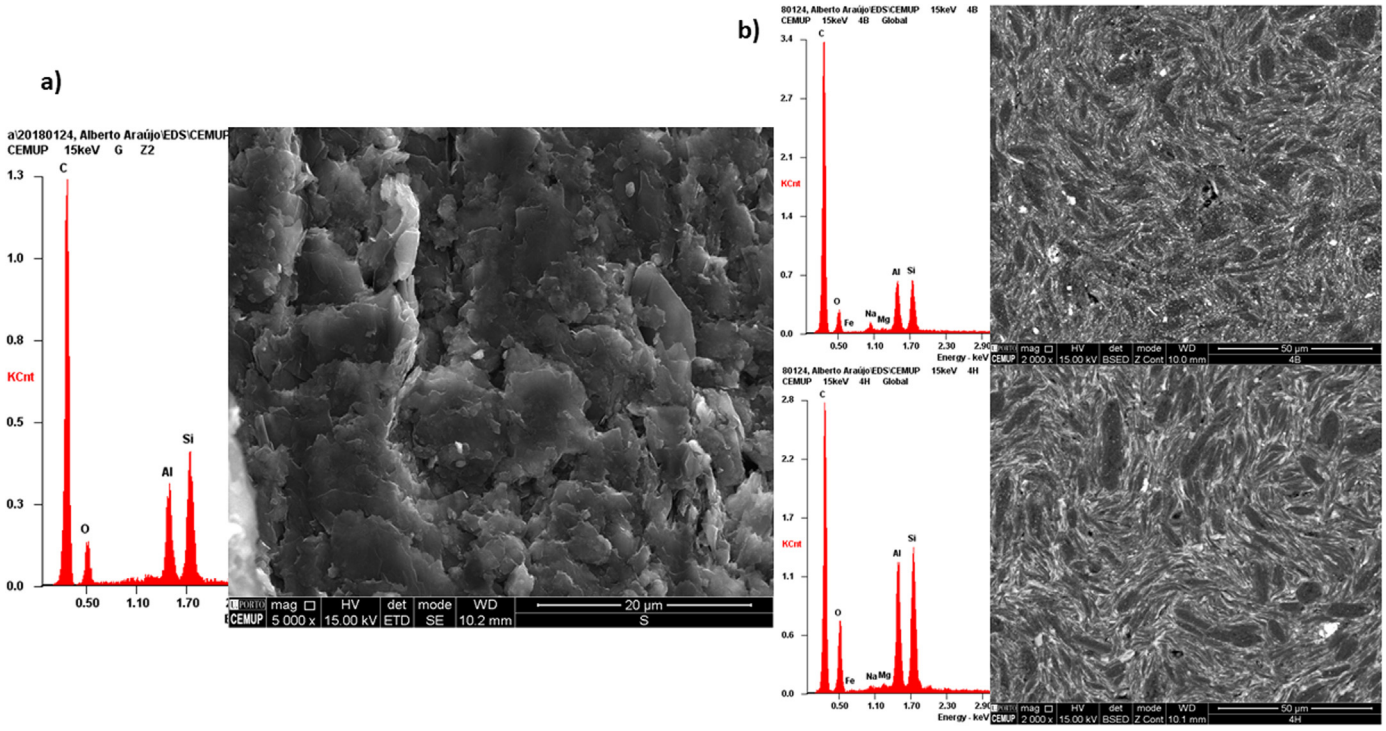


**Fig. 1.** SEM-EDS micrographs of carbonaceous spot in a) Staedtler HB mine and of b) 4B and 4H transverse surfaces after mechanical polishing with 0.05 µm alumina aqueous slurry onto a polishing cloth followed by profuse washing with purified water.

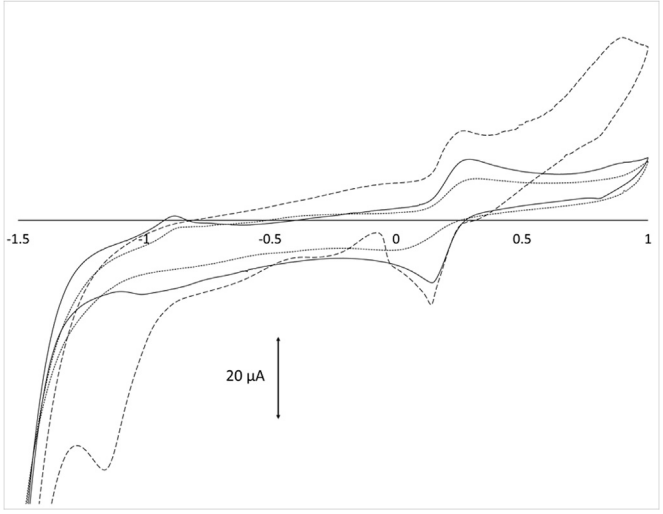


**Fig. 2.** First cyclic voltammograms obtained for Staedtler HB (plain), 4B (trace) and 4 H (dotted) versus Ag/AgCl reference electrode, using a platinum wire as counter electrode. The scans were obtained sweeping potentials first in the reverse direction at 10 mV $s^{-1}$ for a 5 mM potassium $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl electrolyte solution.

potential for a short period of time [32,40–44]. This anodization step seems to be essential to activate the surface and improve the analytical sensitivity. It introduces mediating oxygen functionalities and the formation of a hydrophilic surface readily accessible to the electroactive compounds. Otherwise, treatment provides simple removal of impurities from the polishing and preparation process [39,45]. Özcan group [46] compared the determination of dopamine in blood by using PGEs previously conditioned by two different electrochemical pretreatments (potentiostatic or potentiodynamic). They found that potentiodynamic provided best results by achievement of dopamine signals three times higher. The same conclusions regarding to the effectiveness of the PGE pre-treatment were reached when carcinogen sudan II compound was determined by adsorptive stripping differential pulse voltammetry [47]. The higher efficiency of potentiodynamic over the potentiostatic pre-treatment is explained by the fact that application of negative potentials (cathodization) after the anodization step contributes to the reduction of oxygen passivation, overcoming the inhibitory layer formed during anodization [45]. By means of electrochemical impedance spectroscopy (EIS), some authors evidenced the decrease in the charge transfer resistance ($R_{ct}$) occurring after the electrochemical pretreatment of PGEs [48–51]. The electrolyte used in the electrochemical pre-treatment can also influence transfer process as shown by Özcan [52] who obtained lower $R_{ct}$ and consequently, better oxidation signals for bisphenol A with PGE pre-treated in an electrolyte composed of $LiClO_4$ and NaOH compared to other electrolytes. However, some works omitted any type of treatment performed prior to analysis or modification of the electrode [20,53–55]. Whereas a rough, unpolished graphite surface showed to be beneficial for electrodeposition of copper ions in the nucleation stage [56], a PGE electrochemically pre-treated at + 1.4 V, provided lower signal and lower reproducibility during homocysteine determination [57].

## 2. PGE biosensors

An electrochemical biosensor is defined as an analytical device comprising a recognition surface with a biological entity immobilized over the transducing system. The biological entity may it be an enzyme, whole microorganism, protein or tissue is used with the purpose of detecting specific analytes by converting biochemical energy into a quantifiable electrical signal. The transducer, i.e. the electrode, monitors the biological element in action or processes the product of the biocatalytic reaction and relays it to the amplifier after which the data is displayed [58,59]. An example refers to the immobilization of DNA molecules onto PGE surface to query its interaction with different drugs acting as potential carcinogens. The response is mainly indicated through the variation of the electrochemical signal provided by guanine residues [40,44,60,61]. Main commercially available biosensors are

**Table 1**
Enzymatic PGE biosensors characteristics and analytical performance.

| Analyte | Enzyme (source) | PGE type and diameter | Pre-treatment | Immobilization | Analytical performance | | | | Stability (days) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Linear range (mM) | Sensitivity | | LOD (μM) | | |
| | | | | | | (μA $mM^{-1}$) | (μA $mM^{-1}$ $cm^{-2}$) | | | |
| Glucose | Glucose Oxidase | HB 0.5 mm HB 0.9 mm | mechanical (polishing) | PGE-$POs^{+}NH_2$-GOx | 0 - 10 | - | 1 | - | - | [13] |
| | | 0.5 mm | - | PGE-Nafion-GOx/BSA/PVA-SbQ-MB-CTA | 0.28 - 33.3 | 0.043 | 5.5 | - | 2 | [7] |
| | | H 0.3 mm | - | PGE-PB-GOx/GA-Nafion-PU | 0 - 5.3 | 0.091 | 1.9 | - | 7 | [67] |
| | | HB 0.5 mm | - | PGE-CP-AuNPs-cysteine-DCC-GOx | 0 - 33.4 | 5.06 | - | 22.3 | 45 | [68] |
| | | HB 0.5 mm | - | PGE-CP-AuNPs-cysteine-FcAld-DCC-GOx | 0 - 39 | 2.21 | - | 7.8 | 50 | [69] |
| | | 2B 0.9 mm | mechanical (polishing) Electrochemical (catodization) | PGE-rGO-GOx | 0.04 - 0.6 | 1.8 | 278 | 0.61 | 21 | [72] |
| | | 2B 0.5 mm | Electrochemical (catodization) | PGE-CdS-ZnS-Chit/GOx | 0.01 - 1 | 1.83 | 11.5 | 3 | - | [75] |
| | | 3 mm | - | PGE-poly(GMA-co-VFc)-APBA-FAD-apoGOx | 1 - 17 | 0.27 | 3.8 | 2.7 | 6 | [70] |
| | | 0.9 mm | mechanical (polishing) | PGE-rGO-ZnO/$Cu_2O$-GOx-Nafion | 0.01 − 2 | 0.34 | 54 | 1.93 | 20 | [71] |
| | | B 0.5 mm | Electrochemical (anodization) | PGE-rGO/GOx | 0.01 - 1 | 0.62 | 9.6 | 5.8 | 10 | [73] |
| | | HB 0.5 mm | Electrochemical | PGE-GOx | 0.1 - 8 | 0.03 | 0.9 | 50 | 1 | [74] |
| | Glucose dehydrogenase | 2B 0.5 mm | Electrochemical (catodization) | PGE-CdS-ZnS-BSA/GA/GDH | 0.2 - 8 | 0.118 | 0.74 | 90 | - | [76] |
| | | 2B 0.5 mm | - | PGE-PAMAM-MB-BSA/GA/GDH | 0.01 - 1 | 0.79 | 5 | 4 | 10 | [78] |
| Hypoxanthine | Xanthine oxidase | - | Chemical (acid washing) | PGE-AuFeNPs-XOD | 0.00005 - 0.15 | - | - | 0.05 | 100 | [83] |
| Xanthine | | - | Chemical (acid washing) mechanical (polishing) | PGE-Chit/AuFeNPs-GA-XOD | 0.0001 - 0.3 | - | 1169 | 0.1 | 100 | [82] |
| | | 2 mm | Chemical (organic washing) | PGE-Poly(GMA-co-VFc)/MWCNT-XOD | 0.002 - 0.028 | 16 | 24.3 | 0.12 | 25 | [84] |
| | | - | Chemical (organic washing) | PGE-poly(DTP-alkyl-$NH_2$)-GA-XOD | 0.0003 - 0.025 | 124 | - | 0.074 | 16 | [85] |
| Hydrogen peroxide | Horseradish peroxidase | 6 H 2 mm | Mechanical (polishing) Electrochemical (catodization) | PGE-Chit-AuNPs-HRP | 0.01 - 1.5 | 4.7 | 149 | 2 | 30 | [26] |
| | | 2 mm | Chemical (organic washing) | PGE-Poly(GMA-co-VFc)/rGO-HRP | 0.000006 - 0.0001 | 0.027 | 0.041 | 0.00002 | - | [94] |
| Deferiprone | | - | - | PGE-AuNPs-GA-HRP | 0.1 - 1 | 375 | - | 0.005 | 70 | [96] |
| Oxygen | Laccase (*Trametes versicolor*) | 1.4 mm | Chemical (acid washing) | PGE-Pani-MWCNT-laccase | - | - | - | - | - | [98] |
| | | 5 H 2 mm | Chemical (acid washing) | PGE-Pani-MWCNT-laccase | - | - | - | - | - | [99] |
| | Laccase (*Rhus vernicifera*) | HB, 2 mm | Mechanical (polishing) | PGE-rGO-SWCNT/laccase-solgel | 0 - 0.45 | 4.5 | 132 | 2.7 | - | [100] |
| Ascorbic acid | Ascorbate oxidase (*Cucurbita sp.*) | 2 H, 0.3 mm | - | PGE-PEI-BSA/AOx-PU | 0 - 0.1 | 0.196 | 276 | 0.26 | - | [102] |
| | Ascorbate oxidase (*Cucurbita sp.*) | 2 H, 0.3 mm | - | PGE-SWCNT-PEI-BSA/AOx-PU | 0 - 0.02 | 0.32 | 451 | 0.2 | 11 | [103] |
| | | | | PGE-MWCNT-PEI-BSA/AOx-PU | 0 - 0.02 | 0.486 | 685 | 0.22 | 12 | |
| Cholesterol | Cholesterol oxidase (*Streptomycin sp.*) | HB, 1.5 mm | Chemical (acid washing) | PGE-ChOx | 1.29 - 10.3 | 4120 | 4380 | 90 | 25 | [105] |
| | Cholesterol oxidase (*Brevibacterium sp.*) | - | Chemical (organic washing) | PGE-PTBA-FAD-apoChOx | 0.0008 – 0.0048 | 210 | - | 0.22 | 17 | [106] |
| Triglyceride | Lipase (*candida rugosa*) Glycerol kinase (*Cellulomonas sp.*) Glycerol-3-phosphate oxidase (*Aerococcus viridans*) | 6B 2 mm | Electrochemical (catodization) | PGE-GA/cysteamine/Lipase/GK/GPO | 0.1 - 45 | 185 | 116 | 0.0001 | 240 | [107] |

(*continued on next page*)

**Table 1** (*continued*)

| Analyte | Enzyme (source) | PGE type and diameter | Pre-treatment | Immobilization | Analytical performance | | | | Stability (days) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Linear range (mM) | Sensitivity ($\mu A\ mM^{-1}$) | Sensitivity ($\mu A\ mM^{-1}\ cm^{-2}$) | LOD (μM) | | |
| Ethanol | Alcohol dehydrogenase (*Saccharomyces cerecisiae*) | 2 mm | Mechanical (polishing) Chemical (organic washing) | PGE-SWCNT-PCV-BSA/GA/ADH | 0.0093 - 0.32 | 0.061 | 1.94 | - | 52 | [108] |
| L-lactate | L-lactate dehydrogenase | 6B 2 mm | Mechanical (polishing) Chemical (organic washing) | PGE-GONPs-LDH | 5 - 50 | 3 | 1.6 | 0.1 | 60 | [111] |
| | L-lactate oxidase (*Pediococcus sp.*) | 6B, 2 mm | Mechanical (polishing) Chemical (organic washing) | PGE-PANI-CuNPs/MWCNT-LOx | 0.001 − 2.5 | 1300 | 1009 | 0.25 | 140 | [112] |
| L-glutamate | L-Glutamate oxidase | HB, 2 mm | Mechanical (polishing) Chemical (organic washing) | PGE-ZnO/PPy-Glu-Ox | 0.00002 - 0.5 | 1.4 | 1.1 | 0.00018 | 90 | [114] |
| Uric acid | Uricase (*Arthrobacter globiformis*) Horseradish peroxidase (horseradish) | HB, 0.5 mm | Chemical (organic washing) | PGE-GA/Uricase/HRP | 0 − 0.12 | 2.6 | - | 0.6 | 16 | [115] |
| organophosphate pesticides | Acetylcholinesterase (Electric eel) | HB, 0.9 mm | Chemical (organic washing) | PGE-PEI-PB-GA-AChE | 0 − 0.9 | 0.6 | 6 | - | 60 | [116] |

however typically enzymatic being the most prominent example the glucose sensor. Enzyme biosensors are highly selective for respective substrates and are distinguishable from other types of biosensors by their biological function as catalysers. Nevertheless, responses obtained with the catalyser in solution and upon immobilization are most of times different [62]. The charged nature and hydrophilic/hydrophobic character of the immobilization support determines a new micro-environment. Therefore concentrations of electroactive compounds, ionic strength or pH can differ significantly from those found in bulk solution [63]. Both adsorption and covalent immobilization can impart conformation and steric hindrances to response as well, especially when high loads and crosslinking between the bio-element determines the formation of multiple layers. Unfortunately, most of these aspects are not conveniently addressed in reviewed works, thus limiting envisagement of the most suited protocol for each particular bio-element considered. Thus, literature overview concerning to enzymatic, nucleic acid and whole microorganism PGE-based biosensors will be limited and with emphasis on the pre-treatment used before immobilization and corresponding final analytical performance. The main features are additionally summarized in Table 1.

### 2.1. Glucose oxidase

The fabrication of electrochemical biosensors for point-of-care testing devices used by diabetic patients stands as the most relevant use of glucose oxidase (GOx) in analytical applications. GOx from fungal origin are homodimeric glycoproteins with one FAD cofactor per monomer and shows high selectivity towards β-D-glucose substrate. It is certainly the most used enzyme in research given the high catalytic activity, low cost and availability. The deeply buried cofactor/ active site additionally challenges electrochemists in the search of more effective mediated or direct electron transfer schemes. As molecular oxygen acts as co-substrate by reoxidizing GOx and producing $H_2O_2$ several works resort to such more traditional measuring approach [64,65].

As mentioned earlier, Pishko group [13] was the first to immobilize a biological entity on PGE surface. In low ionic strength medium, the negatively charged GOx was folded by a polycationic redox polymer readily adsorbed onto graphite. The polymer referred as $POs^{+}NH_2$ was synthesised from poly(4-vinylpyridine-*co*-4-aminostyrene) and Os(2,2′-bipyridine)$_2Cl_2$. Thin polished HB leads were encased on heat-shrinkable polypropylene sleeve, and immersed firstly in the polymer suspension which adsorbed strongly ($10^{-8}$ mol cm$^{-2}$) and further coated with glucose oxidase solution (> 100 U mL$^{-1}$, 4 μL). The polymer folding onto glucose oxidase in low ionic strength medium enabled the direct electron shuttle between the buried active catalytic centre of enzyme and the electrode surface. Amperometric measurements of substrate were then performed with a sensitivity value of about 1 μA mM$^{-1}$ cm$^{-2}$. The sensitivity was low but the principle simplicity and ruggedness paved the way for the use of osmium-based redox polymers in latter applications such as in biofuel cells [36]. Two years after a new type of configuration was implemented [7]. The working PGE and an Ag/AgCl reference electrode were inserted inside a hypodermic needle, the latter functioning as counter electrode. The pencil lead was previously electroplated with platinum to allow detection of $H_2O_2$ formed during the GOx catalytic reaction and coated with nafion in order to prevent interferences from ascorbate and urate. The immobilization process consisted in dropcasting the mixture of bovine serum albumin (BSA), stilbazolium polyvinyl alcohol adduct (PVA-SbQ) and the enzyme. In order to avoid a minimal disturbance on enzymatic activity and protein configuration a double crosslinking with glutaraldehyde (GA) vapour followed by photo activation of PVA-SbQ under a fluorescent light was performed according to a previous validated procedure [66]. The setup provided sensitive measurements of glucose with a linear response of 0.06–2 mM, though unfitted with physiological levels. An increase in the analytical range (0.3–33.3 mM) but with

10 times reduced sensitivity trade-off (5.5 μA $mM^{-1}$ $cm^{-2}$) was obtained when a triacetate cellulosic membrane (MB-CTA) was interposed between the sample and the cell. The wide working range of the sensor and its small configuration enabled to hypothesize future application to undiluted blood samples as well as in vivo studies [7].

In 1996 another configuration based on PGE was proposed as implantable biosensor [67]. After electrochemical modification with Prussian blue (PB), an H pencil (0.3 mm ϕ) was placed inside a plastic pipette tip and sealed with epoxy resin. The emergent tip was cross-linked with GOx (12,000 U $mL^{-1}$, 2 μL) using glutaraldehyde, GA (25%), in four repeated cycles. Nafion was then deposited followed by polyurethane (15% w/v). The $H_2O_2$ produced was detected by amperometry conducted at + 0.75 V (*vs* Ag/AgCl) in PBS (pH 7.4). The obtained responses showed a constant sensitivity of 0.091 μA $mM^{-1}$ (1.9 μA $mM^{-1}$ $cm^{-2}$) and a linearity up to 5.3 mM glucose. The electrode sensitivity decreased in plasma samples due to membrane fouling by unspecific components of blood. Its sensitivity was lower when compared with the one described before [7] and was less stable for low analyte concentrations, turning it impeditive for in vivo applications. About two decades later, an HB pencil was covered with carbon paste incorporating gold nanoparticles (AuNPs) to provide chemical anchorage to GOx protein [68]. The biosensor exhibited very good electrochemical performance concerning to stability and durability (reusable after 228 days) though after a fastidious implementation procedure. PGE surface was firstly isolated through coating with carbon paste to avoid interference from native impurities from clay component. The modified surface was sequentially immersed in L-cysteine and *N,N′*-dicyclohexylcarbodiimide (DCC) solution and finally in GOx (865 U $mL^{-1}$). An analytical range up to 33.4 mM glucose, a sensitivity of 5.06 μA $mM^{-1}$ and a limit of detection (LOD) lower than 22.3 μM were obtained. However, the biosensor was this time used in the analysis of hydrolysed extracts of waste tree brunch being the results validated by using HPLC method. A similar approach was performed with ferrocenecarboxaldehyde (FcAld) immobilized onto PGE surface to accomplish the glucose produced in cellulase hydrolysis of waste bamboo chopsticks [69]. Ferrocene derivatives are widely used as diffusional or immobilized electron relays due to their capacity for fast heterogeneous electron transfer at low potentials. So, additional incorporation of FcAld widened the working range up to 39 mM and improved LOD to 7.8 μM when compared to their previous biosensor [68]. However, the sensitivity obtained decreased by half (2.2 μA $mM^{-1}$) showing that mediator immobilization was not entirely beneficial for the analytical performance of the biosensor [69]. In another proposal [70], GOx enzyme was stripped from its native FAD redox centre to yield the apo-enzyme (apoGOx). The PGE was first modified by dropcasting with poly (glycidyl methacrylate-*co*-vinylferrocene), and then immersed in 0.1 M 3-aminophenylboronic acid (APBA) to establish covalent link between the stripped FAD and the polymer. The modified PGE was then soaked in the apo-enzyme solution for effective enzyme reconstitution. A linear range of about 1–17 mM, a sensitivity of 0.27 μA $mM^{-1}$ (3.8 μA $mM^{-1}$ $cm^{-2}$) and a LOD of 2.7 μM were obtained.

The works referred before show that pencil mines provided miniaturized biosensors with good sensitivities owed to enhanced electron transfer at the surface. This aspect is even more evident when metal oxide particles and graphene were used to provide high surface area and enhanced electrochemical properties [71]. Here, the pencil lead was first physically polished with alumina slurry and washed with water. Afterwards, graphene oxide suspension (10 mg $mL^{-1}$) was casted and further electrochemically reduced (rGO). The electrode was then respectively immersed in Zn and Cu solutions and an applied fixed potential modified it with ZnO and $Cu_2O$ nanoparticles. The enzyme was dropcast on the modified surface and left to dry (surface coverage of $3.87 \times 10^{-12}$ mol $cm^{-2}$) before being stabilized with nafion. According the authors, electrostatic forces between the negatively charged enzyme and the positively charged ZnO/$Cu_2O$ particles accelerate the heterogeneous charge transfer process. Given the small electroactive area only a sensitivity of 0.34 μA $mM^{-1}$ was reported but corresponding to a value 54 μA $mM^{-1}$ $cm^{-2}$. Although with a LOD of 1.93 μM, the short linear range (0.01–2 mM) achieved imposed additional sample dilution when human blood serum samples were tested.

Pure electrochemical methods allowing simultaneous reduction of graphene oxide and immobilization of GOx were also described [72]. The pencil lead (type 2B) was first pre-treated by polishing and then applying an oxidizing potential. A solution of graphene oxide (0.5 mg $mL^{-1}$) was applied onto the PGE surface followed by GOx suspension (650 U $mL^{-1}$, 2 μL). Both reduction and immobilization occurred when a potential of −1.5 V for 30 s was applied. This way, a covalent bond between the carboxylic groups of graphene and the side-chain amine groups in the protein were formed. The amperometric biosensor showed a linear response between 0.040 and 0.600 mM, a LOD of 0.61 μM and a sensitivity of 1.8 μA $mM^{-1}$ (278 μA $mM^{-1}$ $cm^{-2}$), for measurements performed at the optimized reduction potential of −0.6 V. Despite the simpler construction process, better general biosensor characteristics were obtained regarding the previously described work [71]. The improvement was due to the increase of specific surface enabled by graphene and its better conductive response in the reduced form (heterogeneous electron transfer of $k = 5.84\ s^{-1}$). However, the linear range upper limit was only 0.6 mM, further away from physiological levels which stand above 4 mM. A very similar procedure as the one described above was adopted, however with the immobilization of enzyme and the GO reduction steps being performed through potentiodynamic means, between −1.3 V and 0.2 V. The sensitivity of 0.62 μA $mM^{-1}$ and LOD of 5.8 μM indicated that the achieved performance was nevertheless poorest [73].

An amperometric biosensor with an automated set up was developed for sequential analyses of 24-well microplates and therefore, perform repetitive analyses without manual operation for several hours [74]. The electrochemical pre-treated PGE (HB, 0.5 mm), containing carboxyl groups was first immersed in EDC and NHS solution followed by immersion in GOx (136 U $mg^{-1}$, 2 mg $mL^{-1}$) solution. In PBS the automated biosensor responded linearly to glucose from 0.1 to 8 mM but only achieving a sensitivity of 0.03 μA $mM^{-1}$ (0.9 μA $mM^{-1}$ $cm^{-2}$) and a one higher order of magnitude LOD of 50 μM.

The combined use of quantum dots (QDs) with GOx have also been proposed showing that electronic and optical properties of the former enhanced the direct electron transfer feature [75]. A 2B pencil mine was first pre-treated by electrochemical anodization followed by two-step electrosynthesis of QDs on electrode surface. First, CdS deposition was performed by immersing in a mixture of $CdCl_2$, $Na_2S_2O_3$, EDTA and mercapto acetic acid solutions and applying −1 V for 1000 s. At last, ZnS was deposited in similar conditions but using a $ZnCl_2$ solution. The modified PGE was incubated in 0.5% chitosan (Chit) and GOx (200 mg $mL^{-1}$) solution for 1 h, dried and rinsed with water before analysis. Accurate detection of substrate concentrations was accomplished in a FIA system. After optimization of hydrodynamic conditions an analytical linear range of 0.01–1 mM was obtained with a LOD of 3 μM and sensitivity of 11.5 μA $mM^{-1}$ $cm^{-2}$ (1.83 μA $mM^{-1}$).

The above described works did not allow identification of a best fitted immobilization process. There is a lack of data to enable comparison of enzyme surface coverage since this influences the diffusion resistance and overall kinetics. As can be seen from Table 1, both approaches proposed by Cheng group [68,69] enabled the most sensitive sensors. Such evidence resulted however from the implementation of typical higher active surface of carbon paste electrodes onto pencil conductive supports and from the very high loadings of enzyme used in the electrode preparation. On the other hand, biosensors proposed by Elahi et al. [71] and Sehat et al. [72] where rGO was used for electron transfer enhancement, an improvement in the LOD was obtained underlining faster heterogeneous electron shuttling process. Surface oxidation to improve wettability plus modification with nanostructures, such as QDs [75], seems to be beneficial since the comparison with similar nanostructured biosensors implemented from HOPG, GC, Au

and ITO, revealed better LOD and sensitivity. This last feature was corroborated after comparison with the works where coverage data is available [13,71].

### 2.2. Glucose dehydrogenase

The glucose dehydrogenase (GDH) is another enzyme seldom used in glucose biosensors. It has similar specificity for β-D-glucose substrate but requires the soluble $NAD^+$ cofactor for catalysis [64] which represents a major drawback to implement third generation biosensors. Recently, a photoelectrochemical biosensor coupled to a FIA system was developed [76]. The anodization pre-treatment of the PGE and modification with CdS-ZnS QDs was performed accordingly to the previous work [75]. The modified PGE was finally immersed in a mixture of GDH (27 kU $mL^{-1}$) and BSA using GA as crosslinking agent. The evaluation of glucose detection was performed by CV with and without QDs irradiation. When the surface was previously irradiated, the peak significantly increased due to facilitated promotion of charges between the valence to the conduction band in the dots. The FIA method under optimized flow conditions and without previous irradiation gave rise to an analytical range of 0.2–8 mM and the obtained LOD and sensitivity was respectively 90 μM and 0.118 μA $mM^{-1}$ (0.74 μA $mM^{-1}$ $cm^{-2}$). When the biosensor was irradiated the sensitivity increased about 2 fold to 0.245 μA $mM^{-1}$ (1.54 μA $mM^{-1}$ $cm^{-2}$) and the obtained LOD decreased to 50 μM [76]. Direct comparison between works where quantum dots were used [75,76] shows that chitosan provided more efficient enzyme immobilization, since both GDH and GOx have similar glucose specificity [77]. The use of QDs was advantageous in terms of biosensor sensitivity although deleterious from the environmental point of view owed to heavy metals used during synthesis. The same research group, in a different immobilization procedure, obtained better results regarding sensitivity (0.79 μA $mM^{-1}$) and LOD (4 μM) using the same enzyme (GDH) and PGE characteristics [78]. The surface was initially modified with polyamidoamine (PAMAM), a dendrimer capable of assisting in enzyme immobilization and electron transfer followed by an electropolymerizable redox mediator, methylene blue (MB). The GDH (338.7 U $mg^{-1}$, 80 mg $mL^{-1}$) was immobilized through cross-linking procedure with GA in the presence of 1% BSA.

### 2.3. Xanthine oxidase

The determination of xanthine and/or hypoxanthine is of great importance in the food industry since it is indicative of freshness especially regarding fish meat. With time of storage, the levels of xanthine increase along degradation. Also in the clinical field the control of xanthine levels in body fluids may be useful for the diagnosis of certain xanthine related diseases such as hypo- and hyperuricemia [79], xanthinuria [80], gout and renal failure [81]. The use of electrochemical biosensors for the detection of xanthine and hypoxanthine may present some advantages over the conventional enzymatic spectrometric and chromatographic techniques since they are less time consuming, cheaper and do not require high-skilled personnel to operate [82].

To the best of our knowledge only Pundir´s [82,83] and Şenel´s [84,85] research groups, have developed PGEs based biosensors where the enzyme xanthine oxidase (XOD) was immobilized. Xanthine oxidase is present in a wide variety of organisms, including humans but is often isolated and purified from bovine milk for scientific purposes. The enzyme is a metalloflavoprotein containing a molybdopterin cofactor, two iron sulphur centres (2Fe-2S) and one FAD cofactor, which catalyses the conversion of hypoxanthine into xanthine and xanthine into uric acid in the presence of molecular oxygen as co-substrate. The substrate is oxidized at the molybdenum active centre and electrons transferred intramolecularly to the co-substrate at flavin site [86,87].

In the initial proposal of these biosensors [82], a mixture of Chit and gold-coated iron nanoparticles (AuFeNPs) was electrochemically immobilized by CV in the surface of a PGE previously treated in acidic solution and mechanically polished. The enzyme XOD (0.15 U, 100 μL) was then covalently bonded via GA reaction. As expected, the nanoparticles decreased the electron transfer resistance through Chit but not compensated the increased upon enzyme immobilization since most biological molecules are poor conductors leading to electrical hindrance. The biosensor presented a LOD of 0.1 μM, linear range from 0.1 to 300 μM and achieved a xanthine sensitivity of 1169 μA $mM^{-1}$ $cm^{-2}$ in phosphate buffer at pH 7.4. The biosensor applicability was tested with fish meat samples and recoveries better than 95% were obtained at two different concentration levels. The functionalization of AuFeNPs with boronic acid improved the covalent immobilization of XOD in a subsequent work [83]. A maximum response for hypoxanthine was obtained at pH 7.2 and 30 °C. In this case a slightly better LOD of 0.05 μM and a shorter linear range (0.05–150 μM) was registered when compared with the previously study [82]. Hypoxanthine levels were successfully determined in fish, chicken, beef and pork samples and corroborated the substrate concentration increase with the storage time [83].

The approach based on the modification of PGE with Poly(GMA-*co*-VFc) reported before for the glucose biosensor [70] was extended to produce a xanthine biosensor [84]. The referred polymer was mixed with MWCNT and dropcasted over the PGE surface after cleaning with acetone and deionized water. After this modification the electrode was oven dried and soaked in a XOD solution of 9 mg $mL^{-1}$ (source and activity not stated but higher that 3.6 U) for 48 h. The addition of MWCNT to the redox polymer led to an increase in peak height and the electrochemical kinetics of the biosensor was proven to be diffusional controlled through CV measurements at different scan rates. The best amperometric results at oxidation potential of + 0.35 V were linear in the range of 2–28 μM, resulting in a sensitivity of 16 μA $mM^{-1}$ (24.3 μA $mM^{-1}$ $cm^{-2}$) and a LOD of 0.12 μM. The sensitivity of the present biosensor is about 48 times lower (1169 μA $mM^{-1}$ $cm^{-2}$) than the one proposed by Devi et al. [82], and reflected in achievement of a shorter linear working range. The biosensor was applied to the determination of xanthine in fish meat stored from 5 to 20 days where a linear increase of xanthine concentration up to 32 μM was found [84]. One year later a biosensor with the enzyme conjugated with an dithieno (3,2-b:2′,3′-d)pyrrole derivative polymer was proposed [85]. The planar structure of the polymer and the electron releasing groups makes it suitable for electron transfer enhancement and overcome the difficulty of enzyme direct electron transfer. Prior to modification, the PGE was cleaned with water and acetone and then modified with poly 10-[4H-dithieno(3,2-b:2′,3′-d)pyrrole-4-yl]decane-1-amine (poly(DTP-alkyl-$NH_2$)) through electropolymerization. The covalent immobilization was obtained by immersing firstly in a GA solution followed by XOD solution in ammonium sulphate for 48 h at 4 °C. Amperometric xanthine measurements in chicken samples were performed at the oxidation potential of + 0.5 V in pH 7.0 buffered medium. The electroactive polymer allowed better response characteristics related to LOD (0.074 μM) and sensitivity (124 μA $mM^{-1}$) when compared with the previously described [84].

### 2.4. Peroxidase

As known, hydrogen peroxide plays important roles in industrial processes where it is used as oxidizing agent in paper bleaching, wastewater treatment and disinfection. Biologically, as reactive oxygen species (ROS), promotes oxidative stress and ageing [88] being also a signalling molecule of ischemia/hypoxia events [89]. Partial reduction of molecular oxygen as by several redox enzymes such as GOx and XOD which utilize $O_2$ as co-substrate in catalysis processes leads to hydrogen peroxide production [90,91]. In turn, $H_2O_2$ is the main substrate of a number of enzymes being horseradish peroxidase (HRP) the best known in the analytical field. As oxidizing agent, it reacts with the heme iron (III) state of the enzyme in a two-electron oxidation process to form an

intermediate compound comprising the Fe(IV) oxoferryl centre and a porphyrin radical cation with concomitant formation of a water molecule [92,93].

A PGE biosensor with immobilized HRP to accomplish $H_2O_2$ determinations was initially proposed by Teepoo group [26]. The pencil mine (6H) was cleaned by mechanical polishing with alumina followed by electrochemical treatment in acetate buffer and then coated with Chit (0.5%). A layer by layer method was implemented for subsequent immobilization of AuNPs along 6 h followed by adsorption of HRP (5 mg $mL^{-1}$) for 12 h. These last two steps were repeated once more to attain best amperometric response. As stated before, AuNPs used together with redox proteins enabled to increase the specific area, conductivity and promote direct electron transfer between the redox centres and the electrode. The performance of the biosensor was evaluated at the optimized potential of −0.2 V with pH of solutions adjusted to 6.0, hence evidencing linear response range from 0.01 to 1.5 mM with a LOD of 2 μM. The biosensor showed a good response to $H_2O_2$ with a specific sensitivity of 149 μA $mM^{-1}$ $cm^{-2}$ (4.7 μA $mM^{-1}$), six times better when compared with a similar biosensor without AuNPs modification. Good reproducibility ( ± 5%) and recoveries between 80% and 104% were achieved in the determination of $H_2O_2$ in hair dye and disinfectant samples.

Other studies evaluated the efficiency of a PGE modified with various carbon additives [94]. The mediator polymer (poly(GMA-*co*-VFc) used in GOx and XOD biosensors [70,84] was this time mixed with carbon additives (carbon black, carbon nanofibers, extended graphite, MWCNT and rGO). Each mixture was dropcasted on a PGE surface, previously cleaned with acetone. After dried it was soaked in 5 mg $mL^{-1}$ HRP solution (pH 7.0) for 48 h at 4 °C under agitation. The best amperometric performance was obtained for the modified PGE doped with rGO and resulted from lower electron transfer resistance as evidenced by electrochemical impedance spectroscopy (EIS). Sensitivity values of 27.4 nA $mM^{-1}$ (0.041 μA $mM^{-1}$ $cm^{-2}$) were however significantly lower than the one determined by Teepoo et al. [26].

Since HRP catalyses the oxidation of a wide spectrum of substrates [93] including pyridine-based compounds [95], direct detection of 3-Hydroxy-1,2-dimethyl-4(1 *H*)-pyridone (deferiprone), an anti-HIV drug, justified the proposal of a new HRP biosensor based on PGE [96]. The pencil mine was first modified with Chit followed by gold nanorods particles. The PGE was then dipped in a 2.5% GA solution to promote crosslinking and finally in a HRP solution (40 mg $mL^{-1}$) for 24 h at 4 °C. EIS studies showed that the two-electron oxidation of the drug catalysed by HRP enhanced the charge transfer rate leading to decreased $R_{ct}$. The response was linear from a concentration of deferiprone of 0.1–1 mM with a LOD of 0.005 μM. These results were also observable by square wave voltammetry where a sensitivity of 375 μA $mM^{-1}$ was obtained. Results validated against a conventional procedure enabled recovery values of about 99% after analyte determination in urine and serum samples thus stressing the usefulness of the described biosensor [96].

### 2.5. Laccase

Some important advantages are pointed out to the ability of enzymes to generate power in fuel cell applications relatively to traditional batteries. In fact, enzymatic biofuel cells do not require use of precious metal catalysts and drive multi-electron redox reactions in less harsh pH conditions. However, produced powers are in the order of microwatts and long-term reduced stability of the biological element are still major issues fostering research. Laccases are blue multicopper oxidases which catalyse the oxidation of several phenolic compounds with concomitant four-electron reduction of $O_2$ to $H_2O$. This last feature attracted researchers on their use as biocathode in fuel cells. Laccases are widely obtained from fungal and plant origins being respectively *Trametes versicolor* and *Rhus vernicifera* the most representative sources. Laccases from fungi are nevertheless mostly referred since they are able to drive reduction of $O_2$ at higher potentials when compared with plant laccases. This way, higher open circuit potential (OCP) and consequently higher power generation is allowed. On the other hand, plant laccases present optimal activity at more neutral pH compared to the more acidic pH of fungal laccases [97]. In this context, an electrode with immobilized laccase from *T. versicolor* was evaluated concerning its efficiency in oxygen reduction [98]. The modification procedure started by ultrasonic assisted cleaning of bare PGE with HCl and finally dried before use. Aniline was electropolymerized (Pani) on the surface of PGE and then immersed in a suspension containing MWCNT (1 mg $mL^{-1}$) and N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride / N-hydroxysuccinimide (EDC/NHS) (1 mg $mL^{-1}$) in order to provide covalent anchorage between the activated carboxyl group of MWCNT and side-chain amine functionalities of laccase in 3 mg $mL^{-1}$ concentrated solution. For comparison purposes, another PGE was prepared in a similar way but without the polymer. In experiments with $N_2$ saturated buffer the OCP measured was low and the generated current density negligible. In the presence of oxygen and ABTS mediator the maximum OCP and current density achieved was respectively + 0.6 V and 296 μA $cm^{-2}$ for the modified PGE, against 229 μA $cm^{-2}$ for PGE without Pani. The higher current density observed for the PGE with Pani was attributed to the highly porous three dimensional nanofibrous structure of the polymer which provided large surface area for enzyme immobilization. Two years later, the same research group repeated the work above but optimizing the type of lead used in the PGE (H, 3 H, 5 H and B) with type 5 H achieving the highest current density, followed by type B [99].

In our group, it was developed and characterized a biocathode comprising the tree laccase *Rhus vernicifera* immobilized in a nanostructured PGE. The electrode was implemented on a HB type PGE casted with a graphene oxide that was further electrochemically reduced. A mixture containing 25 mg $mL^{-1}$ laccase (1.07 U $mg^{-1}$) suspension and 5 mg $mL^{-1}$ single-walled carbon nanotubes (SWCNT) was dropped over the surface followed by the addition of tetrakis(2,3-dihydroxypropyl)-silane monomer sol to provide enzyme physical entrapment. By setting the potential of −0.2 V, the modified PGE enabled a proportional response to molecular oxygen up to 0.45 mM and a sensitivity of 132 μA $mM^{-1}$ $cm^{-2}$ with a LOD of 2.7 μM. Assembled as a biofuel cell, the biocathode was capable to generate the maximum power of 4.5 μW $cm^{-2}$ at + 0.25 V in quiescent oxygen saturated solution [100].

### 2.6. Ascorbic oxidase

Fruits and vegetables are naturally rich in vitamin C compound, further used as natural additive to preserve colour, prevent bacterial growth and extend freshness of other foodstuffs [101]. Thus, analytical strategies based on use of biosensors to this compound usually fit important applications in both industrial and clinical contexts. As indicator of antioxidant capacity and biologically powerful reducing agent, ascorbic acid has firstly captured the attention of Barberis group in a proposal based on PGE biosensing [102,103]. Therefore, a telemetric online system to assess levels in orange juice was devised, which included a biosensor based on ascorbate oxidase (AOx). The system, highlighting all versatility of PGE in electrochemical applications, consisted of four pencil leads (type 2H): biosensor, sensor, auxiliary electrode and a pseudo-reference electrode. The pencil lead used as biosensor had its surface modified with polyethylenimine (PEI) for enzyme stabilization, and was simply immersed in a solution containing 10% BSA and AOx (1 U $μL^{-1}$) and dried. Soaking in a 0.2% polyurethane (PU) solution enabled final enzyme immobilization. The PGE sensor was implemented in the same way but without enzyme. Biosensor response differed in about 30% of that recorded for PGE without enzyme and reflected the selectivity for the substrate oxidized by AOx. In buffered medium, the linear response to ascorbic acid extended from 0 to 0.1 mM with sensitivities of 0.668 μA $mM^{-1}$ (941 μA $mM^{-1}$ $cm^{-2}$)

and 0.196 μA $mM^{-1}$ (276 μA $mM^{-1}$ $cm^{-2}$) for the sensor and biosensor, respectively. However, during analysis of orange juice samples, such sensitivities decreased probably as result of interfering species [102]. Later, the same authors studied the influence of different fullerenes ($C_{60}$ and $C_{70}$) and nanotubes (SWCNT and MWCNT) for electron transfer enhancement in nanostructured sensors. Significant increase in sensitivity towards ascorbic acid in phenols mixture was observed for PGE modified with SWCNT and MWCNT (5.1 times) compared with fullerene $C_{60}$ and $C_{70}$ (1.2 and 1.5 times respectively). Biosensors with both types of nanotubes achieved the same linear range (0–0.02 mM) and LOD (0.2 μM). However, with MWCNT the sensitivity was 0.486 μA $mM^{-1}$ (685 μA $mM^{-1}$ $cm^{-2}$) while for SWCNT was only 0.32 μA $mM^{-1}$ (451 μA $mM^{-1}$ $cm^{-2}$). This biosensor was applied in the determinations of ascorbate and total antioxidant capacity of fruit juices [103].

### 2.7. Cholesterol oxidase

Cholesterol is a sterol molecule essential to structural integrity and fluidity of cell membranes and a major precursor in biosynthetic pathways leading to vitamin D and steroid hormones [104]. As important nutritional parameter directly related with health disorders, cholesterol was quantitatively determined by means of a cholesterol oxidase (ChOx) modified PGE [105]. In this biosensor development, the pencil lead (type HB) was chemically pre-treated with HCl solution followed with $HNO_3$. The treatment aimed either the removal of surface impurities and improvement in the adsorption of ChOx by creating oxygen functionalities that might interact with negatively charged enzyme. After treatment, the PGE was immersed in a ChOx solution and then washed with buffer. The amperometric response was linear for cholesterol in the range of 1.29–10.3 mM and with a LOD of 90 μM. The sensitivity of the biosensor was about 4120 μA $mM^{-1}$ (4380 μA $mM^{-1}$ $cm^{-2}$). The results obtained in serum samples were in good agreement with the standard method.

An apoChOx biosensor have been also proposed for cholesterol determination [106]. The best analytical results were obtained for a PGE modified with poly(thiophen-3-boronic acid) (PTBA) with 10 times higher sensitivity (210 μA $mM^{-1}$) when compared with the other polymer tested, poly(3-aminophenyl boronic acid). The LOD obtained was low (0.22 μM) and the electrode responded to cholesterol in a very short linear range (0.8–4.8 μM) which required a high dilution factor if the biosensor was to be used in blood samples. The reconstitution procedure of the enzyme and immobilization on the PTBA modified PGE was based on previously published work from the same authors [70].

### 2.8. Lipase, glycerol kinase and glycerol-3-phosphate oxidase

The monitoring of triglycerides levels present similar importance as cholesterol in terms of cardiovascular disorders and therefore was the target of an amperometric biosensor comprising co-immobilization of three enzymes [107]. In the proposed biosensor, the co-immobilization of lipase, glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO) allow the breakdown of the triglyceride molecule, triolein and consequently the detection of the $H_2O_2$ produced. The attachment of the enzymes on electrochemically pre-treated PGE (type 6B) was performed through GA and cysteamine linking. Triolein was detected in a wide linear range (0.1–45 mM) with a sensitivity of 185 μA $mM^{-1}$ (116 μA $mM^{-1}$ $cm^{-2}$) and LOD of 0.1 nM.

### 2.9. Alcohol dehydrogenase

Albeit biosensors may be considered an important instrument accomplishing the evolution of ethanol levels in fermentation processes in beverage industries, as far as known only one report aimed the use of a PGE based on alcohol dehydrogenase (ADH) [108]. In the biosensor construction, pencil leads were mechanically treated with alumina, followed by sonication in water and then ethanol for removal of adsorbed particles. The clean surface was further casted with SWCNT (0.1 mg $mL^{-1}$) and then the pyrocatechol violet (PCV) used as an electron transfer mediator was electrodeposited by CV.The immobilization of the enzyme was performed by dipping the PGE in a solution of ADH (30 mg $mL^{-1}$) containing BSA and GA. The biosensor was applied in amperometric measurements of ethanol, obtaining a range of 0.0093–0.32 mM with a sensitivity of 1.94 μA $mM^{-1}$ $cm^{-2}$ (0.061 μA $mM^{-1}$). Ethanol levels were determined in different alcoholic beverages and the results compared with a gas chromatographic method where the relative errors stayed between 2.7% and 4.4%.

### 2.10. L-Lactate dehydrogenase

The production of L-lactate may have an important role in the study of some clinical pathologies such as diabetes [109]. Beyond the presence of L-lactate in plasma, this compound may also play interest in the food industry since it is used as additive due to its preservative properties. Produced by lactic acid bacteria, it is present in milk products being responsible for the sour taste [110].

Two types of L-lactate enzymes (dehydrogenase and oxidase) have been used in two different PGE biosensors by Pundir group [111,112]. In the L-lactate dehydrogenase (LDH) biosensor, a PGE (6B, 2 mm) was mechanically polished and washed with ethanol. Graphene particles were electrodeposited by CV in the treated PGE surface and the carboxylic groups were activated with EDC and NHS for 6 h to further immobilize LDH through the amine bonds by incubating the PGE in the enzyme solution (1 mg $mL^{-1}$, 25 U $mg^{-1}$) overnight. The PGE biosensor achieved a linear range from 5 to 50 mM and a sensitivity of 3 μA $mM^{-1}$ (1.6 μA $mM^{-1}$ $cm^{-2}$) with LOD of 0.1 μM [111].

### 2.11. L-lactate oxidase

The L-lactate oxidase (LOx) biosensor comprised a different immobilization procedure in comparison with the LDH. Here, a similar treated PGE was modified by electropolymerization of aniline to polyaniline (Pani) followed by electrodeposition of a mixture of copper nanoparticles (CuNPs) with EDC/NHS treated MWCNT in the same conditions as Pani. Finally, the modified PGE was immersed in the LOx solution and kept overnight at room temperature. The amperometric response increased linearly with lactate concentration up to 2.5 mM, with a LOD of 0.25 μM and sensitivity of 1300 μA $mM^{-1}$ (1009 μA $mM^{-1}$ $cm^{-2}$). Successful determinations of L-lactate were performed in samples taken from healthy persons and lactoacidosis patients as well as in milk, wine and beer products [112].

### 2.12. L-glutamate oxidase

By being a neurotransmitter, quantitative analysis of glutamate can be relevant to study the neural mechanisms involved in cognition, memory and learning functions [113]. An L-glutamate PGE biosensor was developed as an alternative to chromatographic, spectrophotometric and titration methods [114]. First, the surface of the electrode (type HB) was polished with alumina, rinsed and sonicated in ethanol to remove slurry particles. A mixture of zinc oxide nanorods (ZnO) and pyrrole was electropolymerized (PPy) on the PGE surface. Glutamate oxidase (GluOx) was fixed by immersion in a 5 U $mL^{-1}$ enzyme solution and kept overnight at room temperature. Amperometric studies showed that oxidation current increased linearly with L-glutamate concentration from 0.02 to 500 μM. The calculated sensitivity was 1.4 μA $mM^{-1}$ (1.1 μA $mM^{-1}$ $cm^{-2}$) and the LOD was 0.18 nM. The biosensor was applied lastly in food samples (Chinese soup) where glutamate is responsible for the umami taste, achieving a good correlation with the standard colorimetric method.

### 2.13. Uricase

Uric acid is the final product from purine metabolic breakdown and it is frequently tested in blood to diagnose gout or kidney disorders. Tsai and Wen [115] proposed a biosensor where two enzymes, uricase and HRP, were co-immobilized together with a mediator for detection of uric acid at low potential. Here, uricase enzyme catalyses first uric acid into $H_2O_2$ which is further used by HRP. The electrons generated were then mediated to the electrode by ferrocenemonocarboxylic acid. Before the co-immobilization, the PGE (type HB) was cleaned in methanol and rinsed with water. Then it was incubated in a mixture of uricase (20 U $mL^{-1}$) and HRP (350 U $mL^{-1}$) containing GA as cross-linker. The amperometric current increased linearly with uric acid concentration up to 0.12 mM and the LOD was 0.6 μM. The calculated sensitivity was about 2.6 μA $mM^{-1}$. The results obtained in the analysis of blood serum were accurate when compared with the standard spectrometric method.

### 2.14. Acetylcholinesterase

The detection of compounds at trace levels are generally accomplished by at least one extraction/pre-concentration step in organic medium. Wilkins group [116] evidenced the possibility of dipping high sensitive PGE sensors directly in the organic medium in order to screen organophosphate compounds. Regarding portability, the analysis of environmental samples performed in situ and in a faster way was further hypothesized. The immobilized acetylcholinesterase (AChE), hydrolyses the acetylthiocholine substrate to thiocoline which in turn reacts with the haxacyanoferrate (III) electron shuttle. First, PGE (type HB) was cleaned in methanol and distilled water. The AChE (950 U $mL^{-1}$) was covalently immobilized via GA in the PGE previously modified with PEI and subsequently electro-polymerized with PB. The use of the positive charged PEI polymer created a microenvironment, favouring partition effects regarding the analyte (organophosphate inhibitors). The linear range of the calibration plot was between 0 and 0.9 mM and the sensitivity was about 0.6 μA $mM^{-1}$ (6 μA $mM^{-1}$ $cm^{-2}$) which was about 10 times higher than the sensitivity obtained for biosensor without PEI-PB film. The percentage of the enzyme inhibition increased with the increase in concentration of dichlorvos, diazinon and fenthion pesticides and the LOD for the three pesticides were $0.5 \times 10^{-6}$ M, $0.8 \times 10^{-6}$ M and $1.0 \times 10^{-6}$ M respectively.

### 2.15. Antibodies

Ivnitski and Rishpon research group [117–119] resorted to disposable PGEs to implement the so-called enzyme channelling immunosensor. The viewpoint was that through heterogeneous sandwich immunoreaction at the electrode surface was possible to bring together complementary enzymes regarding a common electroactive substrate thus providing amplified detection in one-step procedure. The enzymes GOx and peroxidase were typically chosen to label the PGE bound and free conjugate antibodies. To further improve distinction between conjugate bound antibody and the one remaining free in the sample medium additional dipping of the PGE in spinning mode was used. The typical procedure for preparation of PGE (HB, 0.5 mm diameter) included wet cleaning with methanol and water prior to dipping in methanol solution containing 2–5% polyethylenimine (PEI). Then 5 mm long PGE surface was immersed in 2.5% glutaraldehyde (GA) for 2 h. GOx or HRP labelled antibody were immobilized on PGE by dipping it in a 1 mg $mL^{-1}$ or 0.5 mg $mL^{-1}$ PBS solution for 4 h at 4 °C. Finally, the PGE was immersed by 1 h in a glycine (0.1 M) solution to block unreacted aldehyde groups. In the presence of glucose GOx produced $H_2O_2$ in turn used as substrate of peroxidase to oxidize iodide ions (mediator) to iodine. The iodine formed is then monitored at a constant reduction potential of −0.07 V (vs SCE) and hence regenerated again to iodide. For practical purposes, human luteinizing hormone (hLH) was determined in blood serum in a 'sandwich' immunoassay by modifying a PGE with avidin. The hLH determination principle was based sandwich formed between the analyte to be detected and two specific monoclonal antibodies, each directed against a different epitope on the hLH molecule. The captured antibodies were conjugated with biotin (αhLH-b), while the other antibodies (used to reveal the reaction) were labelled with HRP (αhLH-HRP). The catalytic current was observed at −0.07 V and the produced calibration curve for hLH showed linearity from 0 to 10 ng $mL^{-1}$ with detection limit of 1 ng $mL^{-1}$ [117].

A similar configuration was implemented in order to study the location of binding site of immobilized calmodulin with target proteins through its immunochemical response [118]. On the other work conducted by Rishpon and Ivnitski [119] the enzyme-channelling immunoassay was tested in different model systems including biotin-advin, HIV viral antigens (CD4-gpl20) and detection of bacteria *Staphylococcus aureus*. For the determination of biotin, PGE immobilized with avidin/GOx was performed in a competitive assay at −0.1 V (vs Ag/AgCl) in a solution containing mediator (KI), glucose and biotin-HRP. A sigmoidal shape response was obtained with increasing concentrations of biotin with current stabilizing with a concentration of 200 ng $mL^{-1}$. The immunosensor was then applied to quantitative determination of HIV glycoprotein gpl20 due to the specific interaction with the immobilized antigen CD4 in the presence of glucose and gpl20-HRP. The current was maximum in the absence of gpl20 and decreased with the increasing concentration. In the last model system, RbIgG was co-immobilized with GOx on the PGE surface promoting the binding of S. aureus cells to the antibody enabling its detection. With increasing concentration of cells in solution more will be attached to the RbIgG in the electrode which in turn will create the binding of RblgG-HRP in a “sandwich” format, generating an increased amperometric signal. S. aureus cells were detected at concentrations as low as 1000 cells $mL^{-1}$.

### 2.16. Living cells

The activity of nitrate reductase in viable bacteria involved in denitrification was assessed in a comparative fashion by using the same mechanical polishing on the surfaces of a PGE (0.79 mm2), one GC electrode (3.14 mm2) and one basal plane HOPG electrode (15 $mm^2$). The same volume of the bacteria strain suspension was dropped over respective surfaces and enclosed with a dialysis membrane fixed to the electrode body with a nylon net. When both the tetramethyl-p-benzoquinone mediator and KNO3 substrate were present in phosphate buffer solution a reduction peak was observed at −0.5 V (vs Ag/AgCl) on forward scans performed at 500 mV $s^{-1}$. The signals obtained were similar for PGE and basal plane HOPG electrodes, 20 μA $cm^{-2}$ but only half of the value reported for GC electrode. After exposure of bacteria modified electrodes to magnetic field (10 mT, 50 Hz) for 24 min peaks similarly decreased by about 20%, due to bacterial death [120]. The specific detection of the virulent K12 strain of Escherichia coli in concentrations above $10^3$ CFU $mL^{-1}$, also showed the ability of PGE in analysis involving whole cells. The terminal 10 mm length of pristine Tombow HB 0.5 mm leads were immersed for one hour in nanoemulsion of CTAB capped gold nanorods in PBS [121]. Before assay the PGEs were incubated for one hour with $10^6$ PFU $mL^{-1}$ bacteriophage T4 dilutions from 1:2 up to 1:10. The equilibration with 100 μL of suspicious suspensions of bacteria for 10 min, followed by rinsing with PBS (pH 7.4), and impedance measurement for further 25–30 min in the presence of 2.5 mM equimolar ferricyanide/ferrocyanide $[Fe(CN)_6]^{4-/3-}$ redox system completed the assay. Phage specificity for the K12 strain was evidenced by the decrease of registered impedance due to lytic action on the bacteria layer adsorbed on the PGE surface. Also, a passive adsorption protocol was adopted to provide immobilization onto PGE surface of 450 nm nanoparticles of diphenylalaninamide grown in aqueous medium by crosslinking with glutaraldehyde [122]. Before assay, the modified PGE sensor was allowed to dry for 15 min and within the biological suspension showed selectivity towards DLD-1

colorectal cancer cells translated by increasing interfacial electron transfer impedance with the same above cited redox probe.

### 2.17. Nucleic acids

Mature microRNAs are 20–30 nucleotides-long, non-coding nucleic acids transcribed from genetic material in cells of living beings. They are able to pair in different extent molecules of messenger RNA in order to silence protein production and regulate gene expression [123]. Profiling of microRNAs in biological samples revealed typical patterns associated with inherited or acquired pathological conditions including tumorigenesis, degenerative, development and addictive diseases. This recent observation has challenged analytical research community to find new selective and expeditious diagnostic tools as alternative to the tedious Northern blot procedure in use [124,125]. In this context, Kilic group [41] exploited the use of disposable PGEs for the direct detection of upregulated microRNA 21 (miR-21) in cell lysates of human breast adenocarcinoma. The PGEs (Tombo HB, 0.5 mm diameter) were initially anodized by applying a potential of + 1.4 V (*vs* Ag/AgCl) in acetate buffer (pH 4.8). Formed carboxylates were then modified to succinimidyl ester groups through dipping in EDC (5 mM) and NHS (8 mM) mixture for 1 h (step deemed as surface covalent activation). The -COOSuc groups on the surface of pencil leads were now able to attach nucleic acids via amide linkage with guanines (N7 position) after 20 min of contact in solution. To ensure specificity and sensitivity the method comprised respectively further hybridization with complementary antimiR21 labelled with biotin, and incubation in solution containing streptavidin labelled with alkaline phosphatase. If hybridization occurred, also the complex biotin-labelled streptavidin remained after a step of surface washing to finally allow the enzymatic conversion of alpha naphtyl phosphate, into the electroactive alpha naphtol easily oxidized and detected by differential pulse voltammetry at + 0.23 V *vs* Ag/AgCl electrode. The procedure was able to detect miR-21 at concentrations 6 pmol in total RNA cell lysates. A biosensor also aimed for the same target was recently proposed, where the PGE surface was initially coated with gold nanoparticles in order to provide higher immobilization yield regarding the probe [126]. Unfortunately, the limit of detection achieved was one order of magnitude higher, 100 pM. The same group proposed a simpler biosensor for detection of miRNA-34a in breast cancer cell lysates [127], which up-regulation promotes both inhibition in cells growth and enhanced sensitivity to drug therapy. The tip of a Tombow HB pencil (1 cm) was initially cleaned and activated at + 1.4 V *vs* Ag/AgCl for 60 s in acetate buffer solution (pH 4.8). Then, it is was soaked in a solution containing the antimiRNA-34a (5 μg $mL^{-1}$) probe and pyrrole added under stirring for 1 min (0.1 M prepared in 0.3 M KCl). Finally, the entrapment of the probe into a polypyrrole film covering the surface of PGE was enabled just by electrochemical scanning from − 0.6 V to + 0.8 V *vs* Ag/AgCl at the scan rate of 25 mV $s^{-1}$. The impedance of the biosensor changed after the hybridization process corresponding to the detection, whenever miRNA-34a exceeded 0.2 μg $mL^{-1}$ in the sample. A similar approach for miR-21 showed the reliability of both the entrapment scheme and transduction principle [128]. DNA molecule also is effectively adsorbed onto PGE surface and provides amplified signal compared to pristine PGE [47,129]. Pividori and Alegret [130] showed that adsorption of DNA on the pre-treated electrodes depends more on the DNA bases than on the phosphate–sugar backbone. In order to achieve the best adsorption of the biological material and/or highest analytic signal optimum anodization potential should be nevertheless optimized. Pournaghi-Azar group [44], found a suitable PGE pre-anodization potential of + 1.7 V in acetate buffer pH 4.8 for the analysis of hepatitis C virus DNA from a range of studied potentials within the − 2 V to + 2.5 V window. They noticed that potentials above + 2 V and more negative than − 1.5 V could lead to the formation of gaseous products from the background electrolyte.

Not only RNA but also single-stranded oligonucleotide DNA sequences can be folded in diverse tertiary structures and then rival monoclonal antibodies regarding the cognate targets or even mimic enzyme catalysis in a wide variety of reactions. At least for compounds ideally bearing positive charge, proton acceptor-donor groups and having planarity features this new wave bio-ligands, known as aptamers, show affinities in the nM to pM range and astonishing selectivity even for simple stereoisomer molecules as is the case between L- and D-aminoacids [131]. Because the synthesis dispenses biological living machinery and can be tailored to the target using a combinatorial chemistry approach on libraries of initial randomized oligonucleotides, several hundreds of reliable aptamers are nowadays available at reasonable costs for heavy metals, antibiotics, toxins, hormones and proteins [132]. Their use in biosensor schemes with electrochemical transduction, may it be of the sandwich type, impedance or structure switching-based assays, showed that immobilization with higher surface densities is potentially enabled due to smaller size of aptamers. Through application of denaturing-renaturing chemical or temperature cycles, the reversibility of the recognition process is also possible [133,134]. Four years ago, a modified PGE for the detection of lysozyme based on the use of an amino-linked anti-lysozyme single-stranded DNA aptamer was proposed [135]. The tip of the pencil lead, 10 mm length, was electrochemically activated as already described above for Kilic group paper [41] and sequentially immersed for 15 min in 110 μL of optimized mixture of graphene oxide in the chitosan polycationic polymer, and for 30 min in equal volume of the aptamer. The high impedance for electron charge transfer due to charge repulsion between the anionic $Fe(CN)_6^{3-/4-}$ redox probe and the negative charged phosphate backbone of the aptamer decreased proportionally in the presence of lysozyme in concentrations above 28.53 nM, being the transduction facilitated by the presence of graphene. Rotring HB pencil leads with 0.5 mm were modified as described above [41] to exhibit -COOSuc groups on the surface and immersed in 40 μL of 15 μg $mL^{-1}$ amino-linked ss-DNA anti-thrombin in Tris-HCL buffer. This biosensor provided at the + 0.23 V *vs* Ag/AgCl electrode for sinusoidal 10 mV signals in the frequencies interval between 1 mHz to 100 kHz, increasing impedances for charge transfer to the probe with the thrombin-aptamer complex concentration [136]. More recently, Ensafi group resorted to a 30 base long ss-DNA with high affinity to insulin, after acquiring a G-quadruplex conformation in the presence of $Mg^{2+}$, to propose a disposable impedance biosensor almost exclusively constructed along successive optimized electrochemical steps [137]. The surface tip of a Pentel HB 0.7 mm lead was in the first step immersed in 0.1 M of sodium carbonate, lithium perchlorate solution and activated through 5 run cycles, 50 mV $s^{-1}$, within the potential limits of − 0.40 and 2.00 V. The active surface was then coated with an electro polymerized film of conductive poly-orthophenylene diamine which in turn served as substrate for gold nanoparticles obtained along more run cycles within − 0.50 to 1.50 V at a scan rate of 2 mV $s^{-1}$. Final dipping in the thiolated aptamer resulted in self-assemblage of the bio-ligand due to the affinity of SH groups for the gold surface of nanoparticles. The biosensor was responsive to insulin in the 1–1000 nM range, using urine or plasma 1:1 diluted with PBS as samples. The assemblage of thiolated anti-dopamine over gold nanostars showed the possibility of having impedance biosensors with sensitivity comparable with the elctrochemiluminescence method for this neurotransmitter molecule. The biosensor was however reusable after immersion for 15 min in aptamer denaturizing solution containing 7 M urea [138]. The gold nanostars were synthesised according to the seed-mediated growth protocol [139] and the bare PGE simply immersed for 15 min in a water suspension of them at 75 °C.

## 3. Conclusions and future perspectives

Pencil mine electrodes paved the way to be considered as valuable alternative to other carbon-based electrodes as evidenced is this review. The thin dimensions and negligible cost enables uses as easily

polarizable and disposable electrodes. Moreover, the ability to offer renewed surface by simple cut, provides good reproducibility as well as implementation efficiency. Several factors may affect PGE performance and the analyst should take them in consideration in order to achieve the best analytical results. The electron transfer efficiency varies according to pencil lead hardness and even may vary according to the pencil manufacturer. Also, the pre-treatment given to the active surface of the electrode plays a crucial role in the outcome of the analysis. A suitable pre-treatment may eliminate background currents by removing impurities or create oxygen functionalities that may enhance the immobilization of the biological entity.

Regarding biosensors, a relevant part of published works refers to enzymes immobilization thus explaining the review organization but with a brief reference to different immobilized biological entities namely, nucleic acids, antibodies and whole microorganisms (bacteria) depending on the envisaged application. In the end of our bibliographic research we have accounted 37 enzymatic and 1 bacteria PGE biosensors applied to the analysis of glucose, xanthine, hydrogen peroxide, oxygen, ascorbic acid, cholesterol, ethanol, L-lactate, L-glutamate, uric acid, drugs, triglycerides, pesticides, bacterial survival, hormones and viral antigens. The enzyme glucose oxidase is the most used enzyme in PGE biosensors due to general commercial success of glucose biosensors and to the clinical significance of the glucose analyte. Studies on laccase activities towards oxygen were also important for the development of enzymatic biofuel cells and self-powered sensors. More recently, pencil leads have been used to draw electrodes and conductive circuits in paper-based sensors making these systems simpler, inexpensive and solvent-free. But as far we know, the application of pencil drawn electrode with enzymes is still limited or even non-existing, predicting a new window of opportunities in research.

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