ECS The Electrochemical Society
Advancing solid state & electrochemical science & technology

Review—Pencil Graphite Electrodes as Platform for Enzyme and Enzyme-Like Protein Immobilization for Electrochemical Detection
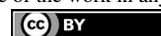
View the article online for updates and enhancements.

Review—Pencil Graphite Electrodes as Platform for Enzyme and Enzyme-Like Protein Immobilization for Electrochemical Detection

Akash Nathani,[=,z] **Nandimalla Vishnu,**[=] **and Chandra S. Sharma**

Creative & Advanced Research Based On Nanomaterials (CARBON) Laboratory, Department of Chemical Engineering, Indian Institute of Technology Hyderabad, Kandi, 502285 Telangana, India

Carbon-based electrodes are being used widely nowadays for biosensor applications, primarily owing to their good electrical conductivity and ease of functionalization. At the same time, the increasing demand for the low cost, disposable and the ease of availability for the do-it-yourself assemblies have provided an opportunity to look beyond conventional carbon materials for electrochemical analysis. In recent time, the pencil lead, entitled as the pencil graphite has been used as an electrode for the enzyme-based electrochemical biosensors. The review highlights the various aspects involved in using pencil graphite electrode (PGE) as a working electrode. This includes the various pretreatment strategies used, which is the first step toward the effective surface functionalization, followed by strategies used for the immobilization of the functional nanomaterials and the enzymes and finally, the integration of the PGE with different types of sensor assemblies. A comprehensive discussion on the latest development in this area also suggests future perspectives based on PGE to develop low-cost point-of-care diagnostics.

In recent times, pencil graphite electrodes (PGE) are emerging as an alternative for the conventional carbon and other metal electrodes. The prime composition of PGE is graphite, which is a most stable form of carbon available in mineral ores like coal.[1] Over carbon and other conventional metal electrodes, PGE, as a disposable electrode gained much consideration among the electrochemical groups for its abundance at ultra-low-cost with various interesting properties like; mechanical rigidity, chemical inertness, low background current, wide potential window, analyte adsorption, ease of miniaturization, and modification.[2,3] Note that sp^2 hybridized carbons of graphite are devoted to the good adsorption and higher conductivity. Thus, graphite composite films on the conventional electrodes are used for the voltammetric determination of genotoxic nitro compounds,[4] organic compounds,[5] antibiotics[6] and nucleic acids.[7] Interestingly, PGEs can facilitate the renewable surface easily unlike, the stringent polishing procedures required for the conventional electrode like glassy carbon electrode (GCE), and yields better reproducibility for the analyses.[2,8,9]

Based on the interesting properties of PGEs and thrust, our group member has already exploited the pre-anodized PGE (PGE*) as surface renewable electrochemical sensor for (i) the detection of total phenolic preservatives in commercially available pharmaceutical insulin[9] (ii) separation-free sensing of dihydroxy benzene isomers in tea dust[10] and (iii) simultaneous sensing of hypoxanthine (Hx), xanthine (X) and uric acid (UA) in fish samples for quality monitoring.[11] Before our attempt, several researchers successfully used PGE for the electrochemical applications and proved that PGEs are the better choice over the other conventional carbon electrodes. For instance, Wang et al. observed that PGE yielded better electrochemical responses over the GCE for stripping based detection of nucleic acids.[12] Similarly, J. K. Kariuki in 2012 explored and compared the physical and electrochemical characteristics of PGE with GCE.[1] He studied the electron transfer rate of PGE and GCE with redox systems (Eg. [Fe(CN)$_6$]$^{3-/4-}$, [Ru(NH$_3$)$_6$]$^{3+/2+}$) and found that PGE provided well defined and promising voltammetric peaks with comparable electron rate transfer to GCE. On the other hand, a screen-printed carbon electrode (SPCE) may act as an alternative to low-cost PGE. For instance, SPCE based electrochemical biosensors are used to evaluate the damage of nucleic acids,[13] antioxidant activity of beer, coffee and tea,[14] and to determine Methocarbamol and Paracetamol simultaneously.[15] Unfortunately, most of the SPCEs are single-use and disposable type electrodes, and its surface cannot be renewed easily.[16] In addition, the overall cost of PGE from a commercially available pencil lead is less than the SPCE.[11]

Among the hetero-structured graphite, pencil lead is one of the low-cost and abundantly available materials used for various purposes in day-to-day life. Note that, as the name suggests, pencil lead does not hold a metallic lead in it and it is an example for an intercalated compound containing a mixture of clay and other particles in a conducting graphite material.[17] However, depending on the hardness and blackness, pencils are categorised from 9H to 9B, here H stands for the hardness and B indicates the blackness.[17,18] For instance, 6B contains 85% graphite, 10% clay and 5% wax.[19,20] EDX of the same displayed 82.9% of C, 6.5% of Si, 6.0% of O, 2.2% of Fe and 2.4% of Al[21] whereas 2B consists of 79% graphite and 21% clay[22] with elemental composition of ((% w/w) O (8.5), P (0.1), Al (2.5), Si (3.0), K (0.18), Mg (0.05), Fe (0.6), C (85.0) and Na (0.18)) thus the main composition being aluminum silicate and HB consists of 68% graphite, 26% clay and 5% wax.[23] However, there is a variation in the composition of a particular grade by different manufactures. Although it seems obvious that the high graphite content serve as a better electrode due to high electrical conductivity which is true in certain cases,[24] the choice for the appropriate grade mostly depends upon the specific interaction between the analyte and the graphite/clay composite. For instance, in the enzymatic sensing of hydrogen peroxide, 6H displayed a superior performance compared to HB, 2B, 1H, 2H, 3H, 4H and 5H by delivering a good reversible redox current.[25] In other cases, where the sensor configuration involves electrodes drawn on the cellulose paper, the ease of delivering sufficient material to the paper dictates the choice of the electrode.[26] On the other hand, several researchers were using the chemically modified pencil leads as PGE based sensors for various electrochemical and biological applications. For instance, Prussian Blue modified PGEs for H$_2$O$_2$ sensing.[27] Likewise, 1-napthylamine polymerised PGE for pH studies in non-invasive body fluids,[28] MoS$_2$ grown PGE for guanine and adenine electrochemical detection[29] and FeS$_2$ grown PGE as an in-expensive tool for detection of UA in human urine samples.[30]

Due to the increasing trend on modified PGE based electrochemical sensors, Akanda et al.,[22] clearly discussed the trends in the fabrication of metal/metal oxide/metal complex nanostructure, carbon nanostructure and polymer modified PGE as chemical or bio-sensors. In addition, Torrinha et al.,[31] discussed the biosensors based on enzyme-modified PGE. However, their discussion limited to research findings of the nanomaterial and enzyme-modified PGE's. In this regard, we are motivated to discuss the biosensors not limited to development

[=]These authors contributed equally to this work.
[z]E-mail: ch14m16p000001@iith.ac.in
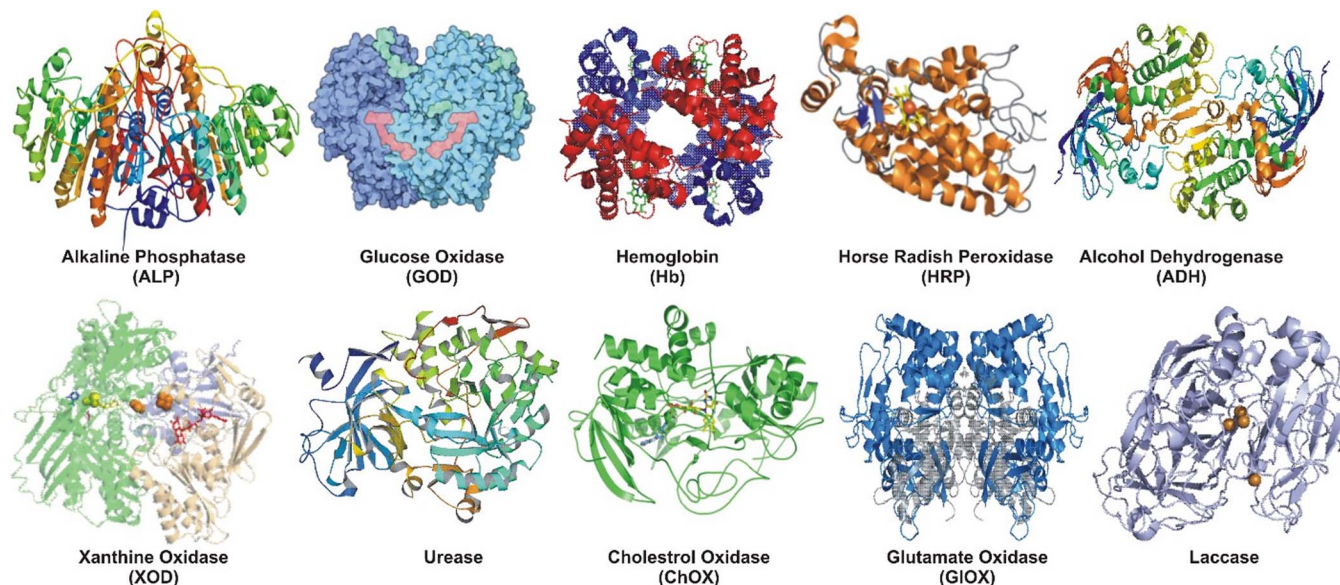
Figure 1. Some of the enzymes and enzyme-like proteins used for immobilization on PGE (Source: Wikipedia).

of biosensors based on enzyme only but also on research trend in the construction of enzyme-like proteins like haemoglobin (Hb) on PGEs. Starting from 1997, enzymes like glucose oxidase (GOx), alkaline phosphatase (ALP), xanthine oxidase (XOD), cholesterol oxidase (ChOx), horseradish peroxidase (HRP), urease, alcohol dehydrogenase (ADH), laccase, glucose oxidase (GlOX), Uricase, Ascorbic oxidase, Lipase, Glycerol Kinase (GK) & Glycerol -3-phosphate Oxidase and proteins like Hb (Figure 1) are used for the modification of chemically modified PGE or bare PGEs and employed as electrochemical biosensors.

Electrochemical biosensors are one of the biosensors where they transform the information of the target biochemical to a readable signal in the current or voltage. These electrochemical biosensors are majorly useful in a wide range of applications like the development of point of care devices, assessing the quality of food and monitoring the environment.[31–37] Fabrication of an electrochemical biosensor includes the two-step procedure, i.e., development of a biocompatible platform and immobilization of the desired enzyme. Immobilization of the desired enzyme on a biocompatible platform and retaining its activity during the analyses are crucial in the construction of a successful electrochemical biosensor. Although enzymes are complex and expensive, immobilization process yields various advantages like reusability, improved stability and reduction in the cost of operation.[38–43] Thus based on the enzyme used for modification of PGEs, the current review article is sub-divided into five main categories, (i) GOx, (ii) ALP, (iii) Hb, (iv) XOD and (v) other enzyme-modified PGEs.

GOx modified PGEs.—Among various enzymes, GOx is used for monitoring glucose. In the development of an electrochemical biosensor for glucose, GOx will be immobilized on the platform constructed by nanomaterial, and it easily catalyses glucose with better sensitivity and improved selectivity. In 1997, Zahir et al.[22] were first to fabricate the mediator-less 2B-PGE based glucose sensor. In this work, 0.003 M of 4- vinyl pyridine (4VP) was polymerized to poly (4- vinyl pyridine) (PVP) on 2B- pencil rod under the constant potential of +0.40 V vs SCE in pH 3 conditions. After successful polymerization, the optimal electrode was immobilized with GOx (i.e., by three different techniques as (i) 5 μL of GOx solution applied for 5 min (ii) the disc was immersed in a GOx solution and stirred for 0.5 hr at room temperature, dried and placed at room temperature, and (iii) 0.2% GOx solution was placed in cell before the 4-vinyl pyridine (4-VP) polymerisation at pH 6.4. Each PVP/GOx electrodes were placed still in

GOx in phosphate buffer solution (PBS) in the refrigerator and prior to use, all PVP/GOx modified electrodes were first rinsed with PBS. Then in an aerated pH 7 solution, at + 0.18 V vs SCE, the glucose current response was measured for 1–14 mM glucose concentrations which yielded a linear relationship with a 0.0325 A M^{-1} sensitivity and 0.5 mM as detection limit (LOD). Although the reusability of the enzyme electrode retained over a week, the developed sensor suffers from some common interferents like lactic acid, ascorbic acid (AA) and 4-aceto amino phenol.

In another case, Cheng et al.[44] fabricated the glucose sensor by using a hydroxyl methyl ferrocene mediator and HB-PGE modified with the carbon paste (CP) and GOx linked nano-Au (AuNP) particles (i.e., HB-PGE/CP-AuNP/GOx). The HB-PGE/CP-AuNP/GOx preparation procedure includes five-steps, in step-I, PGE surface was coated with a 0.8 cm layer of CP and dried for 10 min at 120°C. In step-II, AuNP were electrodeposited on the surface of CP by electro reduction. In step-III, HB-PGE/CP-AuNP was dipped in L-cysteine (CySH) solution for 1 hr at 25°C to create the covalent bonds between the sulphydryl groups of CySH and AuNP. Further, the electrode was washed gently with distilled water to avoid the loosely bounded CySH. To create the effective bonds between the electrode and GOx, electrode was placed in 40 mM solution of N, N'-dicyclohexylcarbodiimide (DCHCDI) chloroform solution for 1 hour at 40°C in step IV. Further, the electrode was dipped for 24 h in GOx in step-V. During the process, an amide bond was created between the carboxyl group and the amino group of CySH and enzyme, respectively. Prior to use, HB-PGE/CP-AuNP/GOx sensor was preserved at 4°C in pH 7 PBS. At 0.33 V, the HB-PGE/CP-AuNP/GOx with a mediator showed an oxidation peak corresponding to glucose. For 45-days, calibration curve by addition of glucose exhibited good linearity at 0–33.41 mM with 5 × 10^{-3} A M^{-1} and 22.3 μM as sensitivity and LOD respectively. The HB-PGE/CP-AuNP/GOX was usable till 228 days and showed 5–8% interference to mannose, galactose and xylitol and 0–1% to cellobiose, xylose, and arabinose. The developed HB-PGE/CP-AuNP/GOx electrode was used for the analyses of glucose produced by the hydrolysis of Cinnamomum caphora tree branch fiber and validated with HPLC. In his next attempt, Cheng et al.[45] used HB-PGE/CP-AuNP/GOx for the flow injection analysis (FIA) of glucose. In this work, the HB-PGE/CP-AuNP/GOx prepared by following the aforementioned five steps and with a new step. This new step was prior to step-IV and in this step the CySH bonds containing electrode was placed in redox mediator, 20 mM ferrocene carboxaldehyde (FcAld; dissolved in EtOH/HCl, 99.5/0.5, v/v) for 1 hr at 75°C and rinsed further with
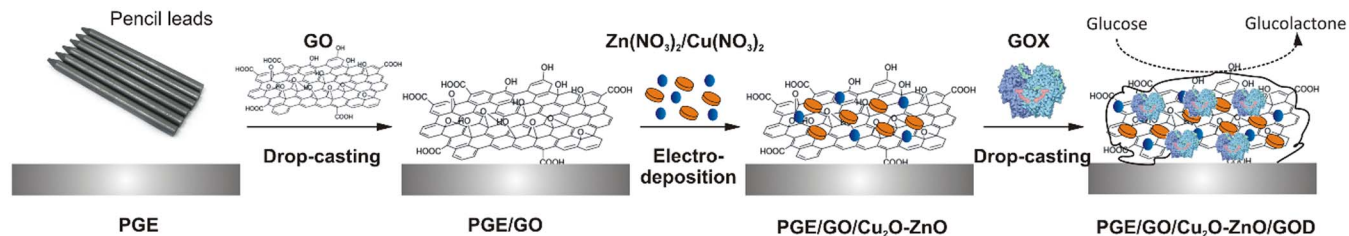
Figure 2. Schematics on the preparation of PGE/GO/ZnO/CuO$_2$/GOx and its reaction to glucose oxidation.[47]

distilled water. Thus, the enzyme immobilized electrode was developed by chemical bonds between the redox mediator, FcAld and GOx modified HB-PGE/CP-AuNP. The new FIA electrode showed the linear response in a concentration range of 0–39.0 mM with 2.21×10^{-3} A M^{-1} sensitivity and 7.8 μM LOD. The FIA electrode can be used for more than 50 days and displayed 5–7% interference to mannose and galactose. The developed electrode was successfully tested for the glucose detection in immobilized enzyme hydrolysate of waste bamboo chopsticks.

Similarly, Dervisevic et al.[46] developed a glucose sensor by immobilizing GOx on a poly(glycidyl methacrylate-co-vinylferrocene) (i.e., poly(GMA-co-VFc)) polymer casted PGE (i.e., PGE/ poly(GMA-co-VFc)-GOx). In the fabrication of electrode, poly(GMA-co-VFc) was drop-casted on the electrode surface of PGE and left to air dry. After drying, PGE/poly(GMA-co-VFc) was dipped in 3-aminophenylboronic acid and in flavin adenine dinucleotide (FAD) to create the linkages between the polymer and FAD. Further, the developed electrode was placed still for 24 h in the enzyme solution to let the reconstitution on the FAD monolayer. Using amperometric i-t technique, current response of PGE/poly(GMA-co-VFc)-GOx to glucose was measured at an applied potential of 0.3 V in pH 7.5 PBS and found linearity in 1–16 mM range with a LOD 2.7 μM (S/N = 3). Further, stability measurements of the PGE/poly(GMA-co-VFc)-GOX revealed that the electrode retained 90% of its original activity during first 13 additions and displayed 65% in last 6 spikes. Prior to the next measurement, the PGE/poly(GMA-co-VFc)-GOx electrode should be stored at 4°C (0.1 M; pH 7.0 PBS) for 5 min. Then the storage studies displayed a stable response for the first week and reduced to 60% in the next 6 days.

In the next phase, Y. Mortazavi et al.[47] started using graphene oxide (GO) and reduced GO (rGO) for the fabrication of GOx based glucose sensors. In the first case, PGE was modified by a GO and ZnO/Cu$_2$O (PGE/GO/ZnO/CuO$_2$). GOx was immobilized on the PGE/GO/ZnO/CuO$_2$ using the electrostatic interaction of positively charged PGE/GO/ZnO/CuO$_2$ and negatively charged enzyme. The PGE/GO/ZnO/CuO$_2$/GOx preparation includes drop-casting of GO suspension on the cleaned surface of PGE (PGE/GO), and by a two-step, electrochemical process, ZnO/Cu$_2$O compounds were prepared on PGE/GO. At first, ZnO was formed by applying a potential of -1.4 V for 200 s and by further by applying -0.7 V for 150 s, Cu$_2$O was formed on PGE/GO to yield PGE/GO/ZnO/CuO$_2$ electrode. The electrode was further cycled between 0 to -1 V vs Ag/AgCl in 0.1 M PBS. On PGE/GO/ZnO/CuO$_2$ electrode, GOx was immobilised by simple drop-casting and air-dried at the room temperature (Figure 2). To prevent the loss of enzyme, Nafion was overlayed on the surface of PGE/GO/ZnO/CuO$_2$/GOx. Under optimal differential pulse voltammetry (DPV) conditions, the interaction between the glucose and enzyme was studied. The current response of GOx was reduced with an increase in glucose concentration. The glucose sensor displayed linearity to glucose over a range of 0.01–2 mM with a LOD = 1.93 μM.

In the next attempt, Mortazavi et al.[48] covalently immobilized GOx on 2B-PGE*/GO and simultaneously GO was reduced by using chronoamperometric and cyclic voltammetric methods (i.e., 2B-PGE*/rGO/GOx). Before 2B-PGE*/rGO/GOx preparation, the 2B-PGE (0.9 mm in diameter with a surface area of 6.36×10^{-3} cm^2)

was polished by a glossy paper sheet and then pre-anodized by applying $+1.8$ V oxidation potential in 1 M NaOH solution for 10 s (2B-PGE*, * = pre-anodized). Then 2 μL of GO solution (0.5 mg/mL) was drop-casted on the 2B-PGE* and kept at room temperature to dry. 2 μL of GOx solution (5 mg mL^{-1} in pH 7 PBS) was drop-cast onto dried GO and was kept at 15 ± 3°C for 5 min. For the two fabricated electrodes, the enzyme immobilization with simultaneous reduction of GO was done by two electrochemical methods. In the first method, 6 continuous cyclic voltammetry (CV) cycles in 0 to -1.5 V potential range at a scan rate of 50 mV s^{-1} was employed, and in the second method, the -1.5 V potential was applied constantly for 30 s. Finally, the as-prepared modified electrodes are identified as 2B-PGE*/rGO/GOx. The developed 2B-PGE*/rGO/GOx electrode showed a pair of redox peaks at -0.521 V corresponding to the enzyme, and by amperometric method, 2B-PGE*/rGO/GOx displayed a 1.77×10^{-3} A M^{-1} sensitivity and 0.61 μM LOD at 0.04 to 0.6 mM glucose concentrations in pH 7 PBS.

In yet another study, Vijayaraj et al. used rGO and GOx with the pretreated Type-B PGE for sensitive detection of glucose in PBS (pH 7).[49] Here, the pretreatment was carried out by running 5 cycles of CV in 0.1M H$_3$PO$_4$ solution at the scan rate of 50 mV s^{-1}. Prior to the pretreatment, the curved surface was covered with Teflon, and the end was polished smooth using weighting paper. The rGO-GOx composite was synthesized on the surface of PGE* in a single step. First, the GO was synthesized using Hummers and Offeman method. Later, the PGE* was immersed in the mixture of GO (1 mg mL^{-1} in DI) and GOx (10 mg mL^{-1} in 0.1 M PBS, pH 7.0) dispersions for 4h followed by the electrochemical treatment of 10 cycles CV from -1.3 to 0.2 V in N$_2$ saturated PBS (pH 5) at 50mVs^{-1} to form rGO–GOx/PGE*. Here, the reduction of GO to rGO and the covalent bonding of GOx to GO was confirmed using fourier-transform infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS). The formation of oxygen functionalities during pretreatment and reduction of GO was systematically investigated by the comparative voltammetry studies of GO–GOx/PGE* and GO–GOx/PGE while the immobilization of GOx by comparing the CV's of PGE, PGE*, GO/PGE* and GO-GOx/PGE* in PBS. The greater reduction in the reduction peak current for GO–GOx/PGE* as compared to GO-GOx/PGE over the successive cycles indicated the presence of a greater number of oxygen functionalities in case of GO–GOx/PGE*. It was observed that the reduction current increases in the order PGE< PGE* <GO/PGE* but later decreases in the presence of GOx i.e., GO–GOx/PGE*, this was mainly due to the consumption of oxygen functionalities to form amide bond with the –NH$_2$ groups of GOx thus confirming the successful immobilization of the enzyme. Finally, the sensing mechanism was based on the reduction of the oxygen reduction peak current (Reaction 1b) in the presence of the glucose. This was probably due to the consumption of the dissolved oxygen by glucose following Reaction 1a. Chronoamperometry at -0.4 V was used to study current response range which was observed to linear in $1 \times 10^{-5} - 1 \times 10^{-3}$ M with dynamic response up to 10×10^{-3} M and the LOD 5.8 μM. No interference was observed in the presence of acetaminophen, AA and UA. Identically prepared four rGO-GOx/PGE* electrode showed a relative standard deviation of 5.6%. Stability was studied by storing the electrode at -4°C in PBS for 10 days which caused a reduction in the current response by 4.98% for 0.5 mM glucose solution.

Reaction 1:

$$Glucose + O_2 \rightarrow gluconicacid + H_2O_2 \qquad [R1a]$$

$$O_2 + 4H + 4e^- \rightarrow 2H_2O \qquad [R1b]$$
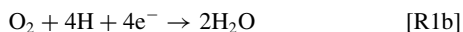
Further, Sağlam et al.[50] also immobilized GOx on quantum dot (QD) (ZnS-CdS) modified PGE, crosslinked with chitosan (CT) for PGE*/ZnS-CdS/CT/GOx fabrication and for the electrochemical detection of glucose through FIA method. For this PGE was activated at an applied potential +1.4 V for 60 s in pH 6 PBS. By electrochemical precipitation, quantum dots (ZnS-CdS) were deposited on the surface of activated PGE (PGE*). The PGE* was placed for 10 min in a mixture solution of 15 mM CdCl$_2$, 8 mM Na$_2$S$_2$O$_3$, 8 mM ethylene-diaminetetraacetic acid (EDTA) and 0.05 mM mercapto acetic acid (MAA) (to avoid the coagulation of quantum dots) containing pH 6 PBS and applied −1 V potential for 1000 s at 30°C. Further, the PGE*/CdS exposed to similar conditions with 15 mM ZnCl$_2$. The obtained PGE*/CdS-ZnS was placed for 1 hr in GOX solution mixed with CT and dried at 4°C in the refrigerator. Using FIA, dried PGE*/ZnS-CdS/CT/GOx was used as an electrochemical detector for glucose at flow rate = 1.3 mL min^{-1}, transmission tubing length = 10 cm, injection volume = 100 µL and constant applied potential = −500 mV vs Ag/AgCl. Under optimal conditions, the glucose sensor showed a linear response in the 0.01–1 mM range with LOD = 3 mM.

Teanphonkrang et al. developed a prototype for the automated robotic amperometric quantification of glucose in 24-well microplates.[51] The device consisted of a PGE working electrode with a computer-controlled stepper motor to move the three-electrode assembly sequentially between the samples. The computer-controlled micropositioner allowed the stepper motor to move the assembly in all three directions, x,y & z, i.e. right-left, backwards-forward and up-down respectively and the 2.5 ml vials of the 24-well plastic microtiter served as the electrochemical cells. The working electrode consisted of HB type PGE with 0.5 mm diameter and 3 cm length, and heat shrink tubing was used to cover the length of the electrode leaving 2 mm of the length to interact with the electrolyte. A systematic study of the pretreatment was carried out considering the untreated and the treatment using continuous and pulsed-potential driven electro-oxidation in 1 M KNO$_3$ solution. The efficiency of the pretreatment was assessed by the ion exchange technique. The H$^+$ ions from the carboxyl groups formed during pretreatment were exchanged with Ag$^+$ ions by immersing the PGE* in 1 M AgNO$_3$ in 0.1 M KNO$_3$ for 1 min. Later the efficiency of the ion exchange and hence the pretreatment was examined by DPV in 1 M KNO$_3$. The DPV reduction of bonded Ag was significantly higher in the case of pulsed treatment as compared to the continuous and untreated electrode, confirming the high efficiency of the pulsed treatment. Later the enzyme immobilization was carried out using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) – N-hydroxysuccinimide (NHS) chemistry for both the types of pretreated electrodes and also the untreated electrode. A comparative sensing study was carried out using chronoamperometry for the reduction of H$_2$O$_2$ formed due to enzymatic reaction at 600 mV by spiking the supporting electrolyte with 1mM glucose solution at regular intervals. The H$_2$O$_2$ reduction current was significantly higher in the case of pulse treated compared to the continuous treated and the untreated electrode. After confirming pulsed treatment as the better technique, the time of treatment was optimized by varying the pretreatment for 2, 5, 15 and 30 min. It was observed that the H$_2$O$_2$ reduction current increased with the increase in the pretreatment period and got saturated at 15 min of pretreatment although the improvement in the slop and the width of the linear region was optimal at 5min of pretreatment, so the same was used for the later studies. Later the automated stability and calibration testing were carried out in the 24-well microplates where the alternate wells were filled with buffer and glucose solution of different concentrations. The buffer solutions were mainly used for the baseline correction and cleaning of the electrode. The signal stability was within 5% of the mean value after the continuous operation for 6.5 h. The assembly offered the evaluation of 4 and 20 samples a per plate run in standard addition and calibration mode, respectively. The linear response was obtained within 0.1–8 mM range and the LOD was 0.05 and 0.1 mM for the manual measurement and automated measurement respectively.

ALP modified PGEs.—In *E. coli*, ALP of molecular weight around 94000 kDa is dimeric and a periplasmic protein. However, it is enzymatically inactive in cytoplasm. Most of the immunoassays use ALP as a label in the analyses. On the other hand, detection by electrochemical methods achieved wide attention due to its precise measurement of current in turbid and coloured samples. In 2005, Ozsoz et al.[52] were the first to report on the hybridization detection of enzyme labelled DNA by covalently immobilization technique by α-naphthyl phosphate (α-NAP) signal. In this work, on PGE surface was immobilized with ALP using the biotin-extravidin (Ex) and electrochemically assayed using a substrate, α-NAP, for the detection of hybridization. Using coupling agents, N-(dimethylamino) propyl- N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) probes were covalently attached on the surface of PGE. For hybridization, the PGE attached with probes are placed in a solution containing the oligonucleotides. ALP labelled extravidin (Ex-ALP) bind to hybrid and α-NAP was added. Consequently, the reaction will occur between the ALP and α-NAP. Post hybridization, the current response obtained by the reduction of α-NAP, i.e., 1-naphtol was measured by DPV (Figure 3).

After DNA hybridization, Ozsoz et al.[53] used a similar technique to detect the mir21 in breast cancer cells (i.e., micro RNA). To construct a sensor, c-probes were attached covalently on 0.5 mm HB-PGE using, EDC and NHS, coupling agents. For hybridization, the HB-PGE attached with c-probes is placed in a solution containing the target probes. ALP-Ex binds to the target due to the interaction between biotin-avidin and enzyme transforms electro-inactive α-NAP (substrate) to electro-active 1-naphtol (product). Post hybridization, the current response obtained by electroactive 1-naphtol was quantified by DPV at +0.23V. Similar to the above work, Erdem et al.[54] and co-authors of the above work, developed protocol for the electrochemical detection of specific Polymerase chain reaction (PCR)-amplified DNA fragments by the introduction of biotin tags into the DNA amplicons during the PCR run in the presence of a biotinylated nucleoside triphosphate. For electrochemical detection, PGE with biotinylated DNA at the surface using the streptavidin-ALP conjugate, are used. The ALP converts the indicator α-NAP which is electrochemically inactive to an electroactive 1-naphthol. The amount of 1-naphthol was quantified by linear sweep voltammetry (LSV).

In another study for the detection of miRNA-21, Mandli et al. used a sandwich hybridization technique using PGE working electrode to overcome the need for modification of the target thus making the technique more suitable for the real samples.[55] In this study, the authors used HB-type pencil lead with 0.5mm diameter and 1.5 cm length of which 1 cm was immersed into the electrolyte. Here the pretreatment was carried out by applying 1.4 V for 60 s in 0.5 M acetate buffer (pH 4.8). The strategy involved functionalization of PGE* with gold nanoparticles (NP) followed by the immobilization of the thiol terminated capture probe (SH-p1). Later the nonspecific sites of the AuNP were covered using 6-mercapto-1-hexanol (MCH) which also helped in the orientation of the probe. After the hybridization of the miRNA-21 (T) with the capture probe, a biotinylated complementary probe (B-P2) was hybridization with the target to form a sandwich structure. Finally, streptavidin-conjugated ALP was immobilized on the B-P2/T/MCH/SH-P1/AuNPs/PGE* by specific interaction with biotin to complete the sensor assembly B-P2/MCH/T/SH-P1/AuNPs/PGE*. The hybridization of the target was investigated with 1 mg mL^{-1} α-NAP in diethanolamine (DEA) buffer wherein, in the presence of the ALP the electro-inactive α-NAP was converted to electroactive 1-naphtol that was sensed by voltammetry. A systematic study of each processing step was evaluated to get the optimal sensor performance. These include the numbers of deposition cycles for the synthesis of Au nanoparticles where the PGE* was cycled between 0.9 to −0.3 V at a scan rate of 50 mV s^{-1} in 0.1 M KNO$_3$ containing 4mM HAuCl$_4$ followed by potential cycling between 0.2 to 1.6 V at 50 mV s^{-1} in
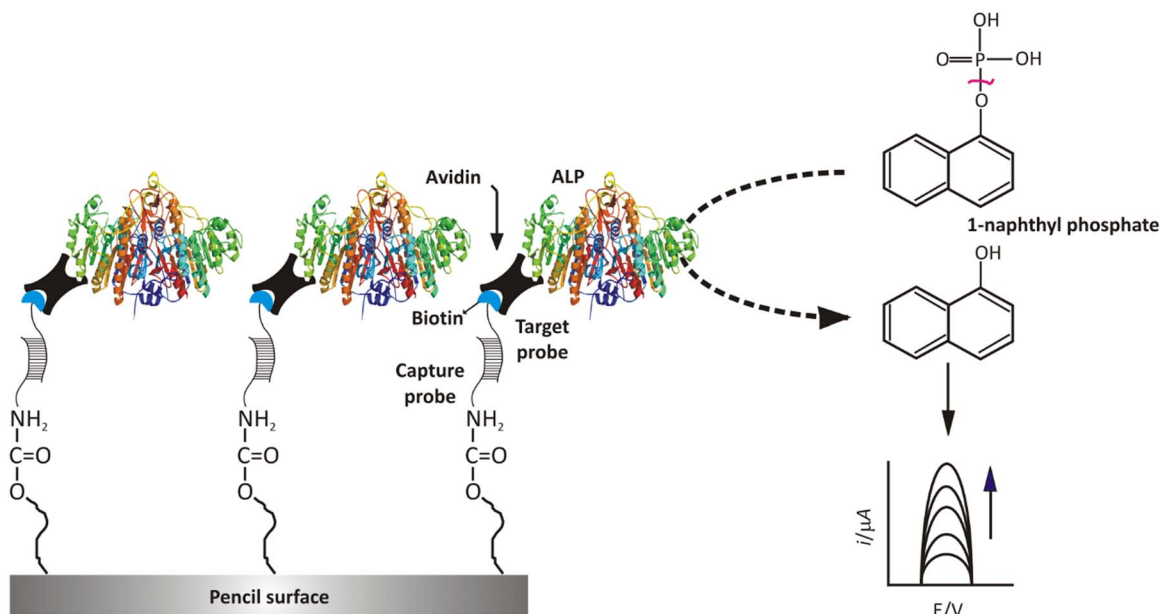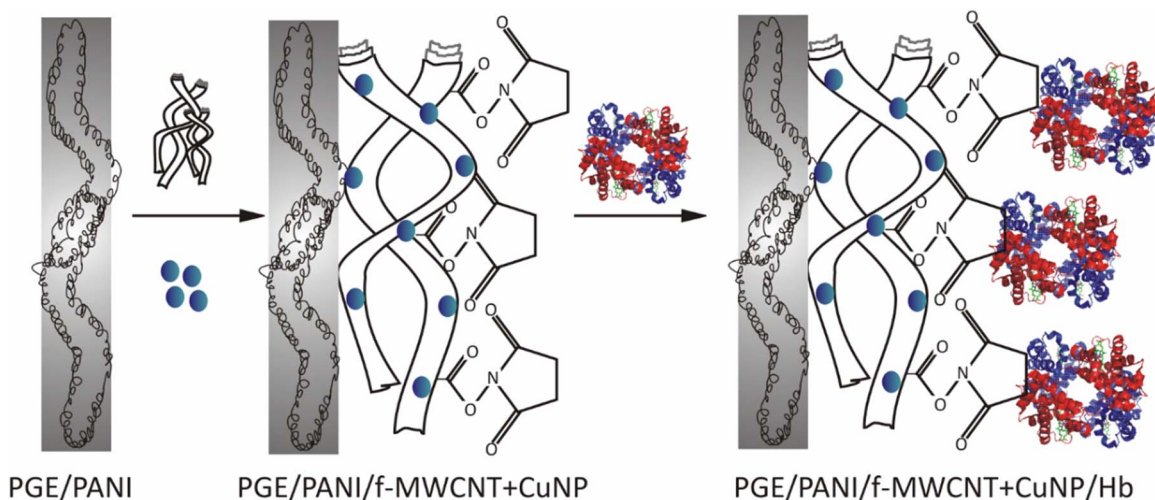
Figure 3. Schematic representation on the sensor preparation for the detection of mir21 in breast cancer cells.[52]

0.5 M H_2SO_4. The maximum CV peak current which could be obtained was tested using 5mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M PBS (7.4) from -0.2 to 0.8 V at 100mV s^{-1}. It was found that the maximum peak current was obtained for 5 cycles of deposition and 6 cycles of acid treatment. Also, the effect of SH-P1 and SA-ALP concentration was evaluated, and the optimal response was obtained for 1.1 μM and 12.2 U mL^{-1}, respectively. With the optimal parameters, the linear range was found to be 200 pM to 388 nM and the limit of detection as 100 pM. Finally, the selectivity of the sensor was demonstrated in the presence of the non-complimentary sequence miRNA-125a, and the reproducibility was demonstrated by fabricating four identical T-P2/MCH/SH-P1/AuNPs/PGE* and tested at different target concentrations where the minimum relative standard deviation obtained was 2.1% and maximum as 9.9%.

Hb modified PGEs.—Hb with a molecular weight of 64,500 is an association of four globular subunits of proteins bonded noncovalently to each other. Though Hb may not play a crucial role as an electron carrier in biological systems, heme proteins of Hb act analogous to peroxidase and display enzyme-like activity. The interactions among the subunits may lead to allosteric properties of the protein. Each subunit consists of a chain of a protein associated with a non-protein heme group. Four iron-heme groups are liable for the electroactivity of Hb displaying the reversibility of Fe(III) to Fe(II).[56] Based on its electroactivity, Batra et al.[57] reported an acrylamide sensor. The central idea behind the sensor was an interaction between the acrylamide and Hb which leads to an adduct which is responsible for the change in electroactivity of Hb. The increase in the concentration of acrylamide-Hb adduct displayed the decrement in the peak current response. This is considered as an analytical signal for detection of acrylamide. In this work, an electrochemical biosensor for acrylamide was developed by immobilization of Hb on a composite formed by conducting polymer like polyaniline (PANI), carboxylated multiwalled carbon nanotube (c-MWCNT) and copper nanoparticle (PANI/c-MWCNT/CuNP). Prior to the immobilization, the composite was electro-deposited on PGE to develop PGE/PANI/c-MWCNT/CuNP and the electrode developed in this work is PGE/PANI/c-MWCNT/CuNP/Hb (Figure 4).

Figure 4. Schematic representation on the preparation of PGE/PANI/CuNP/cMWCNT/Hb.[57]

To fabricate the sensor, aniline was electropolymerised to PANI on the surface cleaned PGE by continuous 20 cycles at a potential window −0.1 to 0.2 V. c-MWCNT was prepared by ultrasonication of MWCNTs in 3:1 ratio of H_2SO_4 and HNO_3 acid for 3–4 hrs. The obtained black coloured c-MWCNT was mixed with EDC and NHS. This c-MWCNT solution is added with CuNP suspension, and the mixture was electrodeposited on the surface of PGE/PANI by 20 continuous cycles at −0.1 to 0.2 V. As an indication of PGE/PANI/c-MWCNT/CuNP formation, the surface of PGE become green. For the immobilization of Hb, the surface of PGE/PANI/c-MWCNT/CuNP electrode was dipped in Hb solution for overnight at room temperature. Then PGE/PANI/c-MWCNT/CuNP/Hb was gently rinsed for 3–4 times in pH 5 acetate buffer and preserved at 4°C until its further use. The developed sensor showed the optimal response in pH 5 acetate buffer at 35°C operated at 20 mV s^{-1}. Under optimal DPV conditions, PGE/PANI/CuNP/cMWCNT/Hb exhibited 72.5×10^3 A M^{-1} cm^{-2} sensitivity and 0.2 nM LOD with a linear range of 5 nM to 75 mM. Further, the sensor was used for 120 times in a span of 100 days and stored at 4°C.

In another case, Majidi et al. reported Hb modified electrodes for the electrochemical detection of nitrite and H_2O_2.[58,59] In the first report, Hb modified H-PGE (H-PGE/Hb) was used for the electrochemical reduction of nitrite. In this work, Majidi et al.[58] prepared H-PGE/Hb where the PGE was tightly covered with a Teflon band leaving the top end for soldering the copper wire for electrical contact and the bottom end was polished and used for the Hb immobilization by simple drop-cast of Hb solution prepared by adding glycerol and Hb in pH 7.4 PBS. The electrode was gently rinsed in pH 7.4 PBS and placed at 4°C until its further use. DPV was carried out to determine the nitrite by H-PGE/Hb. The peaks corresponding the reduction of nitrite were found linear with the concentration of nitrite in a range of 10–220 μM with a LOD of 5 μM. Further, the H-PGE/Hb electrode was tested for nitrite in the spinach and tap water. In another report, Majidi et al.[59] prepared H-PGE/Hb as per their previous report with introducing a new step. Ormosil, prepared by 5 min stirring of methyltrimethoxysilane (MTMOS) and methanol and 0.1 M HCL in 6:3:1 ratio, was drop-casted on the H-PGE/Hb to prevent the loss of Hb. The dried H-PGE/Hb was stored at 4°C for overnight and used for the reduction of nitrite and H_2O_2. Under optimal DPV conditions, H-PGE/Hb showed a linear response to H_2O_2 and nitrite concentration ranging from 5–240 and 10–240 μM with 3 and 5 μM as LOD respectively. Further, the developed PGE/Hb electrode was used for the analyses of nitrite in tap water and H_2O_2 in mother's milk samples.

XOD modified PGEs.—XOD is a molybdopterin-containing flavoprotein that catalyses hypoxanthine (Hx) to xanthine (X) and X to uric acid (UA) by oxidation with molecular oxygen while reducing H_2O.[60] In 2013, Devi et al.[61,62] immobilized XOD on PGE and used for X detection in the first report and Hx detection in another report. In the first report,[61] XOD was covalently immobilized on the surface of a modified PGE by CT and Au-FeNP electrodeposition (i.e., PGE/Au-FeNP/CT/XOD). Prior to the modification, the surface of the PGE wad cleaned using piranha solution [a mixture of H_2SO_4 and H_2O_2 in 3:1] and followed by distilled water. The surface of PGE was polished using a slurry of alumina. On the PGE surface, Au-FeNP/CT was electrodeposited by continuous electrochemical cycling of PGE in a mixture of electrolyte containing the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in 1:1 and Au-FeNP/CT solution between −0.37 V and 0.6 V. The PGE/Au-FeNP/CT electrode was dried at room temperature after gentle rinse in distilled water. On the dried PGE/Au-FeNP/CT, XOD was immobilized via glutaraldehyde (GA) coupling. The GA groups formed on PGE/Au-FeNP/CT help the immobilization of XOD by simple dipping for overnight at 4°C to obtain PGE/Au-FeNP/CT/XOD. Prior to use, the PGE/Au-FeNP/CT/XOD was rinsed thoroughly in pH 7.4 PBS to avoid the excess and unbound enzyme. The XOD sensor was tested by adding X solution in pH 7.4 PBS, and the current response was measured at 0.5 V. The current response was due to the electrochemical reactions between the XOD and X to yield H_2O_2 and it's splitting

to $2H^+ + O_2 + 2e^-$ at 0.5 V. At pH 7.4 and 35°C room temperature, PGE/Au-FeNP/CT/XOD exhibited optimum current response to X at 0.1-300 μM with 1.169 A M^{-1}cm^{-2} sensitivity and 0.1 μM (S/N = 3) as LOD. The PGE/Au-FeNP/CT/XOD was later extended for the analysis of X in meat samples. The stability and reusability of PGE/Au-FeNP/CT/XOD displayed a 75% retention of the sensor activity after its usage of 100 times in 100 days when stored at 4°C. In another report, Devi et al.[62] covalently immobilized XOD (from buttermilk) onto boronic acid-functionalized Au-Fe NPs electrodeposited on PGE via the boro-ester linkages, i.e., the bond between the –NH$_2$ groups of enzyme and free hydroxyl groups of boronic acid. In this work, PGE/Au-FeNP/XOD used boronic acid-activated Au-FeNPs (12 h stirring of 10 g 4-mercaptophenylboronic acid and 6 g Au-FeNPs in 100 mL ethanol yielded a residue which was further washed in diethyl ether to remove 4-mercaptophenylboronic acid from the obtained residue and vacuum dried at 40°C) and these Au-FeNPs were deposited on pretreated PGE in a 22mL solution containing 5 mM of $[Fe(CN)_6]^{3-/4-}$ and 50 mg of Au-FeNP (by 20 continuous CV cycles between −0.6 V to +0.6 V at v 50 mV s^{-1}). The resulting PGE/Au-FeNP modified electrode was rinsed gently in distilled water to avoid the loosely bound material and set aside in dry atmosphere at 4°C. Enzyme was then immobilized as per their previous literature to yield PGE/Au-FeNP/XOD. Then at pH 7.2 and 30°C, PGE/Au-FeNP/XOD showed a linear response to 0.05-150 μM of Hx with a LOD = 0.05 μM (S/N = 3). Further, PGE/Au-FeNP/XOD was tested for Hx levels in various meat samples. The PGE/Au-FeNP/XOD optimal response dropped by 50% after its usage over 100 days by placing it at 4°C.

In another report, an amperometric X biosensor was fabricated by immobilization of XOD on an electrochemically polymerized conducting polymer film (i.e., 10-[4H-dithieno (3, 2-b: 2′, 3′-d) pyrrole-4-yl] decane-1-amine) coated PGE (i.e., PGE/DTP-NH$_2$/XOD).[63] The PGE/DTP-NH$_2$/XOD was prepared as per the following procedure (Figure 5), electropolymerization of DTP-NH$_2$ on a cleaned and pretreated PGE was carried out by applying CV potential in the range of −1.5 to 2.5 V vs Ag/AgCl and at v = 100 mV s^{-1} (PGE/DTP-NH$_2$). Then, the PGE/DTP-NH$_2$ was immersed in 2.5% GA for 3 hrs. To obtain PGE/DTP-NH$_2$/XOD, immobilization of XOD was performed by immersing PGE/DTP-NH$_2$ in 2 U mL^{-1} XOD-ammonium sulfate suspension for 48 h on an orbital shaker set at 150 rpm at 4°C. The fabricated PGE/DTP-NH$_2$/XOD electrode shown an optimal amperometric response at 30°C and at 0.5 V vs Ag/AgCl applied potential. The current response increased linearly with increase in the X concentration ranging from 0.3 to 25 μM and showed the sensitivity and detection limit of 0.124 A M^{-1} and 0.074 μM respectively. In the end, the applicability of PGE/DTP-NH$_2$/XOD electrode was verified to measure the X concentration in chicken meat samples.

Other enzyme-modified PGEs.—Apart from GOx, ALP, XOD and Hb, there are some more enzymes used for the immobilization on the PGE surface. Other enzymes include ChOx, HRP, urease, ADH, lacasse, GlOx, uricase, lactate dehydrogenase, lipase, glycerol kinase and glycerol 3-phosphate oxidase (GPO), glucose dehydrogenase (GDH) and ascorbate oxidase.

Chauhan et al. immobilized ChOx (from *Streptomycin sp.*) on HB-PGE (15 mm diameter and 20 mm long) and used for amperometric determination of serum cholesterol.[64] To fabricate the HB-PGE/ChOx (Figure 6), wooden part of a pencil was removed completely and one end was dipped into 60% HCl for 24 hr followed by dipping in 70% HNO$_3$ for 24 hrs. Then the cleaned electrode was placed for 8 hrs at 4°C in 0.2% enzyme solution (dissolved in pH 7.4 PBS). The electrode was then washed and used as HB-PGE/ChOx. The linear current response was observed at a cholesterol concentration ranging from 1.29-10.33 mM, and LOD was 0.09 mM. The HB-PGE/ChOx electrode was used for 200 times for a span of 25 days, under 4°C storage conditions.

In another case, Teepoo et al. fabricated a renewable 6H-PGE by forming a AuNP and HRP multilayer using layer-by-layer assembly (Figure. 7).[25] Prior to the fabrication, the 6H-PGE was pre-anodized by subjecting 1.8 V for 5 min in pH 4.8 acetate
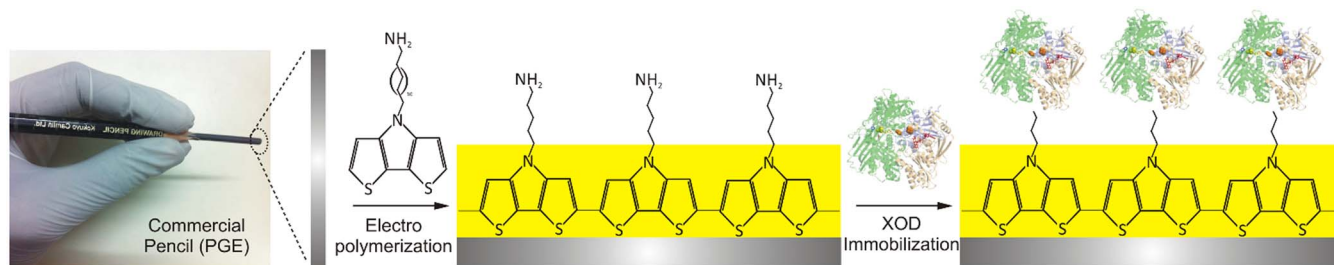
Figure 5. Preparation of PGE/DTP-NH$_2$/XOD electrode.[63]

buffer containing NaCl. The pre-anodized 6H-PGE* was immersed in 0.5% w/v CT solution and subjected to 1.5 V for 5 min (6H-PGE*/CT). Then the 6H-PGE*/CT was dipped in AuNP solution for 6 h (i.e., 6H-PGE*/CT/AuNP) and then placed in HRP for 12 h (6H-PGE*/CT/AuNP/HRP), which were repeated to obtain more number of layers. The developed 6H-PGE*/CT/AuNP/HRP showed a linear current response between 0.01 and 1.5 mM range of H$_2$O$_2$ and displayed 0.15 A M^{-1} cm^{-2}, 0.002 mM sensitivity and LOD respectively and placed in pH 7 PBS at 4°C until further use. The 6H-PGE*/CT/AuNP/HRP was further tested for H$_2$O$_2$ content in disinfector and hair dye samples.

Next, Zhu et al. fabricated pyrocatechol violet electro-deposited on single walled carbon nanotubes (SWCNT)-modified PGE (i.e., PGE/SWCNT/PCV) for electrochemical oxidation of dihydronicotinamide adenine dinucleotide (NADH) at 0.2 V vs saturated calomel electrode (SCE).[65] The PGE/SWCNT/PCV electrode displayed a linear response to 1.3-280 μM of NADH and displayed 0.15 A M^{-1} cm^{-2} and 1.3 μM as sensitivity and LOD, respectively. The PGE/SWCNT/PCV electrode was further fabricated as an ethanol biosensor by immobilizing ADH through GA and bovine serum albumin (BSA) coupling agents (i.e., PGE/SWCNT/PCV/ADH). The feasibility of PGE/SWCNT/PCV/ADH was tested for ethanol in 5 mM NAD$^+$ containing pH 7.5 PBS. At 0.2 V vs Ag/AgCl, amperometric current response of PGE/SWCNT/PCV/ADH toward ethanol in the presence of NAD$^+$ displayed linearity for 9.3-320 μM ethanol and shown a sensitivity of 0.002 A M^{-1} cm^{-2}. The fabricated PGE/SWCNT/PCV/ADH was further used to detect the ethanol content in liquor samples.

Later, Meibodi et al.[66] polymerized aniline on functionalized multiwalled carbon nanotube (f-MWCNT) modified PGE and used to immobilize the urease via physical adsorption and electrochemical entrapment technique and further used for the amperometric determination of urea. To prepare urease biosensor, the PGE was prepared using a pencil graphite cylinder and modelled epoxy resin. On prepared PGE, suspension (sono-dispersion of 2 mg f-MWCNT+0.2 M aniline+0.8 M perchloric acid for 2 h, into 30 mL of solution) was drop-casted and subjected to electrochemical treatment for 30 cycles between 0.1 V and −1 V at v = 40 mV s^{-1}. After this, 0.5%

GA solution was dropped and allowed to dry. Then electrode was placed in 5 mg mL^{-1} urease solution for 90 min. Then the PGE/f-MWCNT-PANI/urease electrode was dried and placed in pH 7.2 PBS and stored at 4°C until its further use. At constant potential (0.3 V vs Ag/AgCl) and in pH 7.2 PBS, amperometric current responses of PGE/f-MWCNT-PANI/urease displayed linearity for 0.07-10 mM and showed 12 × 10^{-3} A M^{-1} and 0.04 mM sensitivity and LOD respectively. But the PGE/f-MWCNT-PANI/urease retained only 50% of original response after 15 days. In another case, Kashyap et al. fabricated the cost-effective bio-electrodes by immobilizing Laccase on PGE@PANI/MWCNT (i.e., PGE@PANI/MWCNT/Laccase).[67] The electrode was fabricated by electro-polymerization of aniline on a pre-treated PGE in the potential range of −0.2-1 V at v = 50 mV s^{-1} (i.e., PGE@PANI). The PGE@PANI was placed in 1 mg mL^{-1} MWCNT+EDC/NHS solution for 2 h. The bio-cathode (PGE@PANI/MWCNT/Laccase) was developed by simple dipping of PGE@PANI/MWCNT into 3 mg mL^{-1} enzyme solution for 24 h under room temperature. Finally, the PGE@PANI/MWCNT/Laccase sensor displayed > 75% of the initial activity over the measurement period.

In continuation, Batra et al. developed l-Glutamate biosensor by immobilization of GlOx on a polymer-modified PGE (Figure 8).[68] To prepare the polymer-modified PGE, the surface of electrode was cleaned and placed in pyrrole (py) & ZnO containing KCl and performed electropolymerisation in −0.25 to 0.8 V potential window for 20 continuous cycles. The resulted PGE/Ppy-ZnO was immersed in GlOx solution (5 U mL^{-1}) for overnight at room temperature for enzyme immobilization. Thus formed PGE/Ppy-ZnO/GlOx was rinsed with pH 7.5 PBS and stored at 4°C. Further, the developed l-glutamate showed optimal response at 0.1 M Tris–HCl buffer of pH 8.5 and 30°C. The PGE/Ppy-ZnO/GlOx displayed 0.1 nM as LOD and was extended to detect l-glutamate in Chinese soups. Even after storage at 4°C, biosensor displayed a 30% loss in the activity over 100 uses in a span of 90 days. Similarly, Tsai and Wen developed a biosensor for UA,[69] a final product of purine metabolism, using uricase and HRP. Here, UA gets converted to H$_2$O$_2$ using uricase, and HRP further detects the generated H$_2$O$_2$. Using the GA coupling technique, the uricase and HRP are co-immobilized on the HB-PGE. Under op-
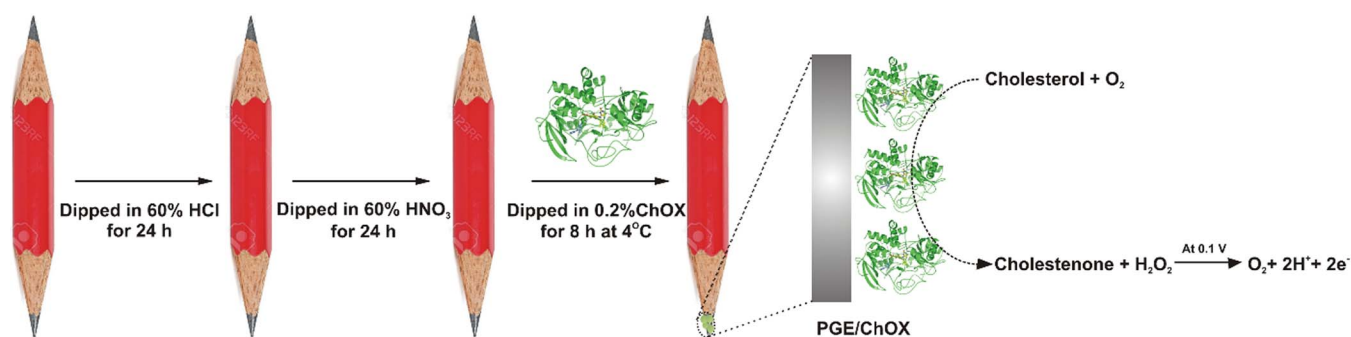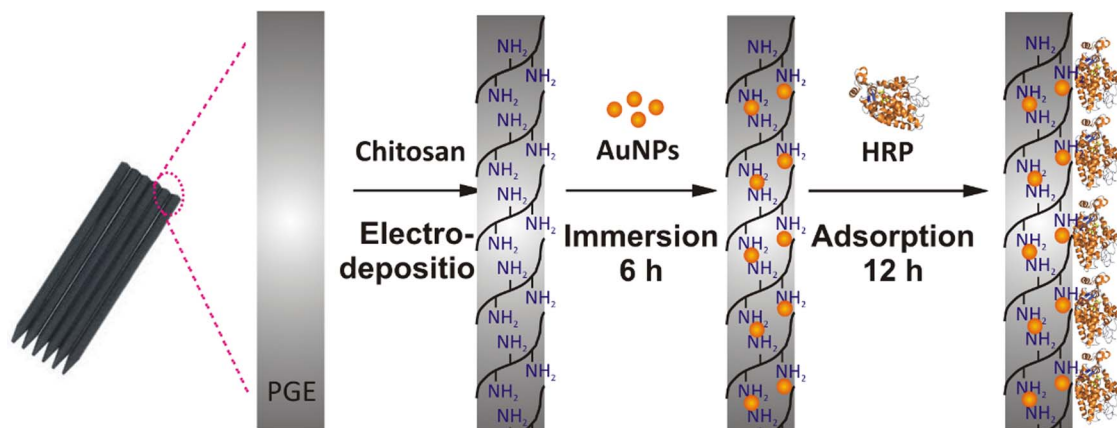

Figure 6. Preparation procedure of HB-PGE/ChOX for serum cholesterol.[64]

Figure 7. Preparation of HRP based sensor on chitosan covered and AuNPs electrodeposited PGE surface.[25]

timal amperometric conditions, using the ferrocene monocarboxylic acid indicator, the electrode displayed linearity up to 0.12 mM with sensitivity and LOD of 2.6×10^{-3} A M^{-1} and 0.6 µM. The developed UA sensor was tested for UA in blood serum.

In another case, Batra et al. developed a lactate dehydrogenase based sensor for the detection of lactic acid.[70] Here the sensing principle was based on the redox signal of NADH in the presence of the enzyme and the lactate. In this work, authors have used graphene oxide nanoparticles decorated PGE to immobilize the enzyme. Firstly, the graphene oxide nanoparticles were synthesized by a modified Hummer's method where HB type pencil rod was grounded in a mortar pestle. Later the graphite powder was mixed with the $NaNO_3$ and $KMnO_4$ and was allowed to react with H_2SO_4 and H_2O_2 in succession followed by washing with H_2O_2, HCl and deionized (DI) water until the neutral pH was attained by the supernatant. The graphene oxide nanoparticles thus obtained were dispersed in the 0.1 M KOH solution by continuous stirring and heating at 92°C. Later the nanoparticles were electrodeposited on the PGE (6B, 2mm diameter) by potential cycling in the dispersion from −0.15 to 2V at 20 mV s^{-1} for 20 cycles. Prior to electrodeposition, the PGE was polished by 0.05 µm alumina slurry on a polishing cloth followed by thorough washing with ethanol and DI water. The enzyme was then immobilized using EDC-NHS chemistry to complete the senor assembly. Various sensing parameters such as solution pH, temperature and time of incubation for enzyme immobilization were optimized to get the optimal sensor performance. Later the sensor performance was evaluated by cyclic voltammetry in 0.1 M sodium phosphate buffer (pH 7.3) from −0.15 to 0.7 V in the presence of 6.6 mM NAD$^+$ and varying concentration of lactic acid. The linear range was found to be from 5 to 50 mM with

LOD 0.1 µM. The sensor performance was also evaluated for the real samples including blood samples from healthy males and females and for the persons diagnosed with lactate acidosis as well as for milk, cheese, curd, yogurt, white wine, red wine and beer. There was no effect on the sensor response due to interferents such ascorbic acid, glutamic acid, citric acid and glucose and 75% of the initial activity was maintained after the regular usage for 60 days when stored at 4°C.

For the detection of triglycerides (TG), Narwal & Pundir used multiple enzymes co-immobilized on the surface of PGE*.[71] In this study, authors have used the enzymes lipase, glycerol kinases (GK) and glycerol 3-phosphate oxidase (GPO) for the detection of TG triolein. Here the enzyme lipase hydrolyzes the TG to glycerol and free fatty acids. The glycerol thus formed is phosphorylated to glycerol-3-phosphate in the presence of adenosine triphosphate (ATP) by GK which gets further oxidized to dihydroxyacetone phosphate and H_2O_2 by the action of GPO. The CV peak current obtained by the electro-oxidation of H_2O_2 is then measured as the sensor response. To prevent the denaturation of the enzyme and maintain their activity and stability, the enzymes were first agglomerated to their respective nanoparticle by glutaraldehyde crosslinking. The enzyme NP's were separately synthesized by desolvation method where absolute ethanol was added to enzyme solutions dropwise followed by crosslinking using a glutaraldehyde solution. The Enzyme NP thus formed were surface-functionalized using cystamine to retain a positive charge on the NP surface and were characterized using scanning electron microscope (SEM), transmission electron microscopy (TEM) and UV-vis spectroscopy. The PGE with 2 mm diameter and 2.5 cm length was pretreated by potential cycling from −1.1 to 0 V in 0.2 M H_2SO_4. Later PGE* was immersed in the suspension of Lipase
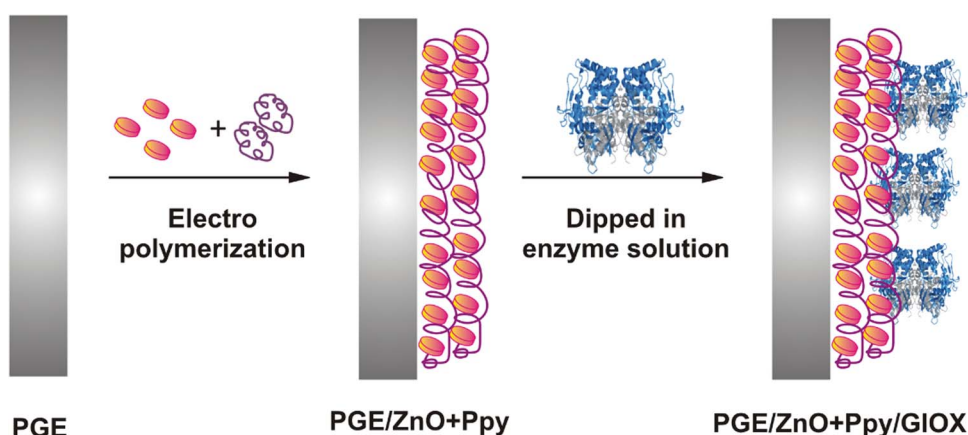
Figure 8. Preparation of glutamate sensor by immobilization of GlOx on ZnO and polypyrrole coated PGE surface.[68]

NPs/GKNPs/GPONPs (1:1:1) for the immobilization to take place via electrostatic interaction between the negatively charged surface and the positively charged –NH$_2$ of cysteamine dihydrochloride. Later the CV studies were carried out for Lipase NPs/GKNPs/GPONPs/PGE* using 0.1 M PBS containing 6.6mM ATP, 10mM MgCl$_2$ and 1mM Triton X100 at 20mV s^{-1} scan rate and the same was compared with Lipase/GK/GPO/PGE*. It was found that the oxidation current obtained for NP's immobilized electrode was much higher as compared to the native enzyme immobilized electrode. The linear range was found to be 0.1 mM – 45 mM with LOD as 0.1 nM. Finally, the study was carried out in the sera of real blood samples collected from 20 apparently healthy adult males and females and also from the 20 subjects diagnosed with hypertriglyceridemia in various age groups. The study with real samples was also carried out with a standard enzymic colourimetric kit, and a good correlation coefficient was obtained between the two methods. Interferants such as urea, uric acid, ascorbic acid, glutamic acid and citric acid did not show any significant effect on the response of the sensor, and it was found the electrode lost 20% of the initial response after regular usage of 240 days when stored at 4°C.

In another study based on the ZnS-CdS quantum dot (QD), Ertek et al. developed QD modified PGE for the development of photoelectrochemical glucose sensor based on the enzyme glucose dehydrogenase and the redox couple NAD$^+$/NADH using FIA system.[72] Initially, the PGE was activated by 1.4 V for 60 s in pH 7 PBS. Later the electrode surface was immobilized sequentially with ZnS and CdS by electrochemical precipitation method in the presence of mercapto acetic acid (MAA) as described by Sağlam et al. When the QD modified electrode is irradiated with the light source, the photoexcitation leads to the formation of electron-hole pair in the conduction-valance band. The transfer of electrons between the electrode material and the holes of valance band of QD with further transfer of electrons between the conduction band of QD and the electroactive molecule leads to the generation of anodic/ cathodic photocurrent. Additionally, the hybrid QD provide better charge separation and high quantum yield compared to individual QD. The CV and impedance studies of bare PGE*, CdS/PGE* and MAA-ZnS-CdS/PGE* in 10.0 mM [Fe(CN)$_6$]$^{3-/4-}$ containing 0.10 M KCl showed that the redox CV peak current decreases and the charge transfer resistance increases with successive immobilization of QDs indicating the semiconducting nature of the QD. To demonstrate the photocatalytic effect of the QD, CV studies of bare PGE* was compared with QD immobilized PGE* with and without illumination in the presence of 2 mM NADH in PBS (pH 7.0). It was observed that while there was a very little increment in the peak current for the bare PGE*, the QD immobilized PGE* showed a significant increase in the oxidation current under irradiation with 250 W halogen lamp. To evaluate the biosensing performance, the CV's of GDH/ PGE* and GDH/MAA-ZnS-CdS/PGE* were recorded in absence and presence of illumination in 0.1 M PBS (pH 7.0) containing 0.1 M KCl and 10 mM NAD$^+$ with 40mM glucose at 20 mV s^{-1}. It was observed that the redox current obtained was the largest for GDH/MAA-ZnS-CdS/PGE* with illumination demonstrating the photocatalytic performance of QD for glucose sensing application. Later, various operating parameters such as transmission tube length, sample volume, flow rate and the applied potential were optimized at 10 cm, 100 μL, 0.6 ml min^{-1} and 800 mV vs Ag/AgCl respectively to get the optimal sensor performance in FIA configuration. For the calibration studies, varying concentration glucose was injected with two injections of each concentration through a 100 μL sample loop. The LOD obtained without (amperometric) and with (photoamperometric) illumination were 0.09 mM and 0.05 mM respectively, and a two-fold increase in the sensitivity was observed for the photoamperometric detection as compared to amperometric detection of glucose. The precision studies carried out by repetitive injection of 7 pulses of 0.5 mM glucose solution gave an RSD of 3.5% and 4.5% for amperometric and photoamperometric detection, respectively. Finally, the interference studies were carried out in the presence of AA, glutamic acid, UA, saccharose, dopamine, CySH and galactose. It was observed that there was no significate change in the oxidation current in the presence

of a hundredfold of glutamic acid and saccharose and a thousandfold of galactose higher than glucose. However, a significant increase in the oxidation peak current was observed in the equimolar concentration of AA, UA, dopamine and CySH mainly due to overlapping of their oxidation peaks with that of NADH.

The antioxidants present in orange juice are well known to protect biological targets from the reactive oxygen, nitrogen and hydroxyl species.[73,74] To distinguish the contribution of the antioxidant capacity of AA and phenols and to quantify the content of AA, Barberis et al. developed a PGE and ascorbate oxidase based telemetric sensor.[75] The authors used a strategy where the oxidation current obtained with (biosensor-BS) and without (sensor -S) presence of the enzyme was evaluated simultaneously by using two working electrodes. The difference in the current obtained at a particular voltage was used to estimate the selectivity index and content of AA and the antioxidant capacity in the juices. The sensor/biosensor assembly involved four 2H-Type PGE (length- 30 mm, diameter- 300 μm) consisting of one pseudo reference, one auxiliary and two working electrodes. The working electrodes were initially coated with the epoxy resin and later polished with alumina wheel attached to a high-speed drill to get a disc-shaped active area. The biosensor electrode surface was initially coated with polyethyleneimine (PEI) by a dip evaporation method, which acts as an enzyme stabilizer. Later the PEI coated PGE was further coated with 25 μL of 10% bovine serum albumin (BSA) solution containing 25 U of ascorbate oxidase. The PEI and enzyme coating steps were alternately repeated 10 times, followed by a final coating layer of polyurethane (PU) which acts as an enzyme immobilizer. The sensor electrode was prepared in a similar fashion except for the use enzyme. All the calculations were carried out based on the assumptions that 1) The total oxidation current recorded by the sensor in more than the biosensor in the presence of AA as some amount of AA gets oxidized by the enzyme before it reaches the PGE surface. 2) The total current recorded by the sensor and biosensor is the same in the absence of the AA. The LOD and limit of quantification were calculated as 0.26 and 0.77 μM respectively for the biosensor. The biosensor displayed good operational stability for 30 measurements, after which the current response decreased to 70% of the initial value. To further enchase the sensitivity and lower the LOD, Barberis et al. immobilized different carbon allotropes including SWCNT, MWCNT, fullerene C$_{60}$ and fullerene C$_{70}$ on the PGE as they are known to have high electron affinity, high specific surface area and display good adsorption capacity for organic molecules.[76] The biosensor-sensor assembly was carried out as described above except prior to PEI coating, the PGE was coated with fullerene (FC) or CNT's by dip evaporation in their dimethylformamide (DMF) dispersion containing 60 mg mL^{-1} fullerene or 10 mg mL^{-1} CNT with 50 tip cycles, besides some preliminary studies were carried out to choose the best solvent and the concentration for FC/CNT dispersion. Later the CV's of S-FC$_{60}$ (sensor-FC$_{60}$), B-FC$_{60}$ (Biosensor-FC$_{60}$), S-FC$_{70}$, B-FC$_{70}$, S-SWCNT, B-SWCNT, S-MWCNT and B-MWCNT were compared for the baseline current and in the presence of 1mM AA. It was observed that for B-FC$_{60}$ and B-FC$_{70}$ the CV's in AA solution completely overlapped with that of the baseline while that for B-SWCNT and B-MWCNT, the current was higher than the baseline. This was mainly due to the complete shielding of AA by oxidation before it can reach the sensor surface in the case of FC while an incomplete shielding effect in the case of CNTs. It is hypothesized that this is mainly due to higher enzyme loading onto the FCs when compared to CNTs due to their high surface/ volume ratio. The hypothesis was confirmed by measuring the CVs in the AA solution and at different concentrations of O$_2$. It was observed that for 0% O$_2$, CV currents for sensors were statistically same as that of biosensors and with the increase in the O$_2$ levels the current for biosensors decreased while that of the sensors remained the same. This was mainly due to the enzymatic oxidation of some quota of AA before it reached the sensor surface. At 21% O$_2$, there was 100% decrease in the current for FC based biosensors while it was 85.5% and 76.4% decrease for B-SWCNT and B-MWCNT respectively. Thus at a given AA concentration, the enzymatic activity depends upon the O$_2$ concentration while at a given AA and O$_2$

concentration, the efficiency of the biosensor depends on the active surface area available for the enzyme loading. The similar studies carried out for the phenols in the absences of AA demonstrated that there was no statistical difference in the response of sensor and the biosensor and also with oxygen concentration, demonstrating negligible enzymatic activity in the absence of AA. The LOD was found to be 0.1 µM, 0.13 µM, 0.2 µM and 0.22 µM for SB- FC60, SB- FC70, SB-SWCN and SB-MWCNT respectively.

Conclusions and Future Perspectives

This review helps us in understanding very categorically that commercially available pencil may act as an electrode for sensor applica-

tions. Besides technical compatibility, the affordable price and ease of availability enable PGE as a low-cost disposable and an alternate to the conventional electrode. Further, PGEs act as a robust platform for the modification of its surface with various redox mediators to enhance the surface area of the electrode and enables the effective immobilization of enzymes. Moreover, the performance of PGE's as an electrode material is influenced by the ratio of graphite/clay and its chemical composition. Table I summarizes the various pencil grades used in the literature with the different enzyme-analyte interactions. For enzyme modification, most of the chemically modified PGEs use coupling and crosslinking agents. Largely, enzyme and enzyme-like proteins modified PGEs are utilised in sensing glucose, 1-naphthol, micro RNA, acrylamide, nitrite, H_2O_2, xanthine, hypoxanthine, serum

Table I. List of various enzymes used for modification of pencil graphite electrode.

S. No	Enzyme/ Protein	Pencil grade	Analyte	Remarks	Ref.
1.	GOx	2B	Glucose	Binder clay initiates the polymerization of 4VP by an alternate mechanism	22
2.	GOx	HB	Glucose	Used as a platform for CPE	44
3.	GOx	HB	Glucose	Used as a platform for CPE	45
4.	GOx	-	Glucose	-	46
5.	GOx	-	Glucose	-	47
6.	GOx	2B (pretreated)	Glucose	Increase in the edge planes with a reduced disorder on the electrode surface lead to faster charge transfer kinetics	48
7.	GOx	B (pretreated)	Glucose	Pretreatment induced oxygen functionalities	49
8.	GOx	2B (pretreated)	Glucose	high electrochemical reactivity, lack of need for polishing, and renewable surface	50
9.	GOx	HB (pretreated)	Glucose	Carboxylate groups generated by the optimized pretreatment used for the immobilization of the enzyme	51
10.	Alkaline phosphatase (ALP)	Pentel or Tombo, Japan (untreated)	biotinylated oligonucleotide – ssDNA, substrate – α-NAP	Surface hydroxyl groups used for the immobilization of capture probe	52
11.	ALP	HB (pretreated)	mir21 (RNA), substrate – α-NAP	Carboxyl groups used for the immobilization of capture probe	53
12.	ALP	Tombow Pencil, Japan (untreated)	Biotinylated DNA fragments, substrate – α-NAP	Immobilization of capture probe by Adsorption	54
13.	ALP	HB (pretreated)	miRNA-21, substrate – α-NAP	High charge transfer kinetics due to pretreatment	55
14.	Hb	- (untreated)	Acrylamide	Used as a platform for electropolymerisation	57
15.	Hb	H	nitrite	-	58
16.	Hb	H	Nitrite, hydrogen peroxide	-	59
17.	XOD	-	xanthine	Used as a platform for electrodeposition	61
18.	XOD	-	xanthine	Used as a platform for electrodeposition	62
19.	XOD	-	xanthine	Used as a platform for electropolymerisation	63
20.	ChOx	HB	Cholesterol	-	64
21.	HRP	6H	hydrogen peroxide	HB, 2B, 1H, 2H, 3H, 4H, 5H and 6H were compared, 6H displayed the best reversible redox current	65
22.	ADH	-	Ethanol, mediator - NADH	Used as a platform for electrodeposition pyrocatechol violet	65
23.	Urease	- (untreated)	urea	Used as a platform for electropolymerisation	66
24.	Laccase	pretreated	Oxygen, mediator - ABTS	Used as a platform for electropolymerisation and adsorption of MWCNT	67
25.	GlOx	HB (untreated)	l-Glutamate	Used as a platform for electropolymerisation	68
26.	Uricase, HRP	HB	Uric acid, Hydrogen peroxide	Enzyme immobilization by glutaraldehyde cross-linking	69
27.	lactate dehydrogenase	HB, 6B	lactic acid	HB was used for the preparation of graphite powder and 6B was used as a platform for electrodeposition	70
28.	Lipase, glycerol kinases, glycerol 3-phosphate oxidase	6B (pretreated)	triglycerides	Enzyme immobilization by electrostatic interaction	71
29.	glucose dehydrogenase	2B (pretreated)	Glucose, mediator - NADH	Quantum dot immobilization by electrochemical precipitation, Enzyme immobilization by glutaraldehyde cross-linking	72
30.	ascorbate oxidase	2H	Ascorbic acid	Platform for enzyme immobilization using polyurethane	75
31.	ascorbate oxidase	2H	Ascorbic acid	Immobilization of CNT's and fullerene by adsorption, enzyme immobilization using polyurethane	76

cholesterol, NADH, urea, lactic acid, l-glutamate, uric acid, triglycerides and ascorbic acid. On the other hand, enzyme-modified electrodes revealed its significance in investigating the hybridization, detection of cancer cells, and as a biocathode in fuel cells. Owing to the commercial aspect and proven impact in clinical samples, the enzyme glucose oxidase was the most used in the construction and development of enzyme-based biosensor. However, the key challenges involving the development of a successful enzyme immobilized PGE in practical electrochemical applications are the mass generation of enzyme-based biosensors on PGE platform with accurate stability and repeatability in the complex sample analyses at normal temperature. Due to inexpensive and simple process, pencil-drawn electrodes on paper are attaining wide attention nowadays. However, the immobilization of an enzyme on such pencil-drawn electrodes received scant attention in the electrochemical community, and this strategy in the near future may possibly open new frontiers in the development of low-cost point of care devices.

Acknowledgments

ORCID

Akash Nathani https://orcid.org/0000-0002-1391-8908
Nandimalla Vishnu https://orcid.org/0000-0001-8832-3274
Chandra S. Sharma https://orcid.org/0000-0003-3821-1471

References

1. J. K. Kariuki, *J. Electrochem. Soc.*, **159**, H747 (2012).
2. K. C. Honeychurch, *Anal. Methods*, **7**, 2437 (2015).
3. P. H. C. P Tavares and P. J. S. Barbeira, *J. Appl. Electrochem.*, **38**, 827 (2008).
4. D. Deýlová, V. Vyskočil, and J. Barek, *J. Electroanal. Chem.*, **717**, 237 (2014).
5. H. Šmejkalová and V. Vyskočil, *Chem. Listy*, **108**, 264 (2014).
6. A. R. Khaskheli, J. Fischer, J. Barek, V. Vyskočil, and M. I. Bhanger, *Electrochim. Acta*, **101**, 238 (2013).
7. V. Vyskočil and J. Barek, *Procedia Chem.*, **6**, 52 (2012).
8. R. Navrátil, A. Kotzianová, V. Halouzka, T. Opletal, I. Triskova, L. Trnkova, and J. Hrbac, *J. Electroanal. Chem.*, **783**, 152 (2016).
9. N. Vishnu and A. S. Kumar, *Anal. Methods*, **7**, 1943 (2015).
10. N. Vishnu, M. Gandhi, S. Badhulika, and A. S. Kumar, *Anal. Methods*, **10**, 2327 (2018).
11. N. Vishnu, M. Gandhi, D. Rajagopal, and A. S. Kumar, *Anal. Methods*, **9**, 2265 (2017).
12. J. Wang, A. N. Kawde, and E. Sahlin, *Analyst*, **125**, 5 (2000).
13. L. Hlavata, K. Benikova, V. Vyskocil, and J. Labuda, *Electrochim. Acta*, **71**, 134 (2012).
14. L. Hlavatá, V. Vyskočil, K. Beníková, M. Borbélyová, and J. Labuda, *Open Chemistry*, **12**, 604 (2014).
15. S. A. Atty, A. H. Ibrahim, and E. M. Hussien, *J. Electrochem. Soc.*, **166**, B1483 (2019).
16. J. Wang and A. N. Kawde, *Anal. Chim. Acta*, **431**, 219 (2001).
17. S. Mamidi, A. Gangadharan, and C. S. Sharma, *Electrochim. Acta*, **310**, 222 (2019).
18. C. A. Mitchell, *Analyst*, **47**, 379 (1922).
19. F. L. Encke, *J. Chem. Educ.*, **47**, 575 (1970).
20. N. Vishnu, A. Gopalakrishnan, and S. Badhulika, *Electrochim. Acta*, **269**, 274 (2018).
21. R. N. Bhowmik, *Compo. Part B-Eng.*, **43**, 503 (2012).
22. M. H. Zahir and S. Ab Ghani, *Anal. Chim. Acta*, **354**, 351 (1997).
23. M. P. Down, C. W. Foster, X. Ji, and C. E. Banks, *RSC Adv.*, **6**, 81130 (2016).
24. S. H. Lee, J. Y. Ban, C. H. Oh, H. K. Park, and S. Choi, *Sci. Rep.*, **6**, 28588 (2016).
25. S. Teepoo, P. Chumsaeng, P. Nethan, W. Prueprang, and P. Tumsae, *Int. J. Electrochem. Sci.*, **7**, 4645 (2012).
26. C. W. Foster, D. A. Brownson, A. P. R. de Souza, E. Bernalte, J. Iniesta, M. Bertotti, and C. E. Banks, *Analyst*, **141**, 4055 (2016).
27. N. Vishnu and A. S. Kumar, *J. Electroanal. Chem.*, **786**, 145 (2017).
28. N. Vishnu, A. S. Kumar, G. T. Pan, and T. C. K. Yang, *Sensor. Actuat. B-Chem.*, **275**, 31 (2018).
29. N. Vishnu and S. Badhulika, *Biosens. Bioelec.*, **124**, 122 (2019).
30. R. Sha, N. Vishnu, and S. Badhulika, *Electroanalysis*, (2019).
31. Á. Torrinha, C. G. Amorim, M. C. Montenegro, and A. N. Araújo, *Talanta*, **190**, 235 (2018).
32. J. Wang, *Chem. Rev.*, **108**, 814 (2008).
33. S. Sharma, R. Jain, and A. N. Raja, *J. Electrochem. Soc.*, **167**, 037501, (2020).
34. H. Razmi, L. Ezzati, and Z. Khorablou, *J. Electrochem. Soc.*, **166**, B961 (2019).
35. A. J. S. Ahammad, T. Akter, A. A. Mamun, T. Islam, M. M. Hasan, M. A. Mamun, S. Faraezi, F. Z. Monira, and J. K. Saha, *J. Electrochem. Soc.*, **165**, B390 (2018).
36. E. Zor, E. Eksin, M. Findik, H. Bingol, and A. Erdem, *J. Electrochem. Soc.*, **164**, B723 (2017).
37. Á. Torrinha, M. C. Montenegro, and A. N. Araújo, *J. Electrochem. Soc.*, **166**, B155 (2019).
38. H. Nguyen, S. Lee, U. Lee, C. Fermin, and M. Kim, *Materials*, **12**, 121 (2019).
39. G. Rocchitta, A. Spanu, S. Babudieri, G. Latte, G. Madeddu, G. Galleri, S. Nuvoli, P. Bagella, M. I. Demartis, V. Fiore, R. Manetti, and P. A. Serra, *Sensors*, **16**, 780 (2016).
40. V. Mani, B. Devadas, S. M. Chen, and Y. Li, *ECS Trans.*, **50**, 35 (2013).
41. Y. Fang, Y. Zhou, and R. P. Ramasamy, *J. Electrochem. Soc.*, **165**, B358 (2018).
42. R. M. Buoro, O. S. Campos, R. P. Bacil, and S. H. Serrano, *J. Electrochem. Soc.*, **163**, G178 (2016).
43. R. Pandey, R. O. Almog, Y. Sverdlov, and Y. Shacham-Diamand, *J. Electrochem. Soc.*, **164**, B168 (2017).
44. C. Cheng, K. C. Chang, C. S. Chena, and D. G. Pijanowska, *J. Chin. Chem. Soc.*, **58**, 739 (2011).
45. C. Cheng, K. C. Chang, and D. G. Pijanowska, *J. Electroanal. Chem.*, **666**, 32 (2012).
46. M. Dervisevic, E. Çevik, and M. Şenel, *Enzyme Microb. Technol.*, **68**, 69 (2015).
47. M. Y. Elahi, A. A. Khodadadi, and Y. Mortazavi, *J. Electrochem. Soc.*, **161**, B81 (2014).
48. A. A. Sehat, A. A. Khodadadi, F. Shemirani, and Y. Mortazavi, *Int. J. Electrochem. Sci.*, **10**, 272 (2015).
49. K. Vijayaraj, S. W. Hong, S. H. Jin, S. C. Chang, and D. S. Park, *Anal. Methods*, **8**, 6974 (2016).
50. Ö. Sağlam, B. Kızılkaya, H. Uysal, and Y. Dilgin, *Talanta*, **147**, 315 (2016).
51. S. Teanphonkrang and A. Schulte, *Anal. Chem.*, **89**, 5261 (2017).
52. P. Kara, A. Erdem, S. Girousi, and M. Ozsoz, *J. Pharm. Biomed. Anal.*, **38**, 191 (2005).
53. T. Kilic, S. N. Topkaya, D. O. Ariksoysal, M. Ozsoz, P. Ballar, Y. Erac, and O. Gozen, *Biosens. Bioelectron.*, **38**, 195 (2012).
54. L. Hároníková, J. Špaček, M. Plucnara, P. Horáková, H. Pivoňková, L. Havran, A. Erdem, and M. Fojta, *Monatsh. Chem.*, **146**, 849 (2015).
55. J. Mandli, H. Mohammadi, and A. Amine, *Bioelectrochemistry*, **116**, 17 (2017).
56. A. Senthil Kumar, P. Gayathri, P. Barathi, and R. Vijayaraghavan, *J. Phys. Chem. C*, **116**, 23692 (2012).
57. B. Batra, S. Lata, M. Sharma, and C. S. Pundir, *Anal. Biochem.*, **433**, 210 (2013).
58. M. R. Majidi, A. Saadatirad, and E. Alipour, *Electroanalysis*, **25**, 1742 (2013).
59. M. R. Majidi, M. H. Pournaghi-Azar, A. Saadatirad, and E. Alipour, *J. Chin.Chem. Soc.*, **62**, 83 (2015).
60. R. S. Pilato, K. A. Eriksen, M. A. Greaney, E. I. Stiefel, S. Goswami, L. Kilpatrick, T. G. Spiro, E. C. Taylor, and A. L. Rheingold, *J. Am. Chem. Soc.*, **113**, 9372 (1991).
61. R. Devi, S. Yadav, R. Nehra, and C. S. Pundir, *Int. J. Biol. Macromol.*, **62**, 629 (2013).
62. R. Devi, S. Yadav, R. Nehra, S. Yadav, and C. S. Pundir, *J. Food Eng.*, **115**, 207 (2013).
63. M. Dervisevic, E. Dervisevic, H. Azak, E. Cevik, M. Şenel, and H. B. Yildiz, *Sensor. Actuat. B-Chem.*, **225**, 181 (2016).
64. N. Chauhan, J. Narang, and C. S. Pundir, *Am. J. Anal. Chem.*, **1**, 41 (2010).
65. J. Zhu, X. Y. Wu, D. Shan, P. X. Yuan, and X. J. Zhang, *Talanta*, **130**, 96 (2014).
66. A. S. E. Meibodi and S. Haghjoo, *Synth. Met.*, **194**, 1 (2014).
67. D. Kashyap, C. Kim, S. Y. Kim, Y. H. Kim, G. M. Kim, P. K. Dwivedi, A. Sharma, and S. Goel, *Int. J. Hydrog. Energy*, **40**, 9515 (2015).
68. B. Batra, M. Yadav, and C. S. Pundir, *Biochem. Eng. J.*, **105**, 428 (2016).
69. W. C. Tsai and S. T. Wen, *Anal. Lett.*, **39**, 891 (2006).
70. B. Batra, V. Narwal, and C. S. Pundir, *Engineering in Life Sciences*, **16**, 786 (2016).
71. V. Narwal and C. S. Pundir, *Enzyme Microb. Technol.*, **100**, 11 (2017).
72. B. Ertek, C. Akgül, and Y. Dilgin, *RSC Adv.*, **6**, 20058 (2016).
73. M. Fojta, A. Daňhel, L. Havran, and V. Vyskočil, *Trends Anal. Chem.*, **79**, 160 (2016).
74. A. Hájková, J. Barek, and V. Vyskočil, *Bioelectrochemistry*, **116**, 1 (2017).
75. A. Barberis, Y. Spissu, G. Bazzu, A. Fadda, E. Azara, D. Sanna, M. Schirra, and P. A. Serra, *Anal. Chem.*, **86**, 8727 (2014).
76. A. Barberis, Y. Spissu, A. Fadda, E. Azara, G. Bazzu, S. Marceddu, M. Schirra, and P. A. Serra, *Biosens. Bioelectron.*, **67**, 214 (2015).