Enzyme-based Amperometric Biosensors: 60 Years Later…Quo Vadis?

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Abstract

Enzyme-based amperometric biosensors represent powerful tools for remote medicine and *in situ* analysis. Nowadays, billions of people are surrounded by enzyme-based amperometric biosensors even considering their relatively young age…only 60 years! In this period, many researchers, dealing mostly with the same target molecules as in early times, have developed novel strategies to tackle electron transfer issues and to realise stable, sensitive, and selective biosensors. Besides marking 60 years from the first enzyme-based amperometric biosensor, this review aims at summarising the technological advancements in the field mainly considering three enzyme families: D-glucose oxidising enzymes, D-fructose oxidising enzymes and L-lactate oxidising enzymes. It is an overview of the past (previous five decades) and current advancements (2010-2020) from the electrode platform tailoring to the technological production and applications (*e.g.*, *in situ* biosensors, Point-of-Care (PoC), wearable biosensors etc.) focused on few enzymes.

Keywords: Enzyme-based biosensors, amperometry, modified electrodes, glucose, fructose, lactate.

1. Introduction

In the last 6 decades, enzyme-based biosensors have been conceived, developed, commercialised, and employed for *in situ* analysis as well as Point-of-Care (PoC) devices being able to provide a reliable output signal quantitatively correlated with a certain analyte concentration. [1–4] The first enzyme-based biosensor has been realised 60 years ago by Clark and Lyons at the Children's Hospital of Cincinnati. [5–8] In this paper, Clark and Lyons reported the first enzyme membrane electrode by trapping a concentrated enzyme layer between two cuprophane membranes wrapped on top of a pH sensing electrode. Specifically, they immobilised glucose oxidase (GOx) within the membrane 'sandwich' enabling glucose

oxidation to δ-gluconolactone that causes an increasing of H^+ concentration, corresponding to pH drop, as shown in figure 1A, which resulted in the variation of potential (1 pH unit for a 10 mg per cent solution of glucose flowing at a rate of 3 mL min^{-1}). Alternatively, Clark and Lyons proposed to use a hydrophobic membrane and an oxygen sensing electrode by exploiting the reaction pathway occurring at GOx, as depicted in figure 1B. [5,9,10] The latter will be then reviewed, cited, and mentioned as prototype of first-generation enzyme-based biosensor. In the next two decades most of the enzyme-based biosensors will be prototyped considering both configurations. In 1984, Cass et al. reported a second-generation biosensor based on ferrocene as electron accepting molecule instead of oxygen, [11] that works as natural electron acceptor for oxidases (*e.g.*, glucose oxidase (GOx), alcohol oxidase (AOx), lactate oxidase (LOx), *etc.*). The authors reported a layer-by-layer construction onto graphite electrode where the ferrocenium ions are acting as oxidants towards GOx. Ferrocene exhibited several features for the development of second-generation biosensors: (*i*) high-rate electron transfer between reduced enzyme and ferrocenium ion; (*ii*) good electrochemical properties of ferrocene; and (*iii*) low water solubility of ferrocene resulting in the effective confinement of the mediator to the electrode surface.

While discussing the advancements of enzyme-based amperometric biosensors, it is important to distinguish among different types of enzymes with respect to their electrical connection with the electrode surface. [12–14] The first group encompasses enzymes based on nicotinamide adenine dinucleotide oxidised (NAD^+) or nicotinamide adenine dinucleotide phosphate oxidised ($NADP^+$) like reductases or dehydrogenases. Both electron carriers are weakly or not bound at all to the enzymatic scaffold acting as diffusing electron relays that can be amperometrically assayed. [15–17] In this case, the electrode should be developed considering that the reaction rate of two-electron transfer reaction should be faster than the corresponding single electron transfer avoiding the formation of radical intermediate species. Moreover, to ensure longer storage stability the enzyme should be physically entrapped or covalently bound considering a certain distance from the electrode that allows for the continuous injection/ejection of substrate/products (electron transfer reaction). Finally, the overpotential needed for the amperometric measurements should be quite small to avoid interferences by other redox molecules. Differently, the enzymes belonging the second group contain redox moieties that are at the edge of the enzyme scaffold enabling direct electron transfer (DET). [18–20] Notably, the enzyme orientation affects DET reaction at the electrode considering different immobilisation protocols. Although this would be the simplest electron transfer

pathway as described, only few enzymes are able to exchange electrons directly with electrode surfaces due to several constrains. [21,22] The third group encloses enzymes with prosthetic groups strongly bound the enzymatic scaffold. When adsorbed on electrodes, their redox centres cannot be oxidised/reduced at potentials not harmful for the enzyme. The electron transfer rate will be affected by the distance between their redox centres and the periphery of their protein or glycoprotein shell. [23,24]

This concept has been transposed to different generations of biosensors as reviewed in many articles. [25–27] Enzyme-based amperometric biosensors can be sorted in four groups: (*i*) 1st generation biosensors where the substrate oxidation reaction (*e.g.*, D-glucose, ethanol, L-lactate *etc.*) relates to the reduction of O_2 (natural electron acceptor for many oxidases) to H_2O_2, both assayed directly at the electrode surface; (*ii*) 2nd generation biosensors based on immobilised electron relays (acceptors/donors) that are able to reduce/oxidise the redox active centre of enzymes during the catalytic process (e.g., pyranose dehydrogenase from *Agaricus meleagris* (PDH), GOx, AOx, *etc.*); (*iii*) 2nd generation biosensors based on diffusive electron acceptor/donor (*e.g.*, NAD-glucose dehydrogenase where NAD^+ acts as electron acceptor being converted to NADH later re-oxidised to NAD^+ *etc.*); (iv) 3rd generation biosensors based on enzymes able to transfer electrons directly from the redox active centre to the electrode surface and *vice versa*, requiring a small overpotential to operate the amperometric detection. [28,29]

Besides marking 60 years from the first enzyme-based amperometric biosensor, this review aims at summarising the technological advancements in the field mainly considering three enzyme families: D-glucose oxidising enzymes, D-fructose oxidising enzymes and L-lactate oxidising enzymes. In the next sections, the enzymatic structures of these enzymes will be presented discussing the electron transfer pathways and how they have been exploited for the development of enzyme-based amperometric biosensors. Then, three sections will follow summarising individually for each substrate (D-glucose, D-fructose, and L-lactate) several biosensing platform by comparing their analytical figures of merit. The present review will also address the future scientific and market development of enzyme-based amperometric biosensors eliciting the main challenges within the field.

2. D-glucose Detection

Most of the research within enzyme-based amperometric biosensors is still focused considering their market within remote medicine devices and personalised medicine. [30,31] Glucose

biosensors are mostly based on glucose oxidation catalysed either by oxidases or dehydrogenases.

2.1 D-glucose Oxidising Enzymes

Glucose biosensors are mostly using the following enzymes as biorecognition elements: *Aspergillus niger* glucose oxidase (*An*GOx), [32] nicotinamide amide dinucleotide (NAD)-glucose dehydrogenase, [33] pyrroloquinoline quinone (PQQ)-glucose dehydrogenase, [34–36] flavin adenine dinucleotide (FAD)-glucose dehydrogenase, [37] cellobiose dehydrogenases from Ascomycetes, [38–40] pyranose dehydrogenase from *Agaricus meleagris* (*Am*PDH) and pyranose oxidase. [41] Their enzymatic structures are reported in figure 2.

*An*GOx was defined as "an ideal enzyme" in the early 1990s. [42,43] Although it is no longer so "ideal", *An*GOx is still widely used in glucose biosensors development. GOx belongs to the CAZy auxiliary activity family 3 (AA3), which encompasses enzymes from the glucose-methanol-choline (GMC) family. [44] AA family enzymes are involved in glycoside hydrolysis and degradation of lignocellulose. *An*GOx exhibits several drawbacks: (*i*) O_2 as natural electron acceptor will always, if present, compete with any artificial mediator introduced into the system to lower the high oxidation overpotential, consequently producing H_2O_2, mainly responsible for enzyme degradation; (*ii*) flavin adenine dinucleotide (FAD) redox centre is strongly but not covalently bound and immobilising *An*GOx on an electrode surface may trigger protein unfolding accompanied by FAD ejection (being wrongly interpreted as the enzyme bound FAD), (*iii*) as GOx oxidises glucose on the C1 carbon, it is only active for the β-form, and (*iv*) the glycosylated wild type enzyme does not exhibit any direct electron transfer (DET) features given the thickness of glycosylation shell (> 18 Å). Most of the researchers focused on DET not only as a scientific challenge but also for practical reasons to simplify biosensors and minimise the influence of other possible interfering components on the amperometric output. [45] However, the electron transfer process through *An*GOx takes place as follows:

FAD accepts the electrons donated from D-glucose molecule being reduced to $FADH_2$ (equation 1);

$$\text{D-Glucose} + \textit{An}\text{GOx}{-}\text{FAD}^{+} \rightarrow \delta\text{-Glucolactone} + \textit{An}\text{GOx}{-}\text{FADH}_2 \qquad (1)$$

The cofactor is regenerated by reacting with O_2, leading to the formation of H_2O_2 (equation 2);

$$An\text{GOx}-FADH_2 + O_2 \rightarrow An\text{GOx}-FAD + H_2O_2 \quad (2)$$

In the last step, H_2O2 is oxidized at a modified electrode (equation 3) either Prussian Blue (PB) or noble metal (*e.g.*, platinum, rhodium *etc.*), being proportional to glucose oxidised molecules (analytical targets). [46,47]

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (3)$$

Indeed, several approaches aiming at minimising the influence of O_2 on *An*GOx have been explored towards the development of glucose biosensors. For instance, *An*GOx can be immobilised within thick osmium redox hydrogels where O_2 is consumed in the outer sensor layer. Although a considerable volume of the hydrogel is not involved in the catalytic response creating a mass-transfer barrier, the absence of O_2 in the inner area does not hinder the electron transfer to osmium atoms. [48,49] Instead, mass-transfer limitations can be avoided by exploiting a layer-by-layer construction of the electrode platform, for example alternating poly-(allylamine) osmium redox mediator and *An*GOx layers. [50]

Alternatively, enzymatic bioengineering can be a viable tool to improve selectively the enzymatic activity towards a certain substrate. [51] In this regard, Marin-Navarro et al. increased the activity and thermal stability of *An*GOx through two mutations: T554M generating a sulfur-Π interaction and Q90R/Y509E resulting in a new salt bridge near the interphase of the dimeric protein structure. [52] In another report, *An*GOx stability was improved by varying the net charge of the enzyme from −67 to +78 via chemical modification of its -COOH groups with polyamines. All chemically modified *An*GOx(*n*) were adsorbed onto graphene oxide (GO) modified electrode. While controlling the net charge, the authors were able to retain *An*GOx activity preventing protein unfolding that may occur during the enzyme operation. [53]

Another research direction explored to decrease O_2 sensitivity of *An*GOx was pursued by replacing the natural flavin with 7,8-dichloro-FAD [54] or through genetic engineering "site-directed mutations on Val464 of GOx from *Penicilium amagasakiense*". [55]

*An*GOx was alternatively replaced with other D-glucose oxidising enzymes. For instance, glucose dehydrogenases (GDHs) (soluble and membrane bound) possessing pyrroloquinoline quinone (PQQ) [56–59] and flavin adenine dinucleotide (FAD) [60–62] as the prosthetic group,

respectively, have been highly focused as a possible ideal enzyme for mediator-type glucose biosensor, because of its high catalytic efficiency (k_{cat}/K_m) and independence of O_2. However, GDHs also exhibit substrate specificity (oxidation of β-form) and thermal stability. [63] The quinoprotein GDH uses PQQ cofactor as electron relays (equation 4):

D-Glucose + PQQ(ox) → δ-Glucolactone + PQQ(red) (4)

Indeed, this ET mechanism does not require any additional electron acceptor like oxygen or NAD^+. PQQ(red) is later re-oxidised at the electrode surface. PQQ-GDH owns a high electron transfer rate system especially while compared to other glucose oxidising systems. PQQ-GDH stability can increased through protein engineering and addition of Ca^{2+} ions that enhance the electron transfer rate from PQQ groups. [64,65]

Regarding PQQ-GDH, there are several claims about its DET features, which can be argued considering PQQ as mediator itself. [66–68] In addition, PQQ-GDH is less selective particularly with respect to maltose (oligomeric form containing two D-glucose units). However, EFCs are mostly based on PQQ-GDH modified electrodes, where selectivity should not be accounted. [69–71]

Besides PQQ-GDH, FAD-dependent GDH can be exploited to develop glucose biosensors that are O_2 insensitive. Several types of FAD-GDHs have been investigated such as FAD-GDH from *A.* sp. (e.g, *A. terreus, A. niger*), *Thermoascus crustaceus* and *Glomerella cingulata* coupled to electrodes through mediated electron transfer. [72–75] Moreover, DET of FAD-GDH has been recently achieved combining *T. crustaceus* FAD-GDH with SWCNT. [76] Alternatively, FAD-GDH has been recently fused with a molecular mediator, so called 2.5th generation biosensor, [77] and with a minimal cytochrome c reported as DET. [78,79]

Despite several papers based on NAD-dependent GDH, this enzyme is not considered a good candidate for glucose biosensors development mainly because of diffusing electron acceptors, not efficiently re-oxidised at the modified electrode surface (*i.e.*, NAD^+/NADH redox couple that exhibits complex electrochemistry in both states). [80,81]

NAD-dependent GDH oxidises D-glucose to δ-gluconolactone transferring two electrons to NAD+ that is reduced to NADH. The latter is re-oxidised at the electrode surface from a mediator/catalyst as schematically reported below (equations 5 and 6):

D-Glucose + NAD^+ → δ-Glucolactone + NADH (5)

$$NADH \rightarrow NAD^+ + H^+ + 2e^- \text{ (catalysed by mediator/catalyst)} \quad (6)$$

Furthermore, many studies focused on alternative FAD-dependent D-glucose oxidising enzymes like pyranose oxidase (glucose 2-oxidase, POx), pyranose dehydrogenase (PDH), cellobiose dehydrogenase (CDH) from Ascomycetes. Differently from previously reported glucose oxidising enzymes, POx and PDH oxidise sugars not at C1 but at C2/C3 because they are anomeric insensitive. In addition, they can oxidise sugars multiple times, and PDH owns a covalently bound FAD-cofactor, ensuring long-term stability, which is usually an issue for enzyme-based amperometric biosensors. [41,82,83]

Cellobiose dehydrogenases (CDHs) are different from the structural point of view, encompassing two different domains: a catalytic FAD domain (dehydrogenase type) and a *heme b*-cytochrome domain as a "built in mediator". [40,84] Only Class II CDHs from Ascomycetes are able oxidise glucose. [85,86]

2.2. D-glucose Biosensors

In this section, the most representative cases of amperometric glucose biosensors based on glucose oxidase, PQQ-, FAD- and NAD-dependent GDH (mostly last decade) are discussed considering pros and cons about the electrode modification and technological advancement.

Considering Clark's research, many glucose sensing electrodes were initially developed considering enzyme-based membranes. They exhibited many advantages like high selectivity (depending on membrane permeability) [87] towards many interferents present in bodily fluids and enhanced linear range mainly due to diffusion limitations. However, they suffered pO_2 variations in blood, hence, needing an additional control with a non-enzymatic O_2 electrode to normalise the current output. [88] Exploiting a platinum electrode, the potential applied for the oxidation of H_2O_2 is quite high (at least 0.6 V vs. Ag/AgCl). Conversely, on modified solid electrodes (without membrane), the overpotential for H_2O_2 oxidation/reduction could be lowered close to 0.0 V (vs. Ag/AgCl) to enhance current output and selectivity. Many examples modifying the electrode surface with Prussian Blue (PB), also known as ferric hexacyanoferrate have been reported. [89–91] Alternatively, the electrode can be modified with several nanomaterials and polymers to facilitate electron transfer processes. [92,93] Horseradish peroxidase (HRP) has been also used as a biocatalyst for H_2O_2 reduction, increasing the selectivity but also increasing the complexity of biosensors. In this case, the stability of the

enzyme layer could be enhanced by using immobilisation strategies relying on physical entrapment (*e.g.*, Nafion layers etc.). [94,95]

Alternatively, to minimise the electrochemical interferents several molecular mediators like ferrocene, [96] neutral red, [97] and osmium polymers [98], have been exploited to develop 2nd generation glucose biosensors. However, the competition between O_2 and other electron mediators should be accounted. For example, thermally reduced graphene oxide was combined with a conducting polymer, namely polyaniline (PANI), to support the immobilisation of GOx physically entrapped within glutaraldehyde layer operating both as 1st (H_2O_2 oxidation at the electrode) and 2nd generation (mediated by diffusing N-methylphenazonium methyl sulfate (PMS)). Indeed, this platform exhibited an extended linear range due to the combination of both electron transfer pathways and the presence of a membrane-like layer limiting substrate diffusion. [99] A similar approach has been exploited by Ramanavicius with the development of reagent-less glucose biosensor based on a nanobiocomposite, namely poly(1,10-phenanthroline-5,6-dione), poly(pyrrole-2-carboxylic acid) covalently linking GOx (*via* EDC/NHS reaction) and mixed with gold nanoparticles. Notably, the nanobiocomposite material was acting both as mediator and as immobilising linker. Also in this case, the extended linear range resulted from the competition of both electron transfer pathways (1st and 2nd generation). Conversely, the presence of gold nanoparticles ensured higher enzyme stability (retained 96% of initial signal after 14 days). [100] Moreover, some efforts have been focused on the immobilisation of GOx by using chitosan (natural linear amine-rich polysaccharide) or other cross-linking agents like glutaraldehyde or thiol linkers to enhance the enzyme loading. [101,102] Recently, GOx based devices have been implemented on alternative scaffolds towards wearable or minimally invasive biomedical applications. For instance, filter paper was proposed as a bioreactor for the synthesis of PB nanoparticles, used as a catalyst for H_2O_2 reduction (produced by GOx upon glucose oxidation). This platform was developed by using both wax- and screen-printing techniques. In particular, the former was used to design the area dedicated for the deposition of PB nanoparticles, while the latter was employed to print the three-electrode electrochemical configuration. Paper Blue biosensor exhibited a linear range up to 5 mM, with a sensitivity of 1.70 μA/mM, and a LOD of 0.17 mM for H_2O_2 detection, while a linear range up to 25 mM for glucose detection. The porosity of the paper was exploited to store the reagents to develop reagent-free PoC devices, as shown in figure 3. [103] Alternatively, a glucose biosensing systems have been also integrated into highly stretchable and microneedles-based platforms towards next generation electronic textile for minimally

invasive and continuous biomarkers monitoring. [104–107] Despite the immobilisation strategy and the integration of nanomaterials and conductive polymers [108–110] as well as in novel minimally invasive platforms, the limit of detection for GOx-based 2nd generation glucose biosensors is still in the micromolar range as well as the dynamic linear range is quite narrow mainly due to the limited amount of enzyme molecules tightly connected to the electron mediator and the presence of O_2, acting as natural electron acceptor for GOx.

To overcome the O_2 dependence, many researchers employed oxygen-independent pyrrole quinoline quinone glucose dehydrogenase (PQQ-GDH). Despite its ability to communicate in DET, PQQ-GDH was initially used with diffusing and immobilised redox mediators, namely ferricyanide [111], 2,4,7-trinitro-9-fluorenone (TNF) [112] and osmium redox polymers [113] obtaining quite extended dynamic linear ranges probably due to the possibility of the enzyme to communicate both directly and through the electronic mediator. To realise a DET-based biosensor ,PQQ-GDH from *Acinetobacter calcoaceticus* was covalently immobilised onto a composite based on polyaniline and MWCNTs deposited on gold electrode exhibiting a wide linear range. [114] due to the properties of conducting polymers improving the electron transfer rate and increasing the enzyme loading. The same research group proposed the assembling of electroactive multilayers assisted by deoxyribonucleic acid (DNA) as a negatively charged building block [115] to achieve an artificial signal chain. Despite tailoring a novel electrode surface that can be potentially used to create biomimicking machinery, this platform has a limited catalytic efficiency as well as analytical figures of merit. Successively, Lisdat and his co-workers reported the electron transfer process of PQQ-GDH onto polythiophene copolymers modified MWCNTs, as shown in figure 3. [116] In this particular case, the authors deposited thiols ended CNTs, followed by the electropolymerisation of different thiophene monomers performed in organic solvent, later thoroughly rinsed with water to avoid any protein inactivation. The bioelectrocatalytic response was linear from 1 μM to 500 μM. Later, the same authors attempted the same electropolimerisation in water-based monomer solution obtaining a wider dynamic linear range up to 2 mM probably due to the variation of apparent Michaelis constant of the enzyme onto the modified electrode. [117]

Alternatively, another O_2 independent GDH (FAD-dependent) from *Aspergillus species* deposited onto MWCNTs modified with chitosan was employed. [118] Specifically, FAD-dependent GDH were extracted from *Aspergillus sp.* and *Aspergillus oryzae*, exhibiting similar performance in MET, with a long storage-stability (6 months) due to the effective connection enzyme-mediator and its insensitivity to O_2.

In another report, Cohen et al. cloned, overexpressed, and purified the FAD-GDH from *T. emersonii* in *E. coli*. After biochemical characterisation, the amperomeric biodevice reported a linear range 0-20 mM by operating both in MET and DET. [119]

Even not being the best O_2 independent candidate glucose oxidizing enzyme, NAD-dependent GDH has been used to develop several sensing platforms. Using several iron-sulfur cluster both to enhance the electron transfer and wire NAD-dependent GDH [120], the authors obtained a linearly dependent response in the range 0.1-100 mM with a LOD varying based on Fe-S structure. The metal-anchorable sulfur atom, the strong π-coordinating iron atom, the favorable redox properties, low cost, and natural abundance make Fe-S an excellent electron-mediating relay capable of wiring redox active sites to electrode surfaces. Alternatively, the combination of GDH with diaphorase onto Osmium Redox Polymers (ORP) bound through avidin-biotin complex on indium tin oxide (ITO) electrodes , successfully employed for glucose sensing in artificial human serum avidin solution (5% w/v). [121]

A novel approach has been proposed by Zebda et al., who reported the integration of NAD^+ dependent GDH with 3D carbon nanotube network modified with poly-methylene green. The proposed electrode reported an onset potential for glucose oxidation at −0.2 V vs SCE, achieving a plateau already at +0.1 V, potentially minimising the effect of several interferents on the analytical response. The pellet-based platform reported a linear range 50 μM-20 mM with a sensitivity of $50{,}8 \cdot 10^{-3}$ A mM^{-1} cm^{-2} and a stability over 1 year. [122]

Dilgin et al. immobilised NAD-dependent GDH from *Pseudomonas* onto pyrocatechol violet (Pcv)-modified graphite pencil electrode (GPE), where Pcv is acting as a catalyst towards the oxidation of NADH (enzymatically generated) to NAD^+. The proposed biosensor exhibited a linear response within the range 5-500 μM with a LOD of 1.2 μM and a sensitivity of 2.78 nA μM^{-1} cm^{-2}. In addition, to minimise the effect of electrochemical interferents like ascorbic acid, uric acid and dopamine, usually present in biomedical and food samples, the authors used a water-insoluble pre-oxidant within the flow injection analysis system, namely sodium bismuthate. This approach allowed to perform a selective glucose detection in real samples without further electrode modification (*e.g.,* selective membrane deposition etc.). [123]

The analytical figures of merit like limit of detection (LOD), dynamic linear range, sensitivity and storage stability are summarised in table 1 for quantitative comparison. The dynamic linear range is limited by two parameters the intrinsic K_M, related to the enzyme properties like substrate affinity or isoelectric point (important for protein conformation and orientation on the electrode surface), and the apparent K_M, mostly affected by the electronic connection of the

enzyme with the electrode (in 1st, 2nd or 3rd generation) and the morphology of the electrode surface (*i.e.*, the presence of nanomaterials, electrode porosity, presence of membranes etc.). Indeed, the presence of membranes or porous electrode structure are hindering the diffusion of the substrate towards the enzyme increasing the apparent K_M, resulting in the extension of dynamic linear range. The sensitivity (considered as the slope of the calibration curve) and LOD are mostly affected by the enzyme loading where immobilization strategies, nanomaterials and electronic mediators are playing an important role towards increasing the sensitivity and decreasing the LOD. Most of the electrode platforms herein reviewed, exhibited a good storage stability (> 50% of initial activity retained after few weeks) mainly due to the presence of nanomaterials acting as stabilising materials.

3. D-fructose Detection

The detection of D-fructose has been relevantly under-taken in the last decades, but high fructose consumption will may cause an endemic obesity status not only because of diabetes type II mellitus (glucose intake related pathology). [124,125] Nowadays, the consumption high-fructose corn syrup (HFCS) beverages as well as soft drinks has become very popular among young generations causing more often the onset of obesity at paediatric level. [126] This may occur because of the increased production of non-alcoholic fatty liver. Over 70% of all foods contain some amounts of added sugar, and consumption of soft drinks increased by five times since 1950. [127] Data analysis suggested that consumption of sugar-sweetened beverages is related to the risk of diabetes, the metabolic syndrome and cardiovascular disease in adults and in children. Drinking two sugar-sweetened beverages per day for 6 months induced features of the metabolic syndrome and fatty liver. In this regard, the development of highly selective and sensitive biosensing platform will play a key role in the early detection as well in the development of personalised treatment based on remotely operating physicians. This will enable a powerful control of this high-risk pathology as well the correct management without the needs of hospitalisation. [128,129]

Most of fructose biosensors, if not all, are developed considering fructose dehydrogenase (FDH) as bioreceptor. Fructose dehydrogenase is a membrane-bound oxidoreductase extracted from bacteria belonging the acetic acid bacteria (AAB) family. In this group, only *Acetobacter* and *Gluconobacter* can oxidise D-fructose producing 5-keto-D-fructose. In the past, it was believed as PQQ-dependent protein, [130] but recently Kano and his group demonstrated the presence of a flavin DH type domain and 3 *heme c* in the cytochrome domain. Hence, the protein now is enclosed in the flavohemoproteins family. [131,132]

FDH, being a heterotrimeric protein, encompasses three subunits: subunit I (DH_{FDH}), which is the catalytic domain, with a flavin group able to oxidise D-fructose to 5-keto-D-fructose in a $2H^+/2e^-$ electron transfer process; subunit II (CYT_{FDH}), acting as a built-in electron acceptor with three heme *c* moieties covalently bound to the enzyme scaffold (only two heme c are involved in the electron transfer process); and subunit III, ensuring the enzyme stability. [4,133–135] Unfortunately, its crystal structure is still under investigation. This would enable the development of highly efficient enzyme-based amperometric biosensors considering the possibility to engineer specific sites within the enzymatic structure.

The electron process occurs in three steps: (*i*) oxidation of D-fructose with the concomitant reduction of FAD to $FADH_2$; followed by (*ii*) $FADH_2$ reoxidation in two sub-sequent monoelectronic processes, eliciting an internal electron transfer (*iii*) that propagates through CYT_{FDH} domain involving heme c_1 and heme c_2..

In the presence of a redox mediator, this can mediate both the electron transfer from the flavin DH domain and from the cytochrome domain. It would be hard to understand the preferred pathway, hence they are classified as 2nd generation biosensors. [136]

Few examples of FDH based biosensors will be reviewed considering the analytical figures of merit, which are resumed in table 2. As reported in table 2, all platforms exhibited a relativelyextended linear range (considering the intrinsic K_M ~8). [137] The sensitivity is mostly affected by the enzyme loading (electroactive area). Most of the FDH-based electrodes have been tested with flow injection analysis (FIA) because of their potential integration in an on-line sensing unit in a manufacturing process. Using biosensors in industrial bioprocess monitoring and control has been hindered by the absence of adequate sampling techniques.

Considering FDH as a membrane-bound protein, other scientists tried to recreate a confortable environment for the enzyme using crystalline cubic phase (LCP) and CNTs (combination of hydrophobic materials) [138] or thermally reduced graphene oxide [139].

The influence of gold nanopores on the bioelectrocatalytic behaviour of FDH investigated. Indeed, the authors reported a wet chemistry based method, notably de-alloying, for the preparation of nanopores with controlled size ranging from 9 up to 62 nm. [140,141] The electrode was further modified either with a self-assembled monolayer of 3-mercaptopropionic acid (3-MPA) or via diazonium salt electrografting of 2-carboxy-6-naphthoyl moieties. FDH was finally cross-linked onto the modified electrode through carbodiimide (CMC), as displayed in figure 5. The proposed biosensors reported a linearity in the amperometric response within the range of 0.05–0.3 mM, with a LOD of 1.2 µM and a sensitivity of 3.7 µA cm^{-2} µM. [142]

By increasing the pore sizes ranging from 10 up to 50 μm (internal nanoporous structure) it was possible to enhance real surface area from 0.073 cm^2 to 24 cm^2 (300 times higher). [143] The proposed biosensor exhibited a dynamic linear range 10 times larger compared to the previous platform probably because of the increased porosity of the electrode surface that hinders the diffusion of the substrate (also controlled through FIA).

Similarly, FDH was immobilised on a porous gold microelectrode to achieve a substrate-transfer-controlled current. Indeed, the anodization of the microdisk electrode allowed to increase the surface area by 170-fold, with pores sizes within the range 20-200 nm. The authors hypothesised that the concave shape of the pores would be able to orient FDH more effectively, resulting in a mass-transfer limited current roughly 300 times higher than the planar electrode. The proposed platform exhibited a linear range up to 10 mM with enhanced selectivity towards both enzymatic and electrochemical interferents. The catalytic current density for the oxidation of 1.0 mM d-fructose is independent of the storage time for up to 6 days. [144] Moreover, FDH could be stabilised by using self-assembled monolayers (SAMs) growth onto NPG electrodes, where the authors realized a site-oriented immobilization based on the study of charge distribution of FDH surface. Electrostatic surface mapping evidenced the distribution of polar/hydrophilic amino acid residues, which shows an overall quite hydrophilic surface with both positive and negative charges nearby the heme 2c. [145]

Alternatively, the possibility to perform a site-oriented immobilisation of FDH was performed through a hydrophobic pocket within the second subunit of the enzyme, being very close to the heme c_1 and c_2 responsible as built-in mediators for the DET mechanism of FDH. The electrode was modified through the electrografting of diazonium salt of 2-aminoanthracene on the surface of single walled carbon nanotubes (SWCNTs). As shown in figure 6, it was possible to isolate the contribution of each heme to the electrocatalytic wave (overall 485 ± 21 μA cm^{-2} at 0.4 V). FDH/2-ANT/SWCNT/GCE platform exhibited a great stability retaining 90 % of the activity after 60 days, with a LOD of 0.9 μM and a very high sensitivity (47 ± 1 μA mM^{-1} cm^{-2}). [146]In 2021, it was attempted as a proof-of-concept the development of 'real' self-powered biosensor trying to exploit the concept of self-charging biosupercapacitors. [147] Notably, the device encompassed two different parts: biocatalytic electrodes and charge storing elements. In this regard, the biocatalytic electrode where working as a normal enzymatic fuel cell depending on D-fructose concentration, while the charge storing part was needed to supply energy to the analytical device. This represents an extremely new concept that enabled not only to obtain a self-standing biosensor (charging time 70 minutes) but also an enhanced sensitivity,

notably 3.82 ± 0.01 mW cm^{-2} mM^{-1}, which is 100 times higher with respect to the same EFC operated as self-powered biosensor.

4. L-lactate Detection

Recently, clinical diagnostics focused also on lactate as biomarker to assess patients' health conditions and actuate a continuous and remote surveillance for sport medicine and food industry. [148–150] The latter had a critical role in the last few years in the development of emerging wearable or minimally invasive technologies ranging from tattoo or smart watches to wrist bands or smart gloves, till microneedles and smart packaging. [151–153] However, the lactate physiological level in blood is within 0.5-15 mM rising to 25 mM under intense workout conditions. [154] This has a key role in the anaerobic metabolism. When there is not enough energy supply from the aerobic pathway, there is an increase of lactate concentration due to anaerobic metabolism activation. [155] The accumulation of lactate could result in pathologic state like lactic acidosis, without adequate clearance by liver and kidney. In healthy humans, lactate levels are strictly controlled by lactate homeostasis. The production of lactate is directly related with the increasing of H^+ within the cell with the contemporary regeneration of bicarbonate to neutralize the protons and prevent the loss of bicarbonate. [156] This allows to keep an internal pH of approximately 7.2-7.4. There are two classes of diseases causing lactic acidosis: the first related with decreased tissue oxygenation like shock, left ventricular failure, sepsis and poisoning with carbon monoxide and cyanide; and the second ones related to the administration of specific drugs responsible for renal and hepatic system failure, diabetes and malignancy or inborn error metabolism. [157]

Lactate biosensors are usually enclosing two families of enzymes, notably lactate oxidases and dehydrogenases. L-lactate oxidase is a globular flavoprotein that can be extracted from several bacterial sources like *Pediococcus*, *Aerococcus viridans*, and *Mycobacterium segmantis*. [158–160] The enzyme contains flavin mononucleotide catalysing the oxidation of L-lactate to pyruvate with the contemporary reduction of O_2 to H_2O_2. Since O_2 is naturally working as electron acceptor, both O_2 and the related product H_2O_2 can be electrochemically monitored to obtain an amperometric output that is proportional to L-lactate concentration. [161]

Although this represents the most viable approach, there are several concerns about the selectivity and reproducibility of the results mainly due to the required high overpotential needed to oxidise/reduce H_2O_2 and the fluctuation of O_2 in the solution, not considering its limited availability while working in bodily fluids (0.22 mM), which would hinder the sensing reaction. Dehydrogenases could be potentially a solution considering their O_2 insensitivity.

[162] LDH is a tetrameric protein ubiquitously present in in animals, plants, and prokaryotes. It has important medical significance as it is present throughout the tissues, such as blood cells and heart muscle. It is released during tissue damage, so it basically acts as a marker of common injuries and disease. L-LDH and D-LDH (EC 1.1.1.27 and EC 1.1.1.28, respectively) catalyse the oxidation of lactate to pyruvate and vice versa in the presence of $NAD(P)^+$/NAD(P)H and represent a good candidate for the development of lactate biosensors without any O_2 sensitivity. $NAD(P)^+$ works as diffusing electron acceptor, hence the electrode surface should be modified with a catalyst specifically oxidising NAD(P)H in order to realise a 2nd generation biosensor based on mediated electron transfer. [163,164]

In table 3, the analytical parameters of few lactate biosensors have been reviewed. Most of the electrode platforms are using LOx in combination with HRP or a catalyst like Prussian Blue (PB) able to reduce H_2O_2. There are also few examples where H_2O_2 is oxidized like platinum electrodes or modified carbon electrodes. In both cases, it was possible to observe narrow linear ranges that are unsuitable to monitor lactate sweat levels that achieve 20 mM during sport activity. However, lactate production is monitored also at level of cell cultures or industrial fermentation processes where concentrations fall within the dynamic ranges reported in table 3. [165–175] Depending on application, several lactate biosensors have been developed as laboratory stand, portable, or hand-held device, and commercialised by several companies. *Nova biomedical* and *Axon Lab* developed a biosensor able to monitor lactate in a small sample of blood, hence not providing continuous monitoring. In 2022, *Abbott Laboratories* developed a 'biowearable' platform able to monitor glucose, lactate, ketones and alcohol based on CGM technology. Alternatively, *Innovative Sensor Technology (IST)* released a multi-parametric sensor based on a small flow cell (1 µL) able to monitor glucose, lactate, glutamine and glutamate simultaneously. For instance, *Pinnacle Technologies Inc.* commercialises a lactate biosensor able to monitor real-time changes in rodents' brain. This type of biosensor is based on most of the previously reviewed platforms encompassing a platinum-iridium electrode, where platinum act as catalyst for H_2O_2 oxidation, further modified with LOx as specific bioreceptor immobilised onto a selective membrane (preventing uneven electrochemical interferences) and bovine serum albumin to prevent aspecific protein adsorption phenomena. The proposed platform exhibits a linear range up to 2 mM and can provide continuous monitoring of brain lactate concentration changes for more than 96 hours. Recently, Gaston Crespo and Maria Cuartero funded *IDRO BV* with the contribution of European Institute of Innovation and Technology (EIT), commercializing their recent platform for lactate monitoring. This platform was developed after a perspective on the impact of lactate

monitoring on sports physiology. [176] IDRO's patch is developed as a flexible and wearable biosensor enabling real-time data and boosting athletes' performance.

Besides the detection of lactate in food and some bodily fluids, more recently, lactate has been widely investigated for the development of many minimally to non-invasive biosensors based on microneedles or tattoos technology. [177–179] In this regard, Wang and his co-workers developed a flexible printed temporary-transfer tattoo reporting a linear range up to 20 mM. This kind of technology allowed to achieve a real monitoring of lactate during prolonged exercise reflecting the variation of sweat production correlated with lactate profile, as shown in figure 7. This electrochemical platform led to the development of several platforms for different analytes useful for the remote medical control not only during sport activities but also for other military and biomedical applications. [151]

More recently, the first 2nd generation microneedle-based biosensor to detect lactate in the interstitial fluid was developed. The latter being in communication with the peripheral blood system contains analytes in a concentration that could be easily correlated with the ones in blood. The authors electrodeposited gold microparticles with carbon nanotubes, followed by methylene blue electropolymerisation. The proposed platform exhibited a LOD of 3 µM, definitely below the ones previously reported and a sensitivity of 178.4 µA cm^{-2} mM^{-1}. [180] This platform has been coupled with a glucose biosensor based on FAD-dependent GDH and used in a simulated model to reproduce the upper layer of skin based on a hydrogel system crossed by a microfluidic system, hence reproducing the exchange between the peripheral blood system and interstitial fluid. [181,182]

5. Enzyme-based Amperometric Biosensors: Technological and Marketing Challenges

As reported in the previous sections, the technological development of enzyme-based amperometric biosensors is evolving towards the online and *in situ* monitoring. Nowadays, biosensors are not only conceived depending on the analytes and analytical figures of merit, but they are encapsulated in polymers integrated in materials and objects commonly used during day life. [183–185] A particular example is the development of glucose biosensors towards the management and treatment of diabetes considering some intravascular and subcutaneous applications. In this case (implanted sensors), there several technical issues related with the biofouling of the electrode surface, as well as potential tissue damage and biocompatibility of all materials employed to modify the electrode surface. [186] This is re-routing the interest of biosensing community towards minimally invasive (microneedles and tattoos) and hand-held biosensors.

There is great engagement on this side also for clinical, environmental, and pharmaceutical applications. What is limiting the technological advancement of biosensors? The key issue is the scalability to produce biosensors that contains several biological elements that are particularly expensive. On the other hand, the limiting aspect is the bioproduction process that does not exhibit the same kinetics of biosensor production. This is important to ensure continuous supply for the production. In this regard, the scientific community is trying to develop biosensing platforms that could be reused multiple times exhibiting a great operational and storage stability over months. [187,188] Another issue is related to the immobilisation of the bioprobe. Recently, most of the preparation protocols endowed the electropolymerisation or electrodeposition process enabling a precise and reproducible immobilisation, which plays a pivotal role in the analytical figures of merit for the biosensors developed. [189]

This is also interesting for the development of micro- and ultra-microelectrodes (UMEs) that are currently used for the development of biosensors. [190]

An important issue/challenge, towards the employment of enzyme-based amperometric biosensors as Point-of-Care (PoC) devices, is the robustness of the electrode platform either in terms of calibration or analysis of real samples. [191] The reliability of enzyme-based biosensors depends mostly on the calibration procedure that should account for the operating conditions. For instance, considering glucose biosensors, for many years the basis for calibration was the assumption that blood glucose concentration was like the one in subcutaneous interstitial fluid. This is not true. In addition, the ratio of blood/tissue glucose is not constant but depends on several factors related to the physiology of glucose and insulin. In this specific case, the reliability of the calibration is low. For other types of substrates, the relationship between blood levels and peripheral bodily fluids might be considerably more complicated. For real sample analysis, enzyme-based electrodes are usually undergoing a progressive denaturation of the enzyme layer and surface fouling. To prevent enzyme denaturation, it is possible to control the rate-limiting step through porous electrode structures or diffusion-limiting membranes that are reducing signal variations even in the case of substantial loss of enzymatic activity. The membrane also serves to protect the active electrode surface from direct contact with the sample and hence minimizes fouling. Antifouling coatings onto electrodes (without enzyme) may also result in robust electrochemical biosensors. Moreover, hydrogel coatings were reported to reduce ionic and bulky interferences.

The development of PoC devices for electrochemical biosensing is one of the main challenges in this field. Currently, much effort has been exerted in the miniaturization of electrochemical transducers and readout instruments to achieve real-time, PoC and easy-to-use analysis in the

field. [192] With respect to biosensor marketing, only PoC devices gained reasonable attention mostly because of their portability, low-cost (depending on the bioreceptor and analytical target), fast response and nowadays also their eco-impact. [193] This means that most of the disposable part of the biosensors like polymers to modify the electrodes as well nanomaterials or supports need to be recyclable. The focus on the ecological impact of PoC devices has been particularly raised during the COVID-19 pandemic and it clearly needs to be addressed carrying on research on the average lifetime of materials and considering if such recycling process is sustainable both from the economic and environmental point of view.

Besides the eco-impact, especially glucose and lactate monitoring are experiencing a shifting from traditional blood self-testing platforms towards wearable technologies, moving from PoC or lab-on-chip devices to lab-on-skin devices. Although electronic textile or e-textile are still at the early stage, such revolutionary epidermal sensing technology will offer the possibility of a continuous monitoring of health and disease status. Many researchers are currently working on the integration through microfluidic apparatus or even directly screen-printing or stencil-printing the electrodes on clothes. Except CGM (as previously reported for lactate), there are no commercial sensing platforms as worn technologies perhaps due to limited sensor stability (related biofouling and receptor degradation) and the limited understanding of the correlation between sweat and blood analyte concentrations. The correlation between epidermal electrochemical sensors and blood biomarkers levels will require large-scale clinical trials and broad validation efforts. [194] Only few examples of microneedles-based platforms are commercialised by start-up companies like *San Diego-based Biolinq*, who reported its first successful human trial with results showing promising correlation and no lag time compared to the venous blood (June 2020). For instance, *Caura (UK)* is focused on monitoring simultaneously glucose and heart rate, while *Melbourne-based Nutromics (Australia)* is working on microneedles for personalised nutrition. Besides continuous biomarkers monitoring, the big challenge for microneedles is about a reproducible skin penetration especially regarding the elasticity of the skin, which results in a counteracting force to the penetration and thus, to a partial or incomplete piercing. Moreover, one should also consider the skin thickness variation depending on the age, gender, body mass index, and ethnicity of the wearer. In addition, researchers need to account for biofouling effects (decreasing sensor reproducibility and lifetime) and the evaluation of the analytical performance to fine tune eventually connected drug-releasing units, operating as a sense-act-treat device. [195,196]

The market of enzyme-based amperometric biosensors is moving towards achieving zero eco-impact of biosensing platform, cost-effective platform and reliable biosensors that could be

used in several fields going from precision and remote medicine till smart food packaging. For instance, global biosensor market size was valued at USD 27.5 billion in 2021 and it is expected to expand annually by 8% between 2022 and 2030. However, the biosensors market will reach USD 49.6 billion by 2030 on a worldwide base, as shown in figure 8. [197] The increasing preference for non-invasive biosensors, research collaboration and agreements between diverse manufacturers raised the popularity of self-testing/medicating devices, consolidating biosensors market. Furthermore, in November 2019, scientists from Brazil and the U.S. developed a biosensor that is a glasses-based device that can give diabetics a less invasive test by measuring blood glucose levels through tears. From a technical point of view, the market is dominated by electrochemical based platforms that captured the largest revenue share approximately of 71.1% in 2021 (figure 8), probably to the ease of biochemical measurement protocols, low detection limits, wide linear response range, excellent stability, and repeatability are all advantages of electrochemical biosensors. They also exhibit robustness, compatibility with new microfabrication technologies, ease of operation, low cost, disposability, independence from sample turbidity, and low power.

6. Conclusions and Future Perspectives

After marking 60 years since the development of the first biosensing platform, the enzyme-based biosensing technology has moved a big step forward considering the technological issues encountered during these six decades. Nowadays, most of the enzyme-based biosensors are employed for the diabetes management and treatment (when connected with insulin pumps) and online target analysis. In addition, many enzymatic biosensors have been also used for pesticides and toxins detection. In this review, we have considered most of the enzymes widely used for biosensors development focusing on glucose, fructose, and lactate detection. In this regard, the electron transfer process has been deeply discussed as well as their importance from the analytical point of view. Afterwards, the analytical figures of merit of several enzyme-based amperometric biosensors were schematically summarised to provide a complete view of the state-of-the-art.

As future perspective, the enzymatic biosensing community will move along two main routes: (*i*) the development of wearable devices that could potentially work as actuators and (*ii*) the possibility to overcome the limit of detection limit, usually within the micromolar range. The first route is currently under investigation. Knowledge about the composition of bodily fluids is crucial for the development of wearable devices. Thanks to real-time biomarkers monitoring, information on wellness and health, and enhancement of the management of chronic diseases

are provided, alerting the user or medical professionals of abnormal or unforeseen situations. To achieve wearable sensing, the (bio)sensing elements must be in direct contact with biofluids, hence they need to comply with body shape (comfortable wearing) by using advanced smart materials that are flexible and stretchable. [198] Although a first report on this technology was published [199], there are plenty of potential developments based on the bio(triggered) drug release, stretchable and flexible platform. The bio(triggered) release could be achieved integrating two-electrode systems encompassing a sensing/actuating electrode and the actuated electrode being often used for drug release or to activate a second sensing process. [200] The former can be activated by O_2 reduction to H_2O biocatalysed by multicopper oxidases (MCOs) that are 'locally' increasing the pH (stimuli). [201] Alternatively, reaction products like H_2O_2 can activate the release from hydrogel layers. In this regard, nanozymes can be easily integrated to replace enzymes and increase the robustness of such platforms. Nanozymes are nanomaterials owning enzyme-like properties, which catalyse the conversion of substrate to product (catalytic activity). In the last decade, 'naked' gold nanoparticles (NPs), functionalized AuNPs, Fe_3O_4 NPs have reported oxidase- or peroxidase- like activity that can be easily employed for the development of (bio)sensing and sense-act-treat devices. [202] They certainly exhibit increased robustness compared to enzymes; however, they lack selectivity as major drawback, additionally, they are unable to reproduce all the reactions catalysed by enzymes. This could be easily implemented exploiting Boolean Logic Gates and "Chimeric" enzymes. [203–207] Recent technological advancements in soft actuators, flexible bioelectronics, and wireless data acquisition systems have enabled the development of ergonomic, lightweight, and low-cost wearable devices. For instance, wearable (bio)sensing gloves merged with sensory feedback devices could enhance hand sensations for healthcare, robotics and virtual reality applications. [208] About the second route, recently Bollella and his co-workers proposed the combination of self-powered biosensor (or enzymatic-fuel cell) with a bipolar junction transistor in extended base configuration. [209,210] In this approach, being the base current controlled, there is the possibility to minimise the signal noise, increase the signal-to-noise ratio and amplify the current output to achieve sub-nanomolar if not lower LOD, which has so far never been achieved with wide-field single molecule electrochemistry approach. The latter can pave the way for unforeseen development for PoC and Point-of-Use devices. Achieving single-molecule diagnostics with electrochemical transduction (considering enzymatic reaction) will have a great impact on PoC and Point-of-Use devices broadening the range of applications where early detection plays a key role.

Acknowledgements

PB acknowledges the following funding: Biosensori analitici usa-e getta a base di transistori organici auto-alimentati per la rivelazione di biomarcatori proteomici alla singola molecola per la diagnostica decentrata dell'HIV (6CDD3786); Research for Innovation REFIN—Regione Puglia POR PUGLIA FESR-FSE 2014/2020.

Authors' Contributions

All authors have thoroughly contributed to conceive and write this article. The final version was approved by all authors.

Conflict of Interest

The authors declare no conflict of interest.

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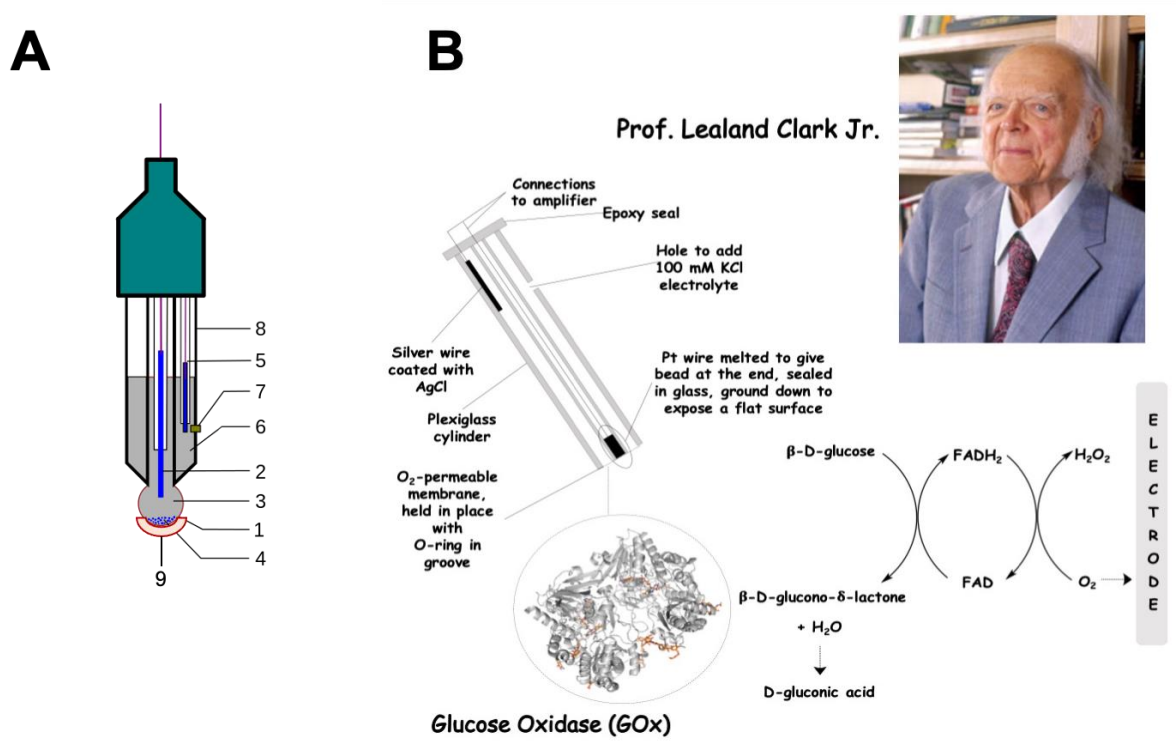


Figure 1. (A) Scheme of pH sensitive glass-membrane electrode modified with GOx cuprophane membrane: 1. pH sensing part of electrode, a bulb made from a specific glass, 2. internal electrode, usually silver chloride electrode or Saturated calomel reference, 3. internal solution, usually 0.1 mol/L MeCl, 4. when using the silver chloride electrode, a small amount of AgCl can precipitate inside the glass electrode, 5. reference electrode, usually the same type as 2, 6. reference internal solution, usually 0.1 mol/L KCl, 7. junction with studied solution, usually made from ceramics or capillary with asbestos or quartz fiber. 8. body of electrode, made from non-conductive glass or plastics, 9. GOx cuprophane membrane; **(B)** Schematic representation of Clark's electrode to monitor O_2 variation modified with a glucose oxidase (GOx) membrane known as the first reported glucose biosensor (inset) picture of Prof. Lealand Clark Jr. Panel A is reproduced from https://commons.wikimedia.org/wiki/File:Glass_electrode_scheme_2.svg under CC-BY-SA https://creativecommons.org/licenses/by-sa/3.0/legalcode. Panel B adopted from [210] with permission of Wiley.

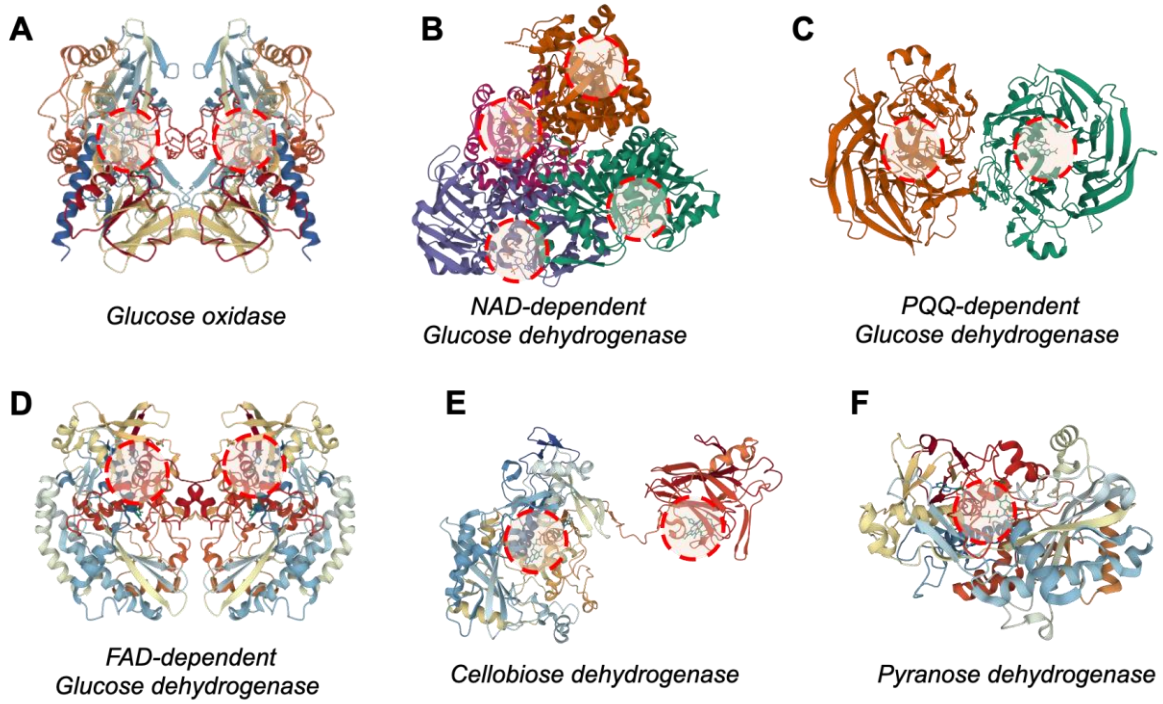


Figure 2. Crystal structures of **(A)** glucose oxidase from *Aspergillus niger* (PDB file: 1GAL); **(B)** *Sulfolobus solfataricus* Glucose Dehydrogenase 1 (PDB file: 2CDB); **(C)** PQQ-dependent Glucose Dehydrogenase from *Acinetobacter calcoaceticus* (PDB fille: 1CQ1); **(D)** *Aspergillus flavus* FADGDH (PDB file: 4YNU); **(E)** Cellobiose dehydrogenase from *Neurospora crassa* NcCDH (PDB file: 4QI7); **(F)** pyranose dehydrogenase from *Agaricus meleagris* (PDB file: 4H7U). The redox centres are highlighted with red circles.

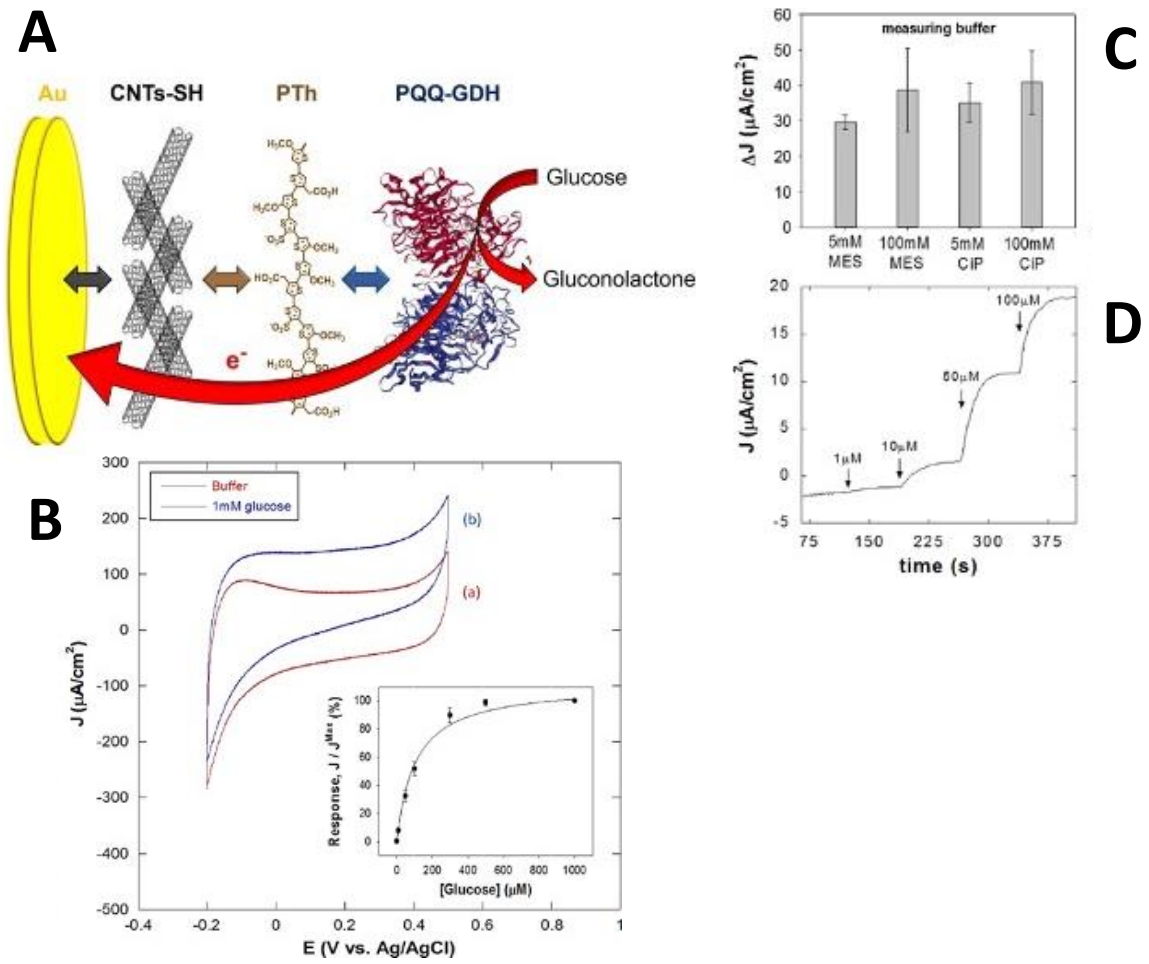


Figure 3. (A) Schematic representation of PQQ-GDH modified electrode; (**B**) Cyclic voltammograms before (a, red curve) and after (b, blue curve) the addition of 1 mM glucose, (scan rate 10 mV/s) and insert plot of the amperometric current density versus the glucose concentration in stirred solution measured by amperometry at 0 V vs. Ag/AgCl; (**C**) Evaluation of the effect of the measuring buffer solution on the bioelectrocatalysis in presence of 1 mM glucose; ((**D**) Amperometric experiment performed at a fixed potential of 0 V vs. Ag/AgCl and measuring the anodic current density with additions of glucose to the stirred solution. Experiments were performed in 100 mM CiP buffer, 1 mM $CaCl_2$, pH 6.5. Error bars refer to the standard deviations calculated from three different electrodes (PQQ-GDH/PTh/MWCNTs/Au). Abbreviations: gold electrode (Au), thiol ended multi walled carbon nanotubes (CNTs-SH), polythiophenes (PTh), PQQ-dependent glucose dehydrogenase (PQQ-GDH), 2-ethanesulfonic acid (MES) and citrate-phosphate buffer (CiP). Panels A-D reproduced from [116] with permission of Elsevier Ltd.

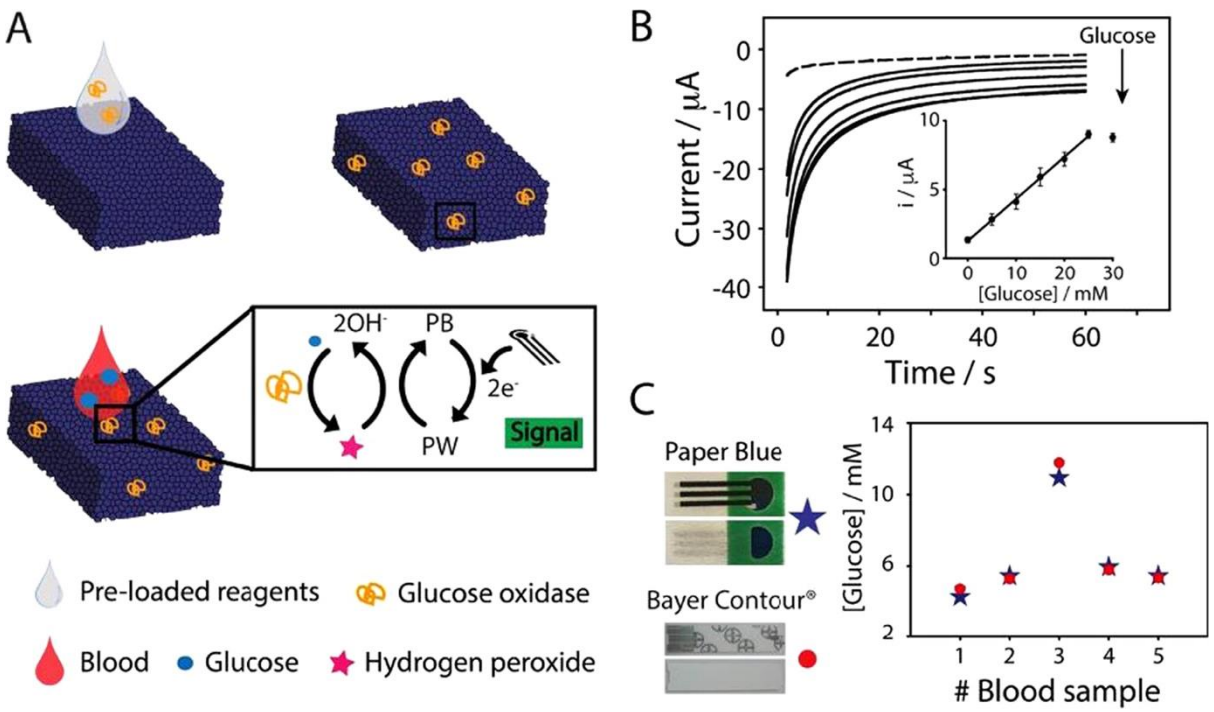


Figure 4. (A) Scheme of Paper Blue biosensor. **(B)** Calibration curve and amperometric responses upon addition of glucose up to 30 mM. **(C)** Comparison Paper Blue with Bayer Contour® on 5 blood samples. Reproduced with permission of Elsevier Ltd from [103].

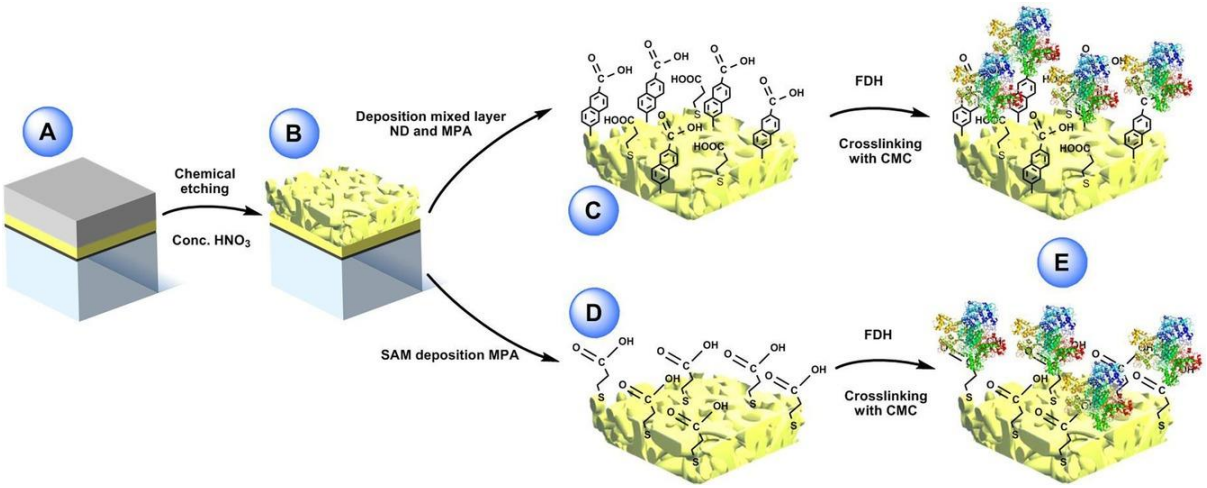


Figure 5. (A) Sputtered glass sheet with layers of titanium, pure gold and gold/ silver alloy (bottom to top). **(B)** Nanoporous gold (NPG) electrode surface post-dealloying by concentrated nitric acid. (C) Electrochemical reduction of ND on the NPG surface utilizing a single scan and subsequent filling of the void spaces with MPA. **(D)** Preparation of a MPA SAM on NPG substrate by immersion over night at 4 °C. **(E)** After adsorption of FDH on the two modified electrodes the enzyme was crosslinked using CMC (sizes not to scale). Panels A-E were adopted from [142] with permission of Wiley.

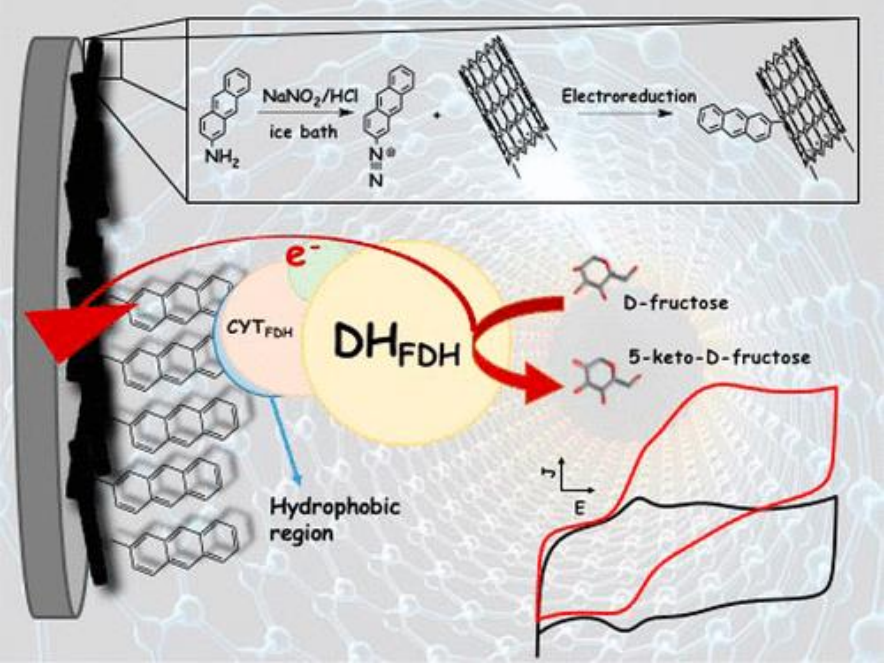


Figure 6. Schematic representation of FDH modified electrode with the electrografting of *in situ* prepared diazonium salt of 2-minoanthracene cation acting as a plug-in for the hydrophobic pocket in the second subunit of FDH. This figure was reproduced from [146] with permission of the American Chemical Society (ACS).

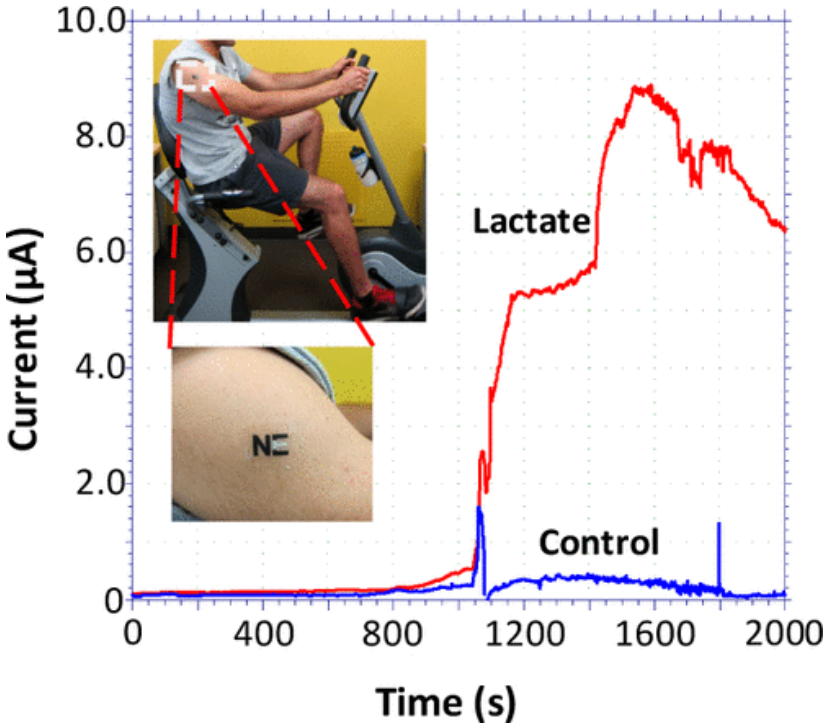


Figure 7. Lactate profile obtained using tattoo-based biosensor during aerobic exercise. Figure reproduced from [151] with permission of American Chemical Society (ACS).

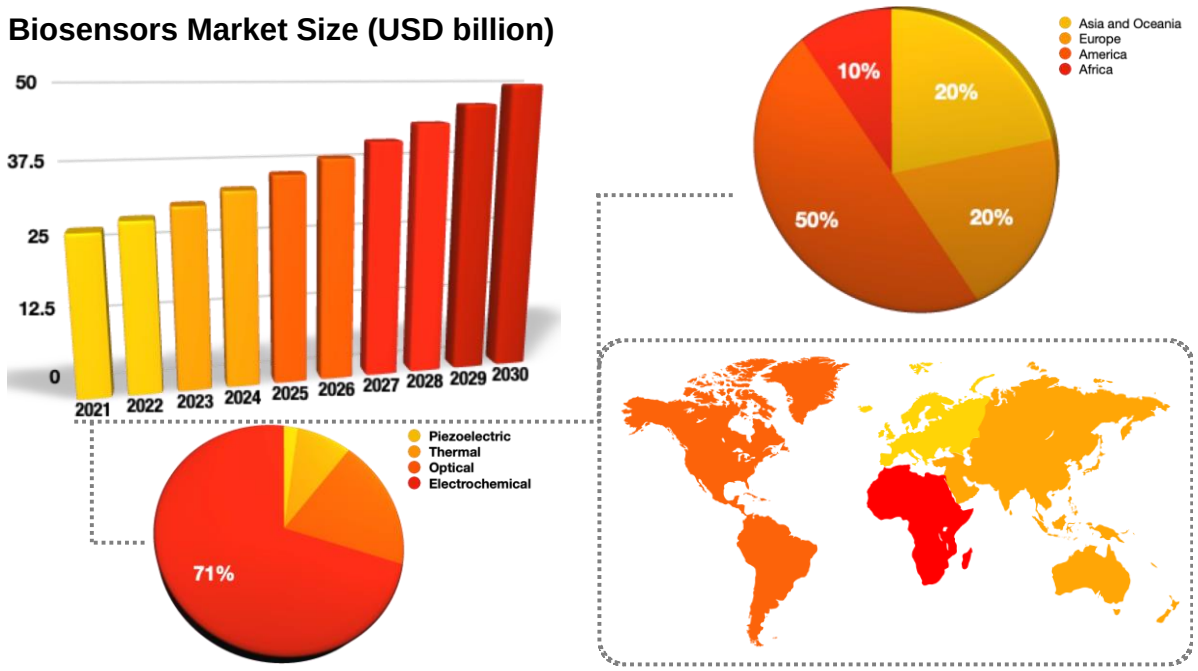


Figure 8. Projection of Biosensors Market Size 2021-2030 with a focus on the distribution by transduction techniques and region for 2021. Data obtained from [197].

Table 1. Analytical figures of merit for reviewed glucose biosensors. Abbreviations: glucose oxidase (GOx), horseradish peroxidase (HRP), poly(toluidine blue) (pTB), reduced graphene oxide (rGO), glassy carbon electrode (GCE), polyaniline:reduced graphene oxide composite (PANI:rGO), graphite (GR), poly(pyrrole-2-carboxylic acid) (PPCA), gold nanoparticles (AuNPs), poly(1,10-phenanthroline-5,6-dione) (PPD), chitosan (Chit), Prussian Blue (PB), gold deposited on styrene-ethylene/butylene-styrene fibers (Au-SEBS), screen-printed carbon electrodes (SPCE), nafion (Naf), Nickel Hexacyanoferrate (NiHx), polyacrylic acid (PAA), poly(3,4-ethylenedioxythiophene) (PEDOT), PQQ-dependent glucose dehydrogenase (PQQ-GDH), thiphene polymer (ThP), thiolated carbon nanotubes (CNTs-SH), gold electrode (Au), NAD-dependent glucose dehydrogenase (NAD-GDH), poly-methylene green (pMG), 3D-carbon network (3DC), pyrocatechol violet (Pcv), graphite pencil electrode (GPE).

Electrode Platform	Linear Range / mM	Sensitivity	Limit of Detection / mM	Stability	Ref
GOx-HRP-PTB/rGO/GCE	0.08-1	0.34 μA mM^{-1}	0.05	50% of the original output retined after 2 weeks	[94]
GOx/PANI:rGO/GR	0.5-50	2.8 μA mM^{-1}	0.089	50% of original output retained after 8 days	[99]
GOx-PPCA(AuNPs)/PPD/GR	0.2-150	0.135 μA mM^{-1}	0.08	96% of original signal after 14 days	[100]
Chit-GOx/PB/Au-SEBS	< 0.5	11.7 μA mM^{-1} cm^{-2}	0.006	8 days	[104]
GOx/PB/rGO/SPCE	< 3 4-8	27.78 μA mM^{-1} cm^{-2}	0.00794	15 days	[106]
Naf/GOx/NiHx/PB/SPCE	2.5-15	109.7 nA mM^{-1}	/	14 h of continuous operation	[107]

Chit/GOx/TiO_2NTs/Ti	0.3-1.5	5.46 µA mM^{-1}	0.07	85% of initial current after 30 days	[108]
GOx/PB/Carbon	0.25-2	2.14 µA mM^{-1}	0.01	72% of initial current after 45 days	[109]
GOx/PAA/PEDOT	0.96-30	2.74 x 10^{-4} A M^{-1}	0.29	30 days	[110]
PQQ-GDH/ThP/CNTs-SH/Au (acqueous electropolymerisation)	0.05-0.5	246 µA cm^{-2} mM^{-1}	0.001	70% of initial signal retained after 15 days	[117]
NAD-GDH/pMG/3DC	0.5-20	50,8·10^{-3} A mM^{-1} cm^{-2}	/	1 year	[122]
NAD-GDH/Pcv/GPE	0.05-0.5	2.78 nA μM^{-1} cm^{-2}	1.2	/	[123]

Table 2. Analytical figures of merit for reviewed fructose biosensors. Abbreviations: fructose dehydrogenase (FDH), carbon nanotubes (CNTs), liquid cubic phase (LCP), thermally reduced graphene oxide (TRGO), 3-mercaptopropionic acid (3-MPA), nanoporous gold (NPG), 2-carboxy-6-naphthoyl (CNPh), 4-mercaptophenol (4-MPh), highly porous gold (h-PG), 2-aminoanthracene (2-ANT), single walled carbon nanotubes (SWCNTs), glassy carbon electrode (GCE).

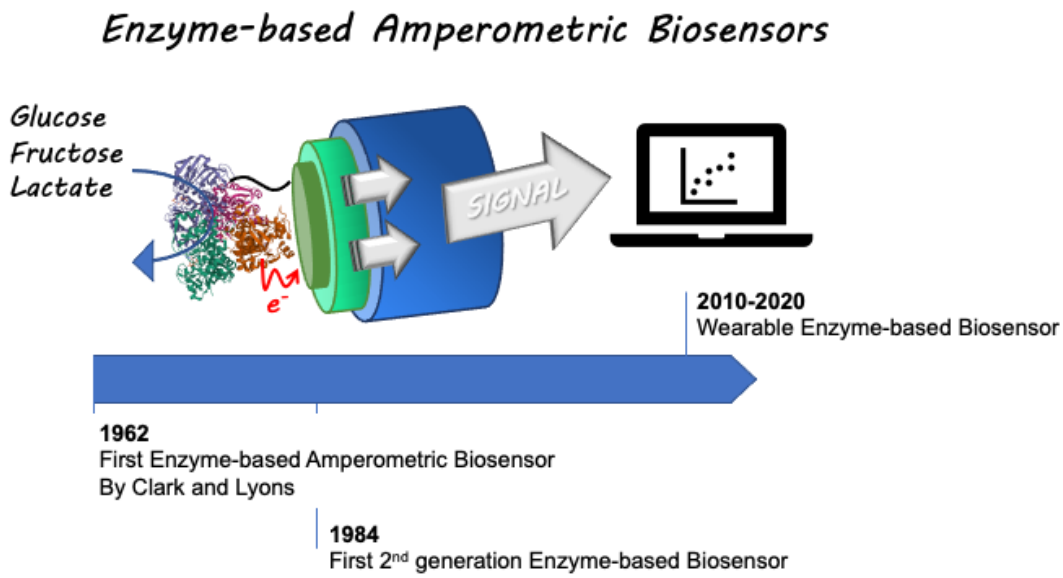
Electrode Platform	Linear Range / mM	Sensitivity	Limit of Detection / mM	Stability	Ref
FDH/LCP/CNTs/Carbon	< 10	4μA mM^{-1}	/	/	[138]
FDH/TRGO/Carbon	0.7-8.8	14.5 μA mM^{-1} cm^{-2}	0.7	10% decrease after 5 days	[139]
FDH/3-MPA/NPG	0.05-0.3	3.7 μA μM^{-1} cm^{-2}	0.0012	25% retained after 6 days	[142]
FDH/CNPh/NPG	0.05-0.3	3.7 μA μM^{-1} cm^{-2}	0.0012	40% retained after 6 days	[142]
FDH/4-MPh/h-PG	0.05-5	175 μA mM^{-1} cm^{-2}	0.0003	90% of original output retained after 90 days	[143]
FDH/2-ANT/SWCNTs/GCE	0.05-5	47 μA mM^{-1} cm^{-2}	0.0009	90% of original output retained after 60 days	[146]

Table 3. Analytical figures of merit for reviewed lactate biosensors. Abbreviations: lactate oxidase (LOx), copper based molecular organic framework (Cu-MOF), platinum electrodeposition (PtE), chitosan (Chit), screen-printed carbon electrode (SPCE), gold nanoparticles (AuNPs), carbon nanotubes (CNTs), reduced graphene oxide (rGO), graphite (G), Poly(3,4-ethylenedioxythiophene) (PEDOT), Prussian Blue (PB), platinum electrode (Pt), muti-walled carbon nanotubes (MWCNTs), methylene blue (MB), gold microparticles (AuMPs), laser-irradiated graphene(LIG), graphene nanoribbons (GNR), 4-aminobenzoic aid (BzA), glassy carbon electrode (GCE), platinum nanoparticles (PtNP), electrochemically reduced graphene oxide (ERGO), poly(allylamine hydrochloride) (PAH), screen-printed electrode (SPE), polyurethane (PU),nanoporous gold (NPG), carbon fibre microelectrodes (CFM), polyaniline (PANI).

Electrode Platform	Linear Range / mM	Sensitivity	Limit of Detection / µM	Stability	Ref
LOx-Cu-MOF/Chit/PtE/SPCE	0.00075-1 4-50	14.65 µA M^{-1} 0.207 µA M^{-1}	0.75 /	50 days	[165]
LOx/AuNPs/CNTs/rGO/Pt	0.05-100	35.3 µA mM^{-1} cm^{-2}	2.3	1 month	[166]
L-LDH/AuNP/ERGO/PAH/SPE	0.5-3 4-16	1.08 µA mM^{-1} cm^{-2} 0.28 µA mM^{-1} cm^{-2}	1 /	75% of initial output after 7 weeks	[167]
LOx/PB/PEDOT/LIG	< 18	2.23 µA mM^{-1}	/	/	[168]
LOx/Bza/GNR/GCE	0.035-28	5.5 µA mM^{-1}	11	30 days	[169]
LOx/PB-RGO-PU/G	< 10	3.58 µA mM^{-1}	400	/	[170]
LOx/PtNP-NPG/CFM	< 1	0.75 nA mM^{-1}	13	95% of initial output after 5 days	[171]

LOx/Chit/Pt/LSG	0.2-3	35.8 µA mM^{-1} cm^{-2}	110	/	[172]
LOx/Pedot/SPCE	0.25-1 1-40	43.42 µA mM^{-1} cm^{-2} 0.32 µA mM^{-1} cm^{-2}	83	30 days	[173]
LOx/SPCE	< 1.5	21.8 µA mM^{-1} cm^{-2}	/	/	[174]
LOx/Pt/PANI/MXene	0.005-5	0.62 µA mM^{-1}	5	30 days	[175]
LOx/MB/MWCNTs-AuMPs/Au microneedles	0.01-0.2	1473 µA mM^{-1} cm^{-2}	2.4	Retained 90% of initial response up to 30 days	[180]

Graphical Abstract



After 60 years, enzyme-based amperometric biosensors still play a key role in a variety of fields, evolving from classical modified electrodes till flexible, *in situ* and wearable technologies.