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Electrochemical biosensors and nanobiosensors

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Electrochemical techniques have great promise for low-cost miniaturised easy-to-use portable devices for a wide range of applications – in particular, medical diagnosis and environmental monitoring. Different techniques can be used for biosensing, with amperometric devices taking the central role due to their widespread application in glucose monitoring. In fact, glucose biosensing takes an approximately 70 % share of the biosensor market due to the need for diabetic patients to monitor their sugar levels several times a day, making it an appealing commercial market.

In this review, we present the basic principles of electrochemical biosensor devices. A description of the different generations of glucose sensors is used to describe in some detail the operation of amperometric sensors and how the introduction of mediators can enhance the performance of the sensors. Electrochemical impedance spectroscopy is a technique being increasingly used in devices due to its ability to detect variations in resistance and capacitance upon binding events. Novel advances in electrochemical sensors, due to the use of nanomaterials such as carbon nanotubes and graphene, are presented as well as future directions that the field is taking.

Introduction

Electrochemical sensors operate by reacting with the analyte of interest to produce an electrical signal proportional to the analyte concentration. A typical electrochemical sensor consists of a sensing electrode (working electrode) and a reference electrode separated by an electrolyte. For most applications, a three-electrode system is used with the reference connected to a high-input-impedance potentiostat and a counterelectrode is used to complete the circuit for current flow. A range of electrochemical techniques can be used for biosensing applications, namely potentiometric (measuring variations in open circuit potential, of which biologically sensitive field-effect transistors is a special type and discussed in Chapter 9), amperometric (measuring currents due to the reduction or oxidation of electroactive species) and impedimetric sensors (measuring the impedance of the system upon immobilisation of biolayers at the electrode surface). Other electrochemical techniques can be used for biosensing, although their application is not as important.

One of the key advantages of electrochemical biosensors relies on their relative simplicity. Inexpensive electrodes can be easily integrated with simple electronics to perform rapid measurements in miniaturised easy-to-use portable systems. The ability to determine the concentration of an analyte within a complex sample at the point-of-care and in near real time is extremely attractive for medical diagnosis, monitoring of existing conditions and environmental monitoring. Amperometric biosensors in particular have been widely used for the monitoring of glucose levels by people with diabetes, where a test can be made within minutes using a small droplet of blood extracted by pricking a finger with a small needle. The use of nanomaterials such as carbon nanotubes and graphene on electrochemical biosensors is lowering the limit of detection (LOD) to unparalleled levels, opening the door to new and exciting biosensing applications.

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Amperometric biosensors

Amperometric biosensors are a class of electrochemical biosensors that transduce the biological recognition events caused by electroactive species at the sensing surface into a current signal for the quantification of an analyte within a sample matrix. The intrinsic simplicity of the transducer lends itself to low-cost portable devices for applications ranging from disease diagnosis to environmental monitoring.

An amperometric transducer is used to study the charge transfer between the interfaces of phases, for example, between two electrodes separated by an electrolyte. Often the term *electrochemical cell* is used to describe the system of phases and interfacial boundaries. One of the half-cell reactions within the electrochemical cell is carefully controlled in order to study the changes in charge transfer at the interface of the other half-cell reaction, usually called the working electrode.

By controlling a fixed or varying potential across the electrochemical cell, an overpotential can be formed, which is the difference between the applied potential and the cell equilibrium potential. On formation of the overpotential, electron transfer becomes thermodynamically viable and oxidative or reductive reactions will ensue. These processes are termed *Faradaic* processes as they obey Faraday's law. Other processes (such as the development of an adlayer) that change the interfacial surface but do not cause charge transfer across the interfacial boundary are termed *non-Faradaic* processes.

The Faradaic current i, is determined by the number of electrons involved in the reaction, n, the Faraday constant, F, the electrode area, A, and the flux of the analyte at the interfacial boundary, j: $i = nFAj$. The flux is of primary concern and describes the rate of the reaction; consisting of the electron transfer heterogeneous rate constant, k_0, which describes the electron transfer kinetics, and the concentration of analyte at the electrode/electrolyte interface, c_0, which is dependent on the mass transport of the analyte to the interface: $j = k_0 c_0$. It is this dependence on the analyte concentration that allows the current to be correlated to the concentration of analyte within the sample matrix for use in biosensing applications. By sweeping the potential, the oxidation and reduction currents can be measured and these can be correlated to the concentration of electroactive species.

An important point to mention is that the slowest process within the system will become the overall reaction rate-determining process. Awareness of factors that detrimentally affect these processes is important should one wish to devise strategies to mitigate them in order to improve the overall biosensor performance. In general, the factors that influence the reaction rate include:

- concentration of the analyte and other species within the matrix *and* at the interfacial boundary
- mass transport (diffusion, convection and migration) of species from bulk solution to the interfacial boundary
- electron transfer across the interfacial boundary
- other chemical reactions occurring within the sample matrix
- other electrode interactions (adsorption, electrodeposition, etc.)
- external factors (temperature, pressure, etc.).

An abundance of literature covering these processes in detail are available (e.g. [1–3]). Different amperometric methods can be used in biosensors: e.g. cyclic voltammetry, differential pulse voltammetry or square wave voltammetry – the latter two tend to be used in most commercial products (glucose being the most common) as they are sensitive only to the Faradaic processes of interest.

Glucose – a model system

A prime example of a commercially successful amperometric biosensor is that of glucose detection for the monitoring of diabetes. First introduced by Clark and Lyons in 1962 [4], the concept has seen significant advances and improvements over the decades [5–7]. Diabetes patients can now accurately self-monitor their blood glucose levels using low-cost handheld devices with rapid analysis times [8].

Given its prevalence, we shall use glucose detection as a model system to explore some of the different architectures of biorecognition layers that can be employed for the enzymatic amperometric determination of glucose. It is quite common for amperometric biosensors to utilise an enzyme or a sequence of enzymes to catalyse the reaction to improve performance. Glucose detection is no different and the enzyme glucose oxidase (GOx) is often used for its high selectivity to its substrate, high catalytic performance, stability and low cost [9].

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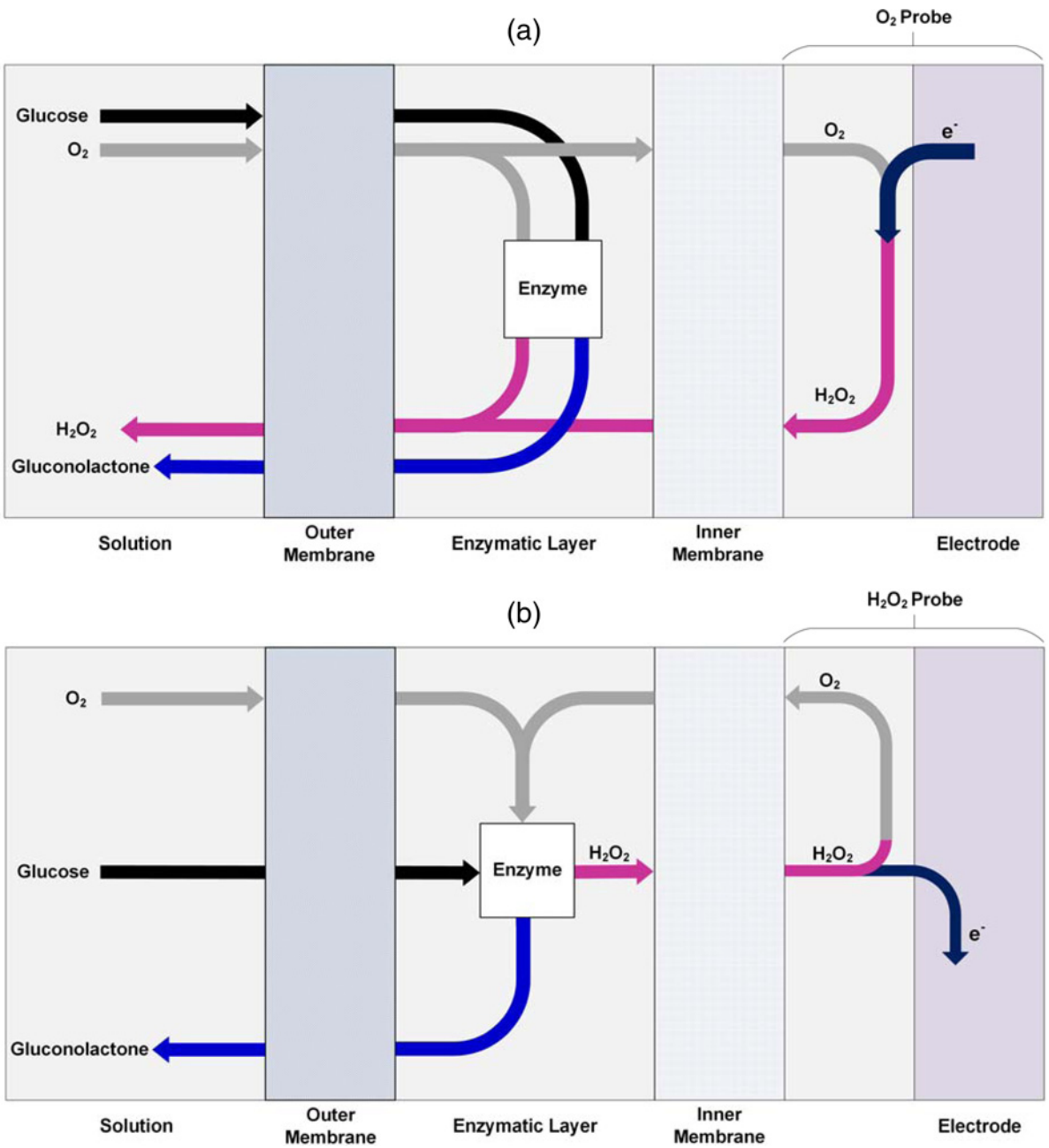

Figure 1. Diagrams of oxygen-linked (a) and hydrogen peroxide-linked (b) first-generation amperometric biosensors for glucose detection

Starting with the simplest architecture, first-generation biosensors (Figure 1) rely on measuring the depletion of substrate (S) or yield of product (P) during the reaction, catalysed by an enzyme (E) such as GOx [4,10,11]:

$$S + O_2 + 2H^+ \xrightarrow{E} P + H_2O_2 \quad (1)$$

In this example, a major issue with monitoring the oxygen depletion is that the natural concentration of oxygen in samples can fluctuate. Furthermore, the wide potential window required for hydrogen peroxide oxidation and oxygen reduction overlaps with the redox potentials of background interferents.

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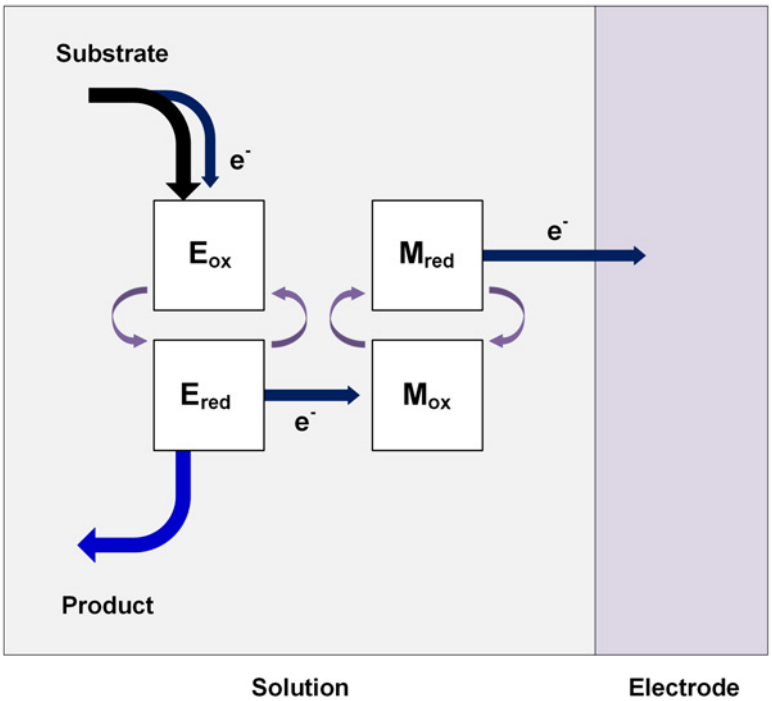

Figure 2. Diagrammatic representation of the architecture of a second-generation amperometric biosensor

In order to overcome the drawbacks associated with first-generation glucose biosensors, Cass et al. [12] demonstrated that a mediator (acting as both a donor and acceptor of electrons to and from the enzyme) could be introduced to improve the electron transfer of the system (Figure 2). This also reduces the necessary potential window of the system, minimising effects from interferents and thus improving the selectivity.

There are several attributes important to selecting a suitable mediator:

- the electron transfer kinetics of the mediator (k_M) should be fast
- mobility within the sample matrix should be high
- it must be electrochemically reversible and stable in both reduced and oxidised states
- it should not be affected by the pH of the sample matrix
- the redox potential should be similar to that of the cofactor(s) of the enzyme
- it should not undergo reactions with interferents within the sample matrix.

Mediators may be freely diffusing, such as ferrocene and phenazine derivatives, quinones and ruthenium complexes [13]. Metal oxides may also be incorporated into carbon pastes or inks. Alternatively, functional groups of the mediator may be used to covalently bond to the electrode, enzyme or within a polymer:

$$S + E_{ox} \overset{k_1, k_{-1}}{\longleftrightarrow} ES \xrightarrow{k_2} P + E_{red}$$

$$E_{red} + M_{ox} \xrightarrow{k_M} E_{ox} + M_{red}$$

$$M_{red} \xrightarrow{E} M_{ox} + ne^-$$

In the final architecture, direct electron transfer between the enzyme and electrode is facilitated by immobilising the enzyme at the electrode surface (Figure 3) [14–16]. Usually a self-assembled monolayer (SAM) is used to perform

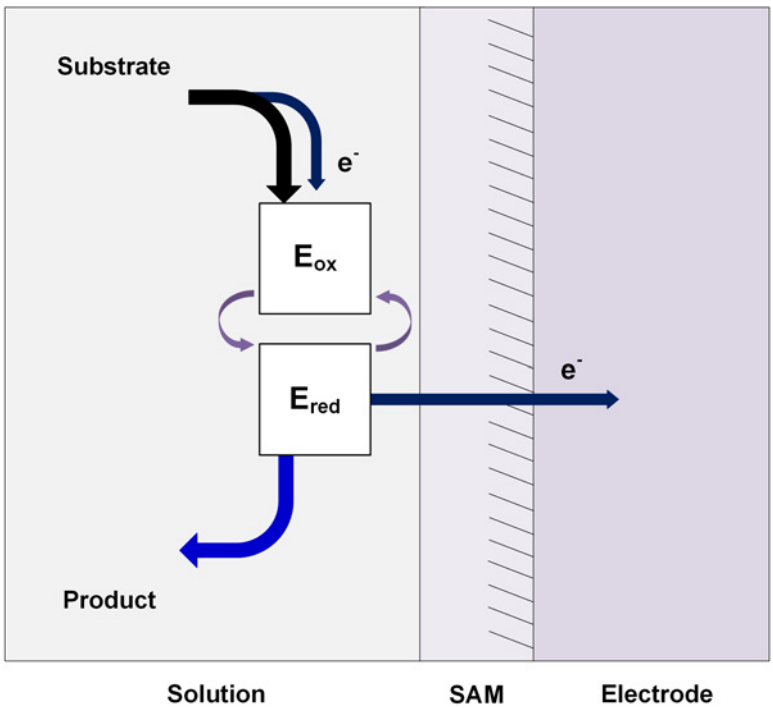

Figure 3. Diagrammatic representation of the architecture of a third-generation amperometric biosensor

this task, allowing controlled spacing and selection of accessible functional groups, permitting the construction of complex biosensor architectures.

In the example of glucose sensing, a conducting polymer, polypyrrole, is used extensively for the immobilisation of GOx [17]. Conducting organic salt electrodes [18] have also been shown to be an efficient strategy in third-generation biosensing, particularly for *in vivo* applications where the low toxicity of the system is appealing.

Impedimetric sensors

The impedance of a generic electrical component is given by dividing the AC potential applied across its terminals by the AC current that flows through it. The impedance is a complex number and, in very simplistic terms, the real part is often linked to resistive processes and the imaginary part to capacitive processes. Electrochemical impedance spectroscopy (EIS) is the most common technique used in impedimetric biosensors, where the impedance is measured over a wide range of AC potential frequencies (typically from 100 kHz to 1 mHz). The frequency-domain response from EIS can provide useful information about the physico-chemical changes that take place when an analyte binds to a bioreceptor immobilised on an electrode. Such information comprises the charge transfer processes from the solution to the electrode surface, solution resistance, as well as diffusion transport of species to and from the bulk solution and double layer capacitance formation [19]. Moreover, the analysis of an EIS experiment allows modelling of the electrochemical double layer with an electrical equivalent circuit, the most used is the so-called Randles circuit (Figure 4). The values of the electrical components are extracted from the equivalent electrical model using least-squares minimisation fitting of the EIS spectrum.

EIS is subdivided in two main categories: Faradaic and non-Faradaic EIS. In the former, redox probes are used in the experiment and the main analysis is focused on charge transfer resistance changes generated by the obstructing presence of the analyte when it binds to the surface. The latter exploits charging currents; redox probes are not used and the analysis is mostly based on the double layer capacitance changes upon target binding. In this respect, capacitive sensors such as interdigitated electrodes (IDEs) have gained particular attention over the last few years.

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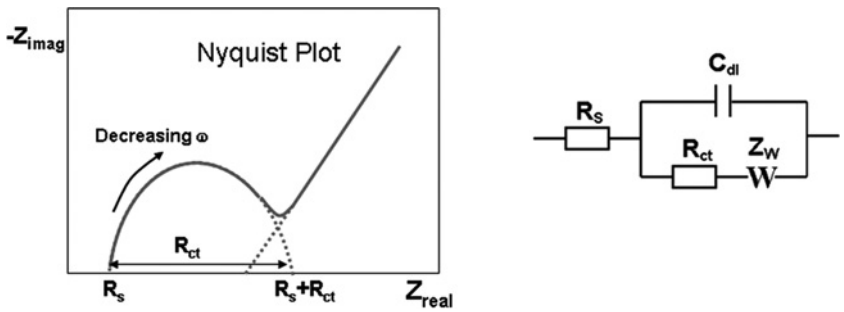

Figure 4. EIS Nyquist plot (Z_{imag} against Z_{real}) and Randles circuit (W is a so-called Warburg element, which accounts for diffusion processes)

EIS has been intensively studied for many years as a characterisation technique, e.g. to confirm the layer-by-layer fabrication processes on to a sensor surface [20]. This could be achieved as standard EIS does not require the addition of any label molecules. Furthermore, as a label-free technique, EIS can monitor the binding affinity in real time. However, the lack of labelling processes caused loss in sensitivity and poor performance of EIS when using real matrixes, such as blood. Nonetheless, EIS has gained increasing popularity in recent years and the above-mentioned advantages, along with improved binding strategies and surface optimisation [21,22], allowed EIS to be used for accurate and sensitive biosensing. As a result, the number of EIS applications in biosensing has rapidly increased over the last decade, making EIS one of the most promising electrochemical techniques. Recent studies reported protein detection down to attomolar (aM) concentrations [23]. EIS-based sensors have been reported for countless applications such as the detection of cancer and other disease biomarkers, bacteria, polluting agents, water contamination and toxins [24]. Furthermore, EIS can be integrated into multimodal detection systems for improved confidence levels [25].

Chronocoulometric sensors

Chronocoulometry refers to the measurement of the charge of electroactive species adsorbed on to an electrode with respect to time. One field of investigation where chronocoulometry sensors are widely used is the quantification of nucleotidic molecules. For instance, the negative charge of the phosphate backbone of deoxyribonucleic acid (DNA) strands can be quantified by measuring the amount of diffusing current originated by a positively charged redox probe, such as $Ru(NH_3)_6^{3+}$, which is needed to counterbalance the DNA charge [21,26]. Diffusion-limited currents are generated by applying controlled potential steps that induce the oxidation of the redox species. In a more general context, chronocoulometry is also used for the determination of diffusion coefficients and for understanding adsorption kinetics.

Carbon-nanotube-based electrochemical biosensors

Carbon nanotubes are formed by sheets consisting of one (single-walled carbon nanotubes – SWCNTs) or more (multi-walled carbon nanotubes – MWCNTs) carbon atom(s), which are organised into tubes (concentric tubes in the case of MWCNTs) (Figure 5). Carbon nanotubes present amazing properties originating from the quantum transport through the crystalline structure of their walls. Table 1 summarises some of these properties and shows how the electrons travelling inside the tube follow ballistic conductivity: considering that the maximum mean length of a MWCNT is a few micrometres, a mean free path of about 25 μm for MWCNT at room temperature means that all the electrons pass through the tube without any interaction with the carbon lattice. Carbon nanotubes have been proposed for a plethora of different applications, including but not limited to touch screens [27], solar cells [28], batteries, supercapacitors and transistors [29], super-strong materials for structural composites [30] and to improve biosensors [31,32].

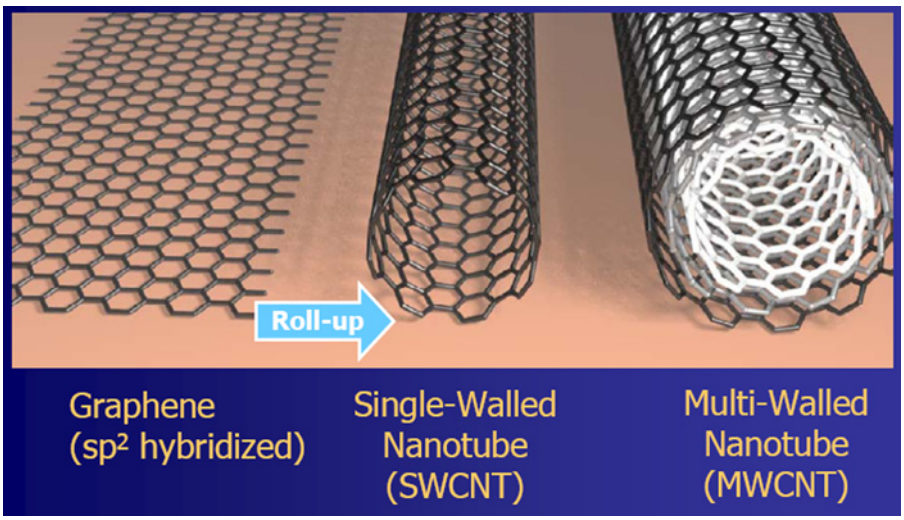

Figure 5. A scheme showing how graphene could be ideally rolled-up to form single- or multi-walled carbon nanotubes (courtesy: K. Banerjee, California University)

Table 1. Some of the transport properties of carbon nanotubes (and comparison with Cu)

	Cu	SWCNT	MWCNT
Maximum current density (A/cm^2)	$<1\times10^7$	$>1\times10^9$	$>1\times10^9$
Thermal conductivity (W/mK)	385	5800	3000
Mean free path at room temperature (nm)	40	>1000	25 000

Carbon nanotubes have been largely used in biosensing since 2000 and MWCNTs can enhance several biosensor features. The easiest to be considered is the increase in electroactive surface area due to nano-structuring of the working electrode. This results in the appearance of thin-layer phenomena [33] that also typically provide a huge increase in the so-called layering effects [31], leading to an increase in the acquired Faradic currents emerging from any redox reaction occurring at the surface of the carbon nanotubes (Figure 6). More often, this increase in the peak current is related to a shift of its Nernst potential as observed in many cases, and this shift in potential is extremely useful, in some cases, to avoid interference with other compounds (e.g. uric and ascorbic acids) when monitoring human fluids [34]. Of course, the provided increase in terms of current collected by redox reactions immediately results in huge improvements of the two main features of electrochemical biosensors: an increase in the sensitivity and a related decrease in the LOD.

For all of these reasons, MWCNTs have been extensively reported to increase the biosensor performance for the detection of many endogenous human molecules including but not limited to glucose [32], lactate [36] and cholesterol [35], and for exogenous human molecules including but not limited to anti-cancer agents [37] and anti-inflammatory compounds [38].

Graphene-based electrochemical biosensors

Graphene is a one-atom thick silk-like sheet made of ordinary carbon which has exceptional properties originating from quantum physics; graphene has been used in a diverse range of fields including touch screens, solar cells, (bio)batteries, transistors, super-strong materials applied in the construction of aeroplanes, cars and satellites, and

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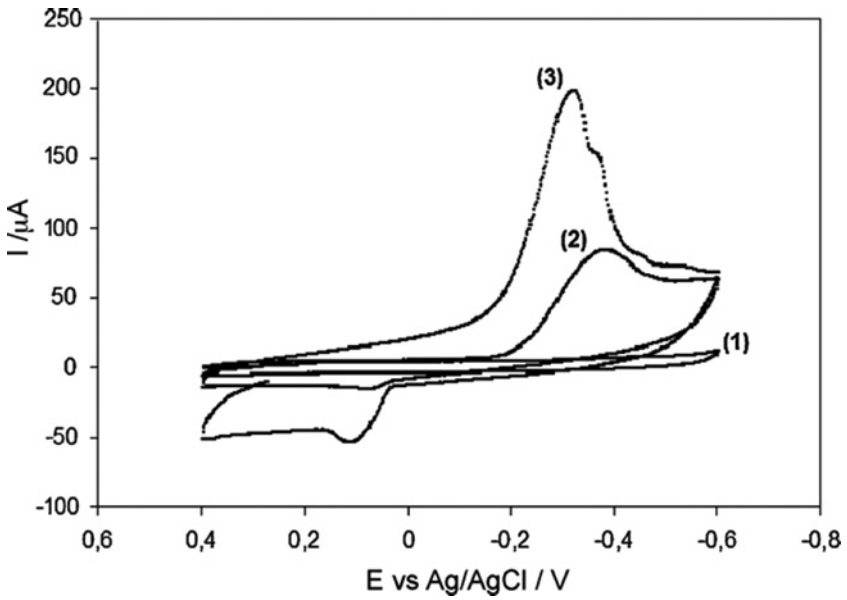

Figure 6. Cyclic voltammograms acquired on screen-printed rhodium–graphite electrodes modified with a metalloprotein: standard electrode (1), modified with gold nanoparticles (2), modified with MWCNTs (3) (reprinted from [35] with permission from Elsevier)

for the construction of biosensors [39]. Graphene properties were first studied in 2004 by Geim and Novoselov (and colleagues) [40] and in 2010 both received the Nobel Prize in Physics for this discovery. Their approach for obtaining graphene flakes is quite interesting – they used graphite, which is found in ordinary pencils, and by peeling off carbon flakes layer-by-layer, using Scotch tape, they finally ended up with a one-atom thick layer of carbon. This was achieved at a time when it was believed that such a thin flake was not stable.

Highly pure graphene sheets needed for special applications are prepared by mechanical cleavage or by chemical deposition techniques, which are quite expensive. A cost-effective way of producing graphene materials is to start with 'graphite oxide' prepared by the oxidation of graphite with strong mineral acids and the subsequent exfoliation of graphene oxide (GO) flakes (Figure 7). GO has a high density of oxygen-containing functional groups and is not very conductive due to disrupted conjugated $\pi-\pi$ bonds; conductivity can be restored by reduction, performed chemically, thermally or electrochemically, and such material is termed reduced graphene oxide (RGO). Although graphene sheets, by definition, should not contain any oxygen, it can reach up to 30 % in GO; however, the amount of oxygen can be decreased to approximately 5–10 % in RGO via reduction [41]. This set of features allowed the development of electrode interfaces capable of hosting high amounts of bioreceptors thereby enhancing the sensitivity of biosensor devices. The lower conductivity of GO compared with graphene can be applied in devices based on impedimetric or field-effect sensing transducing schemes. Carboxyl and other oxygen-containing moieties of GO or RGO can also be used for the covalent attachment of biorecognition molecules either to modify the biosensor surface or to prepare graphene-based bioconjugates for sandwich assay formats.

Graphene has been applied to a wide range of biosensors and, in particular, for affinity-based biosensors (i.e. immunosensors or DNA sensors) for the analysis of high-molecular-mass analytes, such as DNA or proteins. For example, an electrode modified by RGO could detect DNA down to 5 fM, whereas an electrode modified by vertically aligned nanowalls from RGO with a favourable orientation of RGO towards the oxidation of DNA bases could detect the same analyte down to 9 zM (∼5 DNA molecules in 1 ml) [43]. Antibodies and DNA aptamer-based sensors have also been achieved with LODs in the order of attomolar levels.

Graphene-based materials with high surface area and numerous functionalities allow the immobilisation of antibodies and enzymes, which can dramatically enhance the electrochemical read-out by signal amplification. Since GO or RGO is much cheaper compared with other nanomaterials such a strategy can result in the cost-effective preparation of ultrasensitive affinity-based electrochemical biosensors [41].

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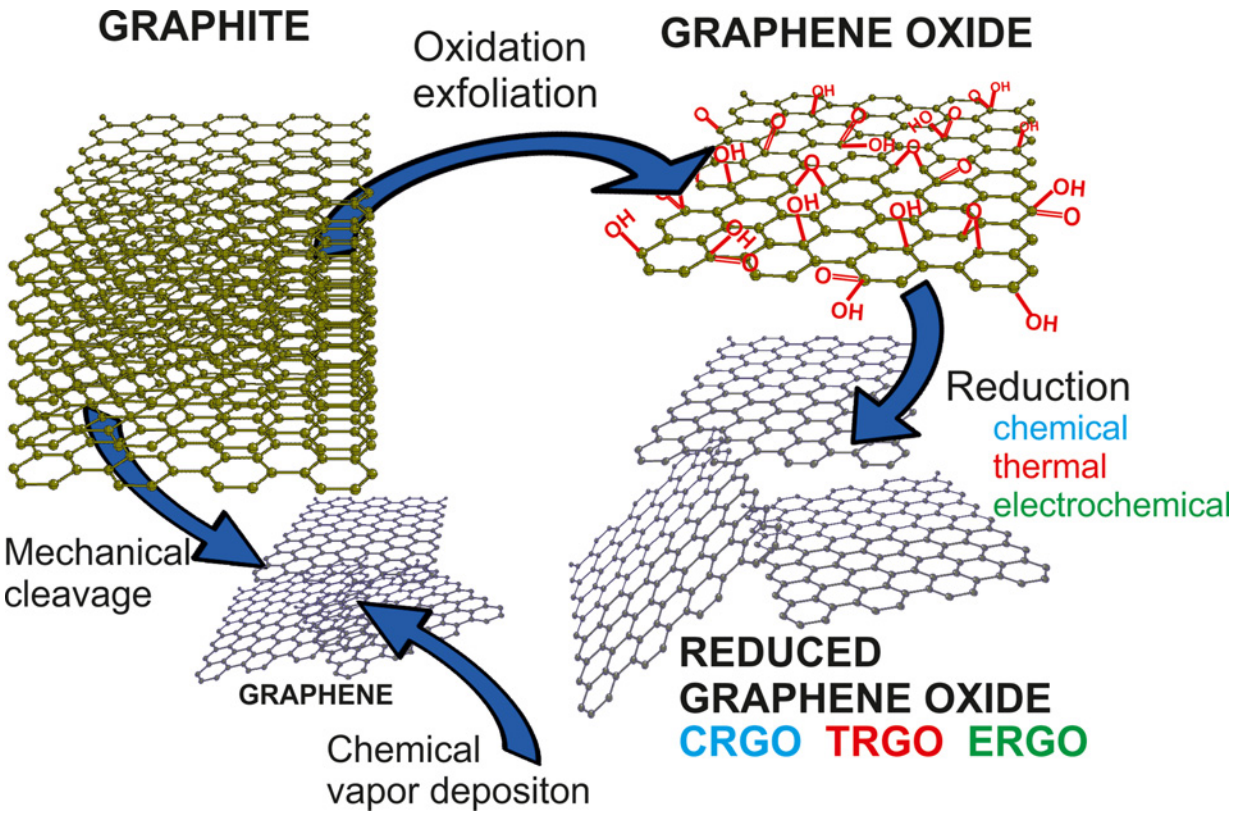

Figure 7. A scheme showing the various ways graphene and graphene-based material can be prepared

CRGO, chemically reduced graphene oxide; TRGO, thermally reduced graphene oxide; ERGO, electrochemically reduced graphene oxide (reprinted from [42] with permission from Elsevier)

Conclusions

Continued work with the plethora of new materials, such as boron-doped diamond (BDD) [44], that offer improvements in solvent and potential ranges, reduced background currents and antifouling properties, will open up new branches of research within electrochemistry. Further development of nanomaterials and the optimisation of fabrication processes should yield improvements in sensitivity and selectivity and the utilisation of other quantum effects [45]. Coupling these systems with micro- and nano-fluidics for sample preparation, processing and introduction will make them more attractive for use in biosensing where reduced sample volumes are desired [46]. As these fields of nanotechnology mature, relatively new techniques such as redox cycling in nanogaps [47] and nano-impact detection [48] may become more established.

The push from industry has seen the cost of microelectronics reach the point where smartphones are now ubiquitous within our culture. These devices offer exciting opportunities to exploit the powerful processing capabilities to be used in conjunction with low-cost point-of-care biosensors.

Although screen-printed electrodes have already been widely adopted in the mass production of low-cost disposable biosensing, further research on surface modification, incorporation of biomaterials and elaborate geometries will probably see their applications broaden. With appropriate validation, the increasing affordability of computing performance has improved the popularity of computational modelling as a tool to increase understanding of the biosensor mechanisms and streamline the optimisation of biosensor design.

The main advantages of the application of nanomaterial-modified electrodes for the construction of biosensors compared with planar electrodes can be summarised as follows:

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- higher surface area allowing the immobilisation of a larger density of biomolecules [49]
- better accessibility (lower diffusion limitations) of analyte molecules to reach immobilised biomolecules [23]
- direct electronic wiring of redox enzymes allowing direct electron transfer between the modified electrode and active site of the enzyme making such enzymatic biosensors more selective [50]
- enhanced catalytic action towards enzymatic by-products (hydrogen peroxide and reduced nicotinamide–adenine dinucleotide being an enzyme cofactor) represented by higher current density and/or analysis at lower overpotential [49,51]
- application of nanomaterials for enhanced loading of secondary biorecognition elements to make a sandwich configuration [41].

The main application niche for electrochemical biosensors is for the analysis of low-molecular-mass analytes indicating the physiological status of the body, such as glucose, lactate and cholesterol, using enzymes (redox enzymes and hydrolases) [52] or high-molecular-mass analytes, such as nucleic acids, using DNA/RNA biosensors or for detecting various proteins. DNA/RNA biosensors could be applied for the analysis of various cancer genes (i.e. bBRCA1, breast cancer gene 1), mRNA (messenger RNA), for the expression of various proteins (i.e. p53, a tumour suppression protein) and microRNAs, which are post-transcriptional regulators of gene expression [53]. The main protein analytes detected by biosensors are the biomarkers of various diseases such as troponin (cardiac disease), glycated haemoglobin (diabetes) and various glycoproteins, such as a prostate-specific antigen, which are cancer biomarkers [54].

Summary

- Electrochemical biosensors are some of the most used biosensors in the market, mainly due to glucose monitoring.
- Electrochemical biosensors are easily miniaturised, inherently inexpensive and require simple electronics for conditioning and read-out, making them ideal for point-of-care applications.
- Amperometric biosensors measure currents due to electroactive species, often using mediators to enhance electron transfer.
- Electrochemical impedance spectroscopy-based biosensors are some of the most promising electrochemical sensors for systems with well-defined charges, such as DNA.
- Electrochemical nanobiosensors with extremely low limits of detection are nowadays being developed thanks to the extraordinary properties of nanomaterials such as carbon nanotubes and graphene.

Abbreviations

EIS, electrochemical impedance spectroscopy; GO, graphene oxide; GOx, glucose oxidase; LOD, limit of detection; MWCNT, multi-walled carbon nanotube; RGO, reduced graphene oxide; SWCNT, single-walled carbon nanotube.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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