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Bioelectrocatalytic systems for health applications

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Abstract

We present a brief overview of bioelectrocatalytic devices for in vitro health applications, including food safety and environmental analysis, focusing on microelectrode- and microfluidic-based biosensors, paper-based point-of-care devices and wearable biosensors. The main hurdles and future perspectives are discussed. We then consider the role of electron transfer between a biocatalyst and an electrode in biosensor design. Brief descriptions of indirect, direct and mediated mechanisms are given. The principal strategies, as well as recent developments for modulation of electron transfer in biocatalytic systems are summarised. In conclusion, we highlight some of the challenges associated with improving these redox systems.

Keywords: direct electron transfer, mediated electron transfer, immobilisation, microbiosensor, nanobiosensor, paper-based biosensor, wearable biosensor, self-powered biosensor.

1. Introduction

Bioelectrocatalytic systems are based on biological entities which catalyse electrochemical processes that concern the interaction between chemical change and electrical energy. Biocatalytic devices incorporate either enzymes, whole cells, parts of cells or tissues as a catalytic element. Such systems can be used for various purposes, such as power generation, bioremediation, chemical synthesis and biosensing. In this review, we focus on biosensing applications of biocatalytic systems in health monitoring, food safety and environmental analysis and mention the application of biocatalytic systems as power supplies for biosensing devices. In biosensors, the biocatalyst is the biorecognition element that recognises the target analyte by chemical interaction and transforms this information into an electrically detectable signal.

Although various biorecognition elements have been used for biosensor construction, enzymes are the oldest (Clark and Lyons 1962) and still most commonly used biorecognition element in biosensing. Of particular interest for electrochemical biosensing is one specific class of enzymes, the oxidoreductases, which catalyse oxidation-reduction reactions along with the subclass, oxidases, which catalyse redox reactions involving molecular oxygen as the electron acceptor. Enzymes are characterised by high turnover rates and high, often specific, selectivity for a desired analyte. These properties, together with the simplicity of enzymatic biorecognition and their relatively low cost, make enzymatic bioelectrocatalytic systems almost indispensable for analysis in complex matrixes, such as biological fluids and environmental samples. Hence, they are extremely important in health applications. Given the prevalence of diabetes and the excellent catalytic properties of glucose oxidising enzymes, it is not surprising that blood glucose biosensors comprise around 85% of the word market for biosensors (Turner 2013). Enzymes, however, also have several disadvantages, such as complex production and purification processes, limited lifetime and difficulties in multianalyte analysis due to the high enzyme specificity.

Microbial cells represent an alternative biocatalyst for biosensing. The catalysis in this case is implemented by enzymes contained within the cell. Whole− cell bioelectrocatalysis was first

reported in 1911 (Potter 1911), but it was applied for biosensing only in 1975 by Divies (Divies 1975). This biosensor was based on the use of *Acetobacter xylinum* with an oxygen electrode. The development of microbial biosensors represents a logical extension of the enzyme electrode concept, since the signal generation is analogous to those systems and is based on enzymatic biocatalytic reactions. In essence, biocatalytic systems using microbial cells, part of cells or tissues, can be considered as a 'bag of enzymes' (Ikeda et al. 1996). Use of whole cells can overcome some of the above mentioned disadvantages associated with enzymatic bioelectrocatalysis. Application of whole cells does not require enzyme purification, supports better stability of biocatalytic systems and permits sensing of multiple analytes using a single biocatalytic element. However, such systems also suffer from a number of limitations compared to enzymes, such as the requirement for nutrient and energy supplies to support living cells, slower rates of signal generation and lack of specificity. The last point can be regarded both as an advantage and a disadvantage of microbial biocatalysis, depending on the nature of the application. For example, use of whole cells is advantageous when a class of analytes needs to be monitored, such as marine toxins (Wang et al. 2015) or heavy metals (Chouteau et al. 2005). For detection of individual analytes, such as glucose (Noiphung et al. 2013) or lactate (Kim et al. 2014) in complex matrices, application of enzymes is undoubtedly more effective.

Bioelectroatalytic systems are dependent on electrochemical contact between the biocatalyst and an electrode. Enzymatic bioelectrocatalysis is possible indirectly via electroactive intermediates in the reaction, via direct electron transfer (DET) between the active site of the biocatalyst and the electrode, or via mediated electron transfer (MET), where small molecules that are artificially introduced into the system, shuttle electrons between the enzyme and the electrode. Whole cells establish electron transfer with electrodes using similar mechanisms. DET is accomplished via cytochromes located in the cell outer membrane (Lovley 2008) or by specific biological nanostructures (Reguera et al. 2005). Biosynthetic redox mediators, such as flavins, phenazines and quiones (Freguia et al. 2012) or chemical exogenous mediators (Schröder 2007) are used for

MET. Due to the direct influence of the electrochemical contact between the biological and physical part of the sensor on its analytical performance, this issue should be given special attention when designing new biocatalytic systems for biosensing applications. Therefore, in the second part of this review, we will summarise some well-known methodologies to create efficient electron transfer in biocatalytic systems as well as the key recent findings in this area, focusing mainly on examples of enzymatic bioelectrocatalysis.

Biosensors for health applications

Biosensors have achieved considerable success in both the commercial (US$ 13 billion annual turnover) and academic arenas and the need for new, easy-to-use, home and decentralised diagnostics is now greater than ever. It is rapidly becoming apparent that such sensors can contribute substantially to reducing healthcare costs and to enhancing the quality of life for our citizens. Healthcare spending is growing unsustainably and according to the WHO, has already exceeded 18% of GDP in the USA and 9.5% of GDP in Europe. Maintaining a healthy and sustainable environment is also top of the political agenda and food safety and personal security are key concerns. New thinking is crucial to finding effective solutions that deliver the high quality of life rightly demanded by our ever ageing population while leveraging technology to deliver this in a cost-effective manner. Several key drivers are coming together to form a "perfect storm" that may just finally catalyse change to our 2,500 year-old model of healthcare delivery and health maintenance. Personalised medicine recognises that every individual is different and needs a tailor-made health package; these differences can only be identified with an appropriate suite of diagnostics. Individuals are increasingly recognising that data about their bodies should be owned by them and that they should have the choice to use and supplement this information. This generates consumer choice and drives evidence-based payment, where regimens and treatments are paid for on the basis of successful outcomes, which consequently need to be measured. Focus on the individual and their needs drives decentralisation and the possible radical restructuring of how we deliver health management both nationally and

internationally. We already see “health rooms” in pharmacies, but the next step will be health rooms in your home, in your pocket or on your wrist. These advances are underpinned by technologies facilitating mobility and data processing. At the core of all this, however, is rapid, convenient and easy ways to measure our body chemistries at the genomic, proteomic and metabolomic levels and the body’s associated interaction with the environment and the food we eat. Next generation diagnostics fabrication is targeting fully-integrated platforms such as the all-printed biosensing systems, integrated sampling and wearable devices. Further development will result in cost reduction and a diversity of formats such as point-of-care tests, smart packaging, telemetric strips and print-on-demand analytical devices. Realisation of these paradigm-changing scenarios requires new business models, the effective harnessing of emerging technology, inspired vision from clinical partners or others “users”, and leading-edge engineering and design, to produce functional systems in appropriate volumes at the right cost to meet society’s needs. Bioelectrocatalytic systems will play a key role in this future. In the following section, we will discuss some key publications appearing in the last decade which in the opinion of the authors indicate trends in the development and main achievements in biocatalytic systems for health monitoring.

2. Trends in biosensing technology

2.1 Miniaturisation

Miniaturisation is one of the most prominent trends in biosensor technology and provides a series of advantages, such as mass production at low cost, easy storage and distribution, improved analytical performance, the possibility of fabricating multi-analyte biosensors and facilitating the analysis of small volumes, which is especially crucial for biomedical applications. A variety of miniaturised biosensing systems have been developed, but devices based on electrochemical transduction mechanisms have been the most successful and have the most promising future due to cost-effectiveness, portability and the simplicity of the construction and operation (Eggins 2002; Turner et al. 1987b). An electrochemical biosensing device consists of an electrochemical cell, usually formed by a two- or three-electrode system, with at least one of the electrodes functionalised with a biorecognition element, and an electrochemical detector (Turner et al. 1987b). The electrodes can be easily miniaturised to dimensions in the order of micro or nanometers (Compton et al. 2008; Feeney and Kounaves 2000; Huang et al. 2009). The electrochemical detector and instrumentation required for its control, can also be efficiently downscaled by applying micromachining and microfabrication technologies (del Campo 2014; Wang 2002) and microelectromechanical (MEMS) and nanoelectromechanical (NEMS) systems can be fabricated for biosensing. Another promising possibility for miniaturisation and simplification of the instrumentation required for electrochemical detection is the adaptation of mobile phone platforms for signal detection, processing and analysis (Vashist et al. 2015; Velusamy et al. 2013).

2.1.1. Micro- and nanoelectrode-based biosensors

Scaling the electrode size down to micro dimensions leads to a number of improvements for electroanalysis. The establishment of radial diffusion, rather than the planar diffusion at macroelectrodes, enables higher selectivity, lower detection limits and shorter response times, due to enhanced mass transport at the electrode surface (Compton et al. 2008; Vagin et al. 2014).

Moreover, such electrodes can be used in resistive (unsupported) media without additional pretreatment, and this is particularly useful for analysis of aqueous environmental samples. Micro- and nano-electrodes are widely used for monitoring of neurotransmitters and other important analytes such as glucose, lactate and glutamate *in vivo*, but these sensors for *in vivo* analysis fall outside the scope of our review. Comprehensive reviews on these topics can be found elsewhere (Kotanen et al. 2012; Mirzaei and Sawan 2014; Vaddiraju et al. 2010). For *in vitro* analysis, utilisation of micro- and nanoelectrode arrays rather than single electrodes are more common, since a single electrode produces low currents, which are difficult to detect with conventional, inexpensive electrochemical equipment. Such an array may be formed either as an ordered distribution of electrodes (Arumugam et al. 2009; Joseph et al. 2014; Ng et al. 2015) or by forming ensembles of randomly distributed electrodes (Choudhry et al. 2010; de Souza et al. 2014; Duay et al. 2014; Fletcher and Horne 1999). Microelectrode arrays (MEAs) may consist of individually addressable electrodes (Lin et al. 2008; Taurino et al. 2014) or interconnected electrodes (Arumugam et al. 2009; Ng et al. 2015; Vagin et al. 2014). Fabrication of interconnected electrodes is technically simple, however individually addressable MEAs provide many advantages for biosensing technology, such as high spatial resolution and the possibility of multi-analyte sensing. MEAs allow the integration of biological catalytic systems into lab-on-a-chip devices (LOC), which represent an emerging field for health applications. The theory of and fabrication methods for microelectrode and nanoelectrode arrays have been reviewed in detail, for example, by Compton's group (Compton et al. 2008; Huang et al. 2009; Ordeig et al. 2007) and will be not discussed further here.

The first MEA-based biosensor was reported in 1994, by Ross and co-workers (Ross and Cammann 1994). Different model enzymes such as glucose oxidase (GOx), lactate oxidase (LOx) and choline oxidase were immobilised in a polypyrrole film electrodeposited on a platinum array produced by photolithography. Amperometric responses from all three biosensors were monitored under optimised conditions. Since this first publication, enzymatic MEA

biosensors have been continually developed. Xu et *al*. (Xu et al. 2008) described glucose sensing MEAs using a carbon MEMS microfabrication technique for 3D microstructures involving the pyrolysis of patterned photoresist (Fig. S1A). The technique was utilised for high density and high aspect ratio 3D carbon post-microarray production, which were then further functionalised by electrocopolymerisation of conducting polymer and GOx, as a model enzyme. Numerical simulations based on enzymatic reactions and analyte diffusion kinetics, were used to obtain optimum geometric design rules for the biosensor. In a further work (Lupu et al. 2013), tyrosinase, an enzyme specific to phenolic compounds, was immobilised in an electropolymerised polymer, poly(3,4-ethylenedioxythiophene), on the surface of a gold MEA. The biosensor was used for simultaneous monitoring of dopamine and catechol using a bipotentiostat. This approach resulted in both increased sensitivity and selectivity of the biosensor for environmental analysis. Whole cells are frequently used as a biorecognition elements in bioelectrocatalytic systems based on MEAs. Wang et *al.* (Wang et al. 2015) demonstrated a cardiomyocyte-based potential biosensor for marine toxin monitoring. Cardiomyocytes were grown on a self-assembled monolayer-modified nano-platinum electroplated MEA. The portable wireless 8-channel MEA system was used for noninvasive monitoring of electrical activity of cardiomyocytes in a natural environment, and after addition of increasing concentrations of the marine toxins, saxitoxin and brevetoxin. Signal parameters i.e. spike amplitude, firing rate and 50% of spike-potential duration, extracted from the extracellular field potential signals of the potential biosensor, were analysed to quantitatively evaluate the toxicological risk.

Screen-printing as a standard technology for production of low cost electrochemical devices has also been adapted for the fabrication of miniaturised biosensors. Since the minimum feature size in screen-printing is about 50-100 μm, which is not small enough for efficient fabrication of microelectrodes in the lateral dimension, a strategy of cross-cutting has been used to create microband geometries. Hart's group (Pemberton et al. 2009) fabricated a microband glucose

biosensor for glucose monitoring, by screen-printing a water-based carbon ink containing redox mediator and GOx enzyme, followed by an insulating layer. Then the structure was cut perpendicularly to expose a 20 µm thick and 3 mm-long working electrode edge. The system was shown to be suitable for continuous process-monitoring applications, with the possibility for automated biosensor recovery by cutting, in situations where glucose consumption and perturbation of the system must be minimised. In our group (Vagin et al. 2014), we used a similar approach for microband array fabrication (Fig. 1A). Monolayer microband arrays with different band length and multilayer arrays were produced by screen-printing followed by crosscutting. The fabricated structures were characterised by physical and electrochemical methods and used as a versatile platform for the development of mediated biosensors.

Microband electrode arrays are commonly used for biosensor construction in the form of interdigitated arrays (IDAs). IDAs consist of two pairs of working electrodes, which represent parallel strips of conducting material that are interdigitated and separated by an insulating material (Sanderson and Anderson 1985). The main advantage of IDAs for biosensor technology is lower detection limits compared to MEAs, due to amplification of the current signal resulting from recycling of the oxidation-reduction of the product of the electrocatalytic reaction or mediator that occurs when different potentials are applied to the two electrodes. Pavinatto et *al*. (Pavinatto et al. 2015) reported a gold IDA directly inkjet-printed on a plastic substrate for construction of a flexible and all-printed impedimetric biosensor for polyphenols in wine and olive oil (Fig. 1B). A tyrosinase-containing active layer was deposited on the printed structures using the large-area and high throughput printing method of gravure. Special attention was devoted to optimisation of the printing conditions to allow deposition of electrodes with sub-100 µm features and to the development of an enzyme-containing ink for gravure. IDAs are considered to be the best transducers for conductimetric miniaturised biosensors (Jaffrezic-Renault and Dzyadevych 2008). In work by Chovelon and co-workers (Chouteau et al. 2005), the microalgae *Chlorella vulgaris* was immobilised in bovine serum albumin membranes, cross-

linked with glutaraldehyde vapours and deposited on platinum conductimetric IDAs for heavy metal ions and pesticide detection in water samples. Changes in local conductivity caused by algae alkaline phosphatase and acetylcholinesterase inhibition were monitored.

Conductive boron doped diamond (BDD) MEAs (Fig. S1B) are currently being extensively investigated as interesting transducer candidates for biosensing applications (Hebert et al. 2014; Qureshi et al. 2009). Boron doped diamond possesses unique electrochemical properties, such as extremely low background current, large electrochemical potential window, fouling resistance and chemical inertness (Qureshi et al. 2009). BDD can be deposited as a robust, extremely thin nanocrystalline film on silicon and other microelectronic-compatible substrates. The main obstacles for application of BDD in biosensing technology are its chemical inertness, which prevents efficient and easy bio-functionalisation of the electrode, and the lack of a low-cost, versatile and applicable mass production method to obtain BDD microstructures. The first obstacle was partially resolved by Hamer's group (Yang et al. 2002) and Garrido's group (Hartl et al. 2004) in 2002 and 2004, respectively; they first suggested a photochemical method for nanocrystalline diamond film functionalisation for attachment of short synthetic DNA sequences and, later, green fluorescent protein and catalase. These breakthroughs resulted in the development of a series of bioelectrocatalytic systems for biosensing based on macroscale BDD electrodes (Bai et al. 2014; Zhou et al. 2007). However, to the best of our knowledge BDD MEA based biocatalytic sensors are yet not reported in the literature. We believe further development in diamond thin film synthesis and new chemical approaches for biomolecule immobilisation may lead to the development of an entirely new class of BDD MEA electrochemical biosensors with exceptional sensitivity and stability.

Since the fabrication of micro- and nano-electrodes is currently more costly compared to macrostructures, and due to difficulties associated with the immobilization of the biorecognition element, real-life applications of such systems have so far been limited. However, microelectrode-based biosensors are undoubtedly advantageous in cases where detection of low

concentrations of analytes with high sensitivities are required, e.g. water analysis, cell metabolism analysis or when sample volume is limited, e.g. in tears, saliva, sweat, etc.

2.1.2. Biosensing in microchannels

Integration of bioelectrocatalytic systems with microfluidics for the development of miniaturised lab-on-a-chip devices, is a new emerging field of biosensing technology. Biosensing in microchannels offers numerous advantages for electrochemical analysis, such as improved sensitivity due to: the confinement of the analyte near to the detector in a small volume; increased selectivity for multi-analyte detection due to the possibility to integrate additional separation steps; rapidity due to flow analysis; and significant reduction of reagents and sample volumes. Microfluidic electrochemical biosensing was recently broadly reviewed by Rackus et *al.* (Rackus et al. 2015). Here we will mention some of the most interesting examples of bioelectrocatalytic systems used for biosensing in microchannels, excluding paper-based systems which will be discussed in a later section.

Itoh et *al.* (Itoh et al. 2012) demonstrated a portable, droplet-based microfluidic device for on-site electrochemical determination of fish freshness, using adenosine-5'-triphosphate (ATP) as a marker (Fig. 1C). A bi-enzymatic system immobilised in a Nafion layer on a microelectrode was utilised to measure ATP concentration. During the recognition process, glycerol is first phosphorylated by glycerol kinase to glycerol-3-phosphate in presence of a stoichiometric amount of ATP. Then, glycerol-3-phosphate is oxidised by phosphate oxidase, with production of hydrogen peroxide, which is quantified amperometrically at +0.7 V vs an on-chip Ag/AgCl electrode. The device showed good correlation with conventionally used high-performance liquid chromatography. Compact disk (CD)-based microfluidics is a fast developing class of miniaturised device, which uses centrifugal force for reagent and test solution transport and thus combines various laboratory functionalities in a portable format. The main advantages of the centrifugal pumping of solutions compared to electroosmotic pumping, are its relative insensitivity to physicochemical properties of the solution, such as pH, ionic strength or chemical

composition, and the simplicity of flow-rate control by changing rotation speed. Rattanarat *et al.* (Rattanarat et al. 2015) described a CD-based microfluidic device equipped with an electrochemical detector for the determination of glucose in human serum. The system was fabricated from poly(dimethylsiloxane) and consisted of a reservoir, mixing chamber, spiral channel, carbon-paste electrode and waste reservoir. During rotation, the sample solution and GOx were mixed in a spiral channel to produce hydrogen peroxide, which was then detected amperometrically at +0.4 V vs a pseudo-reference carbon-paste electrode. While the CD-based platform demonstrated in this paper does not have any obvious advantage for glucose analysis in blood compared to conventional systems, the approach can be easily adapted for simple reusable detection of a variety of analytes via enzymatic assays, with a wide number of possible applications in healthcare, food quality control and environmental analysis in a laboratory environment.

Microfluidic electrochemical biosensors facilitate on-line analysis of cell cultures, which is extremely important for drug screening, cancer research and environmental analysis. Weltin *et al.* (Weltin et al. 2014) reported a multi-parameter microphysiometric system for the dynamic online monitoring of T98G human brain cancer cell metabolism. The system was based on a glass chip and combined a cell cultivation chamber, microfluidics and series of chemo- and biosensors for monitoring of pH, oxygen, glucose and lactate. GOx and lactate oxidase were immobilised in an UV curable poly(2-hydroxyethyl methacrylate)-based membrane on the surface of indium thin oxide microelectrodes, that were placed in a microfluidic system downstream of the cultivation chamber. Hydrogen peroxide was detected amperometrically at +0.45 V *vs* an on-chip Ag/AgCl reference electrode. The authors demonstrated application of the system for drug screening by detecting the alteration and recovery effects of cellular metabolism induced by the addition of substances to the medium. Microfluidics have been successfully combined with MEAs for direct culturing of cells on the surface of electrodes for monitoring cell metabolism. Matharu et *al.* (Matharu et al. 2013) presented a biosensor consisting of hydrogel

microstructures with entrapped horseradish peroxidase (HRP) immobilised on gold MEAs. The system was used for probing the effect of ethanol and antioxidants on hepatocytes, which play a key role in triggering responses to alcohol toxicity by producing inflammatory signals such as cytokines and reactive oxygen species (Fig. 1D). A MEA was integrated into a microfluidic device and modified with collagen to facilitate hepatocyte adhesion. Hepatocytes exposed to ethanol and hydrogen peroxide were monitored on a MEA by cyclic voltammetry over 4 hours. Incubation of hepatocytes with antioxidants prior to addition of ethanol, resulted in a ~5 fold decrease of detected peroxide, suggesting the biosensor may be applied to monitor oxidative stress and thus, to investigate effects of antioxidants.

2.1.3. Problems of miniaturisation and future perspectives

In spite of the rapid development of miniaturised bioelectrocatalytic systems, immobilisation of biorecognition elements on micro- and nanostructures with high spatial resolution, is still a challenge. This is particularly required for multi-analyte devices, to provide efficient electron transfer between the electrode and biological elements of the sensor, and still remains one of the main obstacles for microbiosensor development. Another problem for biosensor miniaturisation is complications related to the extreme reduction of electrode size down to nano-dimensions, such as mass transport limitations, noise issues related to baseline drift, and the need for more advanced and expensive equipment for nanoscale resolution in the fabrication process. Miniaturisation is generally considered to be an obvious advantage and the aforementioned difficulties are rarely discussed in literature, except in a few reports (Dahlin 2012; Squires et al. 2008). However, the use of microsensors rather than nanosensors often offers a good compromise between the advantages and complications of miniaturisation for applications in medical diagnostics, food safety and environmental monitoring. To further improve the analytical performance of microsensors and reduce the probe volume needed for analysis, implementation of microbiosensors with continuous flow, rather than decreasing the electrode

size, might be a better solution. Hence we believe that there are unrealised opportunities for microfluidics combined with electrochemical microbiosensors.

Miniaturised electrochemical biosensors are expected to further decrease in cost and size with new advances in microtechnology, enabling fabrication of microstructures using new materials such as polymers and nanomaterials. Convergence of various fields of research may bring some unexpected and fascinating new directions in biosensor technology. One such direction may be an extension of the 'smart dust' concept (Bachand et al. 2009; Fischer et al. 2009) for biosensing in environmental, biodefence and biomedical monitoring. This involves a network of distributed electronic devices with millimetre dimensions, which are capable of sensing their environment and of communicating with each other and the user. Another rapidly growing trend is combination of smartphone technology and compact electrochemical biosensors (Lillehoj et al. 2013). Such systems are expected to increase the availability and accessibility of diagnostic testing, especially in developing countries that lack adequate resources and laboratory facilities. Following early work on apps for diabetes management by companies such as Lifescan, commercial amperometric glucose sensors were first interfaced with mobile phones by AgaMatrix, whose Nugget™ iPhone plug-in glucose meter gained FDA 510(k) on 7 December 2011, and was subsequently marketed by Sanofi Aventis with the iBGStar™ app in 2012. To date, however, most academic reports have focused on the application of mobile phones as image devices for biosensors based on optical transduction mechanisms. However, we expect a growth in the future in the number of publications concerning the innovative adaptation of smartphone platforms for electrochemical biosensing and the use of RFID and near-field transmission systems.

2.2 Paper-based devices

Paper has re-emerged recently as a promising material for point-of-care (POC) testing devices since it is light, cheap, environmentally friendly, easily printed and coated, biocompatible and thus, suitable for fabrication of disposable and safe single-use tests. Paper-based analytical

devices have the potential to bring biosensor technology to the developing world as they suit the World Health Organisation requirements for diagnostics for developing countries as defined by the ASSURED acronym: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users (http://www.who.int/std_diagnostics).

Paper-based colorimetric detection in the form of dipsticks and lateral-flow analysis is a well-established technique, such as the dipstick test for glucose in urine (Free et al. 1957) and the human pregnancy test (http://uk.clearblue.com/). In spite of the attractiveness of naked-eye colorimetric detection for simple and inexpensive paper-based assays, the increasing demand for higher sensitivity and, in particular, transmission of data in health applications challenges this detection mechanism. This has resulted in the development of paper-based devices coupled with instrumental detection techniques, such as mobile-phone colorimetric detection, fluorescence, chemiluminescence and electrochemistry (Yetisen et al. 2013). In addition to the aforementioned advantages of electrochemical detection for miniaturisation, it also has advantages for paper devices, being insensitive to ambient light, illumination and colour contamination, and highly compatible with printing technologies on paper (Maxwell et al. 2013; Turner 2013). There are many recent reviews on paper-based diagnostics (Hu et al. 2014; Maxwell et al. 2013; Yetisen et al. 2013), and so we will focus on paper-based catalytic biosensors for health applications, with an emphasis on electrochemical detection rather than the microfluidic component, and on fully integrated paper-based devices.

2.2.1. Paper-based electrochemical biosensors

Bioelectrocatalytic systems accommodated on electrochemical paper-based analytical devices (EPADs) have been utilised in various health applications, since the first "wicked" commercial electrochemical glucose sensors were introduced by Medisense (Abbott) in 1995 (Precision QID™). Following these commercial introductions, somewhat belated academic reports of paper-based microfluidics appeared in 2009 and 2010 (Dungchai et al. 2009; Nie et al. 2010b). There were two main strategies to combine paper-based platforms with electrochemical

detection. The first was the construction of electrodes directly on paper using printing techniques (Dungchai et al. 2009; Kit-Anan et al. 2012) or deposition of metal via sputtering (Shiroma et al. 2012), and the second was by placing paper sheets or "wicks" containing immobilised biorecognition systems onto conventional electrodes, usually screen-printed electrodes (Kong et al. 2014; Noiphung et al. 2013). Microfluidic channels are often introduced in EPADs using photolithography (Apilux et al. 2010; Dungchai et al. 2009), wax screen-printing (Dungchai et al. 2011; Nie et al. 2010b), wax dipping (Noiphung et al. 2013; Songjaroen et al. 2011) and wax printing (Lu et al. 2010b) forming electrochemical microfluidic paper-based analytical devices (EμPAD).

Henry and co-workers (Dungchai et al. 2009) published the first academic paper on a bioelectrocatalytic system integrated with a μPAD. The device was utilised for determination of glucose, lactate and uric acid in human serum with three electrodes modified with GOx, LOx and uricase solutions, respectively. Electrodes were screen-printed on filter paper with microfluidic channels introduced via photolithography. All working electrodes were printed using a Prussian Blue (PB) (hydrogen peroxide sensitive catalyst) containing carbon ink. The determination of analytes in serum samples was performed using chronoamperometry at 0 V *vs* on on-chip Ag/AgCl reference electrode. Two years later, the same group reported a similar paper-based device for glucose determination in human serum, but with microfluidic channels produced by wax screen-printing (Dungchai et al. 2011), an idea that could be linked to the paraffin impregnation method reported by Müller and Clegg in 1949 (Müller and Clegg 1949). Another related paper (Noiphung et al. 2013) described an example of a paper sheet with integrated plasma isolation, that was placed on carbon screen-printed electrodes, for determination of glucose in whole blood samples (Fig. 2A). A dumbbell shaped piece of paper contained two blood separation zones, designed to separate plasma and generate flow to the middle detection zone modified with GOx, and which were formed by a wax dipping method. Hydrogen peroxide generated as a result of the biocatalytic reaction was detected at -0.1 V, *vs* a printed Ag/AgCl

electrode, at a PB-modified working electrode underneath the sheet of paper. These reported biosensor systems demonstrate the advantage of paper-based approaches for the fabrication of disposable sensors, since it allows reduction in cost and environmental impact of the devices. Paper-based microfluidics can be considered as a future alternative technology for development of single use biosensors, for example for glucose analysis in blood.

Screen-printed carbon is the most often used material for electrodes in EPADs. However, there are some examples of using other materials for electrode fabrication such as pencil graphite (Kurra and Kulkarni 2013; Santhiago and Kubota 2013), gold nanoparticles (Kong et al. 2014; Liana et al. 2013; Määttänen et al. 2013) and carbon-based nanomaterials (Kong et al. 2014; Liana et al. 2013). Paper represents an ideal surface for pencil drawing, allowing conducting tracks to be created that are suitable for paper-based sensors, microfluidic devices and energy storage devices (Kurra and Kulkarni 2013). Kubota *et al.* (Santhiago and Kubota 2013) demonstrated the coupling of a graphite pencil electrode with an EμPAD. Wax printing was again used for formation of three circular microzones: one for filtration, one for an enzymatic reaction and one for electrochemical detection. The reaction zone was modified by the addition of a redox mediator and enzyme solution. Pencil graphite was used as the working electrode with silver ink (back face) as the reference and auxiliary electrode. Folding of the device when not in use, prevented contamination of the enzyme in the reaction zone. This type of device provides a low cost lab-on-a-paper technology for sequential multianalyte analysis using the same working electrode, by fabrication side-by-side on paper of many circular electrochemical detection spots modified with different enzymes.

The sensitivity of paper-based biosensors still remains an issue for their successful application in POC devices. Modification of paper-based devices with high surface area materials such as metallic nanoparticles (Kong et al. 2014; Määttänen et al. 2013) or carbon-based nanostructures (Määttänen et al. 2013; Ruecha et al. 2014) is one of the possible ways to overcome this issue. Määttänen *et al.* (Määttänen et al. 2013) demonstrated a paper-based biosensor with inkjet-

printed nanoparticle-based gold working and counter electrodes, and an inkjet-printed silver nanoparticle electrode. Various types of electrode functionalisation were performed, such as self-assembly of a monolayer of alkanethiols, electropolymerised polyaniline (PANi) for potentiometric pH sensing, electropolymerised poly(3,4-ethylenedioxythiophene) (PEDOT) with two different dopants Cl^- and GOx. Amprometric glucose sensing was executed at +0.85 V vs. a Ag/AgCl electrode. In another report (Ruecha et al. 2014), carbon screen-printed electrodes of an EμPAD with wax-printed microfluidic channels were functionalised via electrospraying using a graphene, polyvinylpyrrolidone (PVP) and PANi composite. The presence of PVP increased the dispersibility of the graphene in the nanocomposite, thus increasing the sensitivity of the amperometric detection of hydrogen peroxide at +0.6 V vs a printed Ag/AgCl, with a 3-fold increase compared to an unmodified electrode. Cholesterol oxidase was drop-cast on the surface of a nanocomposite working electrode for measurements of cholesterol in human serum. Hydrogen peroxide produced by the enzymatic reaction was monitored amperometrically. The combination of gold nanoparticles and graphene was also shown to increase the sensitivity of the paper-based biosensor (Kong et al. 2014). In the example given, a screen-printed carbon electrode (SPCE) was coupled with a paper disk for detection of glucose in human blood. The SPCE was modified with a G/PANi/Au nanoparticle/GOx biocomposite coated with a Nafion membrane, and then covered by a Whatman chromatography paper disk impregnated with the sample. Only 2 μl of the sample was needed for analysis, which is close to the current commercial standard of 0.3 to 1 μl, routinely delivered by commercial home blood glucose sensors. Differential pulse voltammetry was used for analyte determination. The system has the advantages of low cost, environmental amity and high adaptability for analysis of various analytes, compared to commercially available biosensors for glucose analysis in blood.

Another way to improve the sensitivity of paper-based biosensors is by the integration of microelectrodes into a device. Microelectrodes have many advantages for biosensor construction as discussed in the previous sections. Henry's group reported the first attempt to introduce a

microelectrode in an EμPAD (Santhiago et al. 2013) (Fig. 2B). The microelectrodes were fabricated by back-filling holes, made in polyester sheets using a laser-engraving system, with carbon paste. To make electrical contact, the working electrodes were combined with silver screen-printed paper in a sandwich type two-electrode configuration. Fabrication of a carbon paste MEA was also demonstrated by the authors. The device was used for amperometric detection of cysteine as a proof-of-concept and showed a low limit of detection (4.8 μM). Crooks's group introduced gold micro-wires and carbon fibre electrodes in a paper-based device (Fosdick et al. 2014). The authors reported that micro-wire electrodes provided a number of advantages in addition to sensitivity improvement for PADs, as they can be placed at any location within the device, for instance suspended within the channel or between two channels, and can easily be modified with e.g. self-assembled monolayers prior to integration in the device. Hence, the use of microelectrodes can apparently bring many advantages for fabrication of paper-based bioelectrocatalytic POC devices. However, as far as we know, biosensors using microelectrodes incorporated in PADs have not so far been reported.

2.2.2. Integrated paper-based devices

The potential of EμPADs for applications in cheap POC devices is restricted by the need for an external electrochemical reader to perform the analysis. Here we discuss some recent attempts to tackle this limitation. There are two main directions of research to solve this problem. The first is the development of cheap devices (or adaptation of existing ones), including mobile phones, that can be connected to, or communicate with, simple paper-based disposable strips. The second approach is the incorporation of a reader into the paper-based device itself (Maxwell et al. 2013). Whitesides's group demonstrated the possibility of combining an EμPAD with a commercially available glucometer (Fig. 2C) (Nie et al. 2010a). The EμPAD was fabricated on chromatographic paper and consisted of microfluidic channels formed by wax printing, four screen-printed electrodes (carbon working and counter, two silver references) and electrical interconnects printed with silver ink. The use of the EμPADs integrated with the glucometer was

demonstrated for analysis of glucose, cholesterol, L-lactate in human plasma and ethanol in aqueous solutions. These measurements were based on amperometric quantitation of the reduced electron-transfer mediator, ferrocyanide, generated in an enzymatic reaction catalysed by GOx, cholesterol oxidase, LOx or alcohol dehydrogenase, respectively. The chemical reagents needed for analysis were either stored in the detection zone of the EμPADs in dried form (in the cases of glucose and ethanol detection) or mixed in a separated tube with analyte (for analysis of lactate and cholesterol). The integrated device was less accurate than commercially available test-strips, but it was suggested that it would be suitable for application in cheap POC devices after further development. Another group developed an inexpensive, hand held, open source potentiostat called 'CheapStat' that has potential for applications in resource limiting settings (Rowe et al. 2011). The authors demonstrated the device for food and drug testing, environmental monitoring and biosensing utilising various electrochemical techniques such as cyclic, square-wave, linear-sweep and anodic-stripping voltammetry. Further research is needed to adapt this platform for integration with EμPADs. Chen *et al*. (Chen et al. 2013) presented a paper-based electrochemical biosensor array integrated with an electrochemical reader which could perform eight tests in a single run. As a proof-of-principle study, the authors used the integrated device for amperometric measurements of glucose, L-lactate and uric acid, based on ferrocyanide-mediated enzymatic reactions of GOx, LOx and uricase, respectively. The EμPAD was fabricated employing the same procedure as reported by Whitesides's group (Nie et al. 2010a).

Attempts in another direction were made by Crooks and co-workers, who reported the first paper-based platform integrated with a power source (Liu and Crooks 2012). They presented the EμPAD coupled with electrochromic read-out in the form of a PB spot and a metal/air battery for the detection of glucose and hydrogen peroxide in artificial urine samples. The addition of sample activated the battery that powered the electrochemical sensor and the read-out. The device had two parts: a sensor section and an Al/air battery section, separated from each other by a wax barrier. For glucose measurements the paper reservoir of the sensor section was filled with

dried GOx and $Fe(CN)_6^{3-}$ was used as an enzyme mediator. As a result of the enzymatic oxidation of glucose, $Fe(CN)_6^{4-}$ is formed which was then oxidised back to the reduced form on the lower indium tin oxide (ITO) electrode. This reaction leads to reduction of PB to colorless Prussian White (PW) on the upper ITO electrode. Thus, semi-quantitative information about glucose concentration can be obtained from changes of the PB spot color intensity. Hydrogen peroxide detection was achieved in a similar fashion to the glucose measurements, using the enzymatic reaction of horseradish peroxidase and following oxidation of PW to PB. This concept of integration of a paper-based device with a battery and read-out has major implications for the future development of paper-based POC integrated devices. Thom *et al.* (Thom et al. 2012), at almost the same time, demonstrated another example of integration of a paper-based device with a power source. A Galvanic cell, referred to as a 'fluidic battery', was integrated into a microfluidic channel and was capable of powering a surface-mounted UV LED to conduct an on-chip fluorescence assay. The device could be adapted to power an electrochemical reader.

Printed electronics (PE) or hybrid printed electronics (Hy-PE), based on fully printed elements or printed elements combined with silicon-based components, is a promising approach for incorporation of an electrochemical reader into a paper-based device for fabrication of cheap POC systems (Beni et al. 2015; Turner 2015). The fabrication of individual electronic building blocks using printing technology has already been demonstrated. However, the use of PE for the construction of a complete device is currently restricted by the poor performance of printed transistors as microprocessor components (Beni et al. 2015). Hy-PE devices, at the present time, present a more realistic way forward for the fabrication of integrated POC paper-based electrochemical assays. Geng *et al.* (Geng et al. 2012) developed a paper-based bio-patch using a combination of CMOS technology and inkjet-printing for multichannel recording of bio-signals from a patient. The bio-patch consisted of printed electrodes, interconnection and antenna for data transmission, coupled with a soft battery and a CMOS processor. The system however required an external display. Our own group demonstrated the first fully integrated Hy-PE paper-

based device for amperometric measurements (Fig. 2D) (Beni et al. 2015). This approach combined the sophistication of advanced electrochemical biosensors with a simple manufacturing technique to create an entire, use-and-throw instrument. The system was manufactured under ambient conditions. All interconnections were printed and an anisotropic conductive glue was used for interconnection between the chip and the conductors. A screen-printed manganese dioxide battery and a printed vertical electrochromic display were incorporated in the instrument. The display is paper-like in the sense that it works in reflective mode, that is, no backlight is used to light up the pixels. This integrated biosensing platform forms a workhorse in our hands for a variety of diagnostic systems including amperometric enzyme electrodes, electrochemical affinity assays and general electroanalysis. Development is ongoing to incorporate RFID or Bluetooth communication with mobile devices or area networks, and to create wearable formats with integrated sampling. Potentiometric and electrochemical impedance-based devices are also under development.

2.2.3. Disadvantages of paper-based devices and future perspectives

Paper-based analytical devices have a great potential for providing cost-effective solutions for POC diagnostics and monitoring in resource-limited conditions. However, paper-based devices should be further improved in terms of analytical performance (Hu et al. 2014). The lack of device robustness generally results in insufficient performance for actual use in health applications. There are two main causes of instability. The first is related to the need for more effective strategies for immobilisation of the biorecognition element to retain fast electron transfer between the biosensing element and the electrode(s). Recent progress in this direction will be discussed in following sections. The second is the influence of temperature and humidity on migration forces of liquid in microfluidic channels that can lead to signal variations. Further research in engineering of EμPADs should be done to overcome this problem. Low sensitivity is another disadvantage of paper-based devices (Parolo and Merkoci 2013). Some recent attempts to increase sensitivity by incorporation of nanomaterials and microelectrodes in EμPADs were

discussed earlier. Further development of existing amplification strategies or investigation of new ones, such as hollow-channel EμPADs (Glavan et al. 2013; Renault et al. 2014), is required in order to extend the range of analytes feasible for detection in real samples.

The requirement for cheap integrated platforms in POC diagnostics and monitoring should drive further breakthroughs in the development of inexpensive and simple portable electrochemical readers as well as PE and Hy-PE devices on paper, such as energy-storage devices, transistors and other active elements. Merging of self-powered biosensors (Villarrubia et al. 2014) or solar cells (Barr et al. 2011) as an alternative power supply with EμPADs, will allow the cost to be decreased and sensitivity of integrated paper-based devices to be increased. The progress in self-powered biosensors will be discussed in the next section. Coupling of bioanalysis with modern telecommunications is an emerging theme of future POC devices for health applications (Maxwell et al. 2013; Turner 2013). Due to the aforementioned advantages, electrochemical paper-based technology is well suited for integration with mobile-phone-based systems. Some examples of integration of paper-based bioelectrocatalytic systems with a smartphone as a signal reader for optical measurements have been already demonstrated (Chun et al. 2014; Wu et al. 2015).

2.3 Wearable biosensors

The development of wearable, sensor-based systems for health monitoring has attracted a lot of attention over the past decade as indicated by the increasing amount of research published in this area (Bandodkar and Wang 2014; Pantelopoulos and Bourbakis 2010; Windmiller and Wang 2013). The growing popularity of wearable sensors mirrors a major trend in health monitoring involving a shift from centralised hospital-based care systems to home-based personal medicine, since the latter can greatly reduce healthcare cost as well as delivering a better service to the individual. Considerable progress has been achieved in development of wearable physical sensors for monitoring of parameters such as heart rate, blood pressure, skin temperature, bodily motion, etc. (Hammock et al. 2013). However, wearable non-invasive biosensors for monitoring

of chemical information, which can provide much more data on patient health status, are still at an early stage due to challenges in accessing appropriate samples, detection of low concentrations of analyte, small sample volumes, biofouling and biocompatibility of the sensors (Bandodkar and Wang 2014).

Electrochemical transducers offer many advantages for wearable biosensors for physiological and environmental monitoring, since they can be easily miniaturised to perform highly sensitive analysis of small sample volumes, and can be integrated onto textile materials or directly on the epidermis (Windmiller and Wang 2013). There are many reviews published on wearable electrochemical biosensors (Bandodkar and Wang 2014; Windmiller and Wang 2013), including textile based (Choudhary et al. 2015) and epidermal (tattoo-based) biosensors (Bandodkar et al. 2015a; Kim et al. 2011). Here we will highlight some of the most interesting examples of wearable electrochemical catalytic biosensors and focus more on power generation technologies available for wearable biosensors, with emphasis on self-powered biosensors as an emerging field of biosensor technology.

2.3.1. Wearable bioelectrocatalytic systems

Wearable electrochemical biocatalytic sensors for *in vitro* monitoring can potentially measure target analytes in tears, saliva, sweat and skin interstitial fluid (Bandodkar and Wang 2014). Arguably the most important example of biosensors for tear analysis, is the biosensor embedded in a contact lens. Parviz and co-workers first demonstrated immobilisation of GOx on the surface of a contact lens for glucose monitoring (Yao et al. 2011) (Fig. 3A). The glucose biosensor was fabricated by creating microstructures on a polymer substrate and then shaping it into a contact lens. For amperometric detection of glucose, GOx was immobilised in a titania sol-gel membrane and then covered with a Nafion layer to reduce interferences. Hydrogen peroxide generated by the enzymatic reaction was monitored at +400 mV *vs* a Ti/Pd/Pt reference electrode. In the example, the sensor was hardwired, but in their further work the authors demonstrated a biosensor with integrated wireless electronics for data recording (Yu-Te et al.

2012). The system can potentially work as a POC device with the possibility of near-field communication with mobile phone for power and data transfer. A similar device is currently being developed by Google™ and has attracted considerable attention in the popular scientific press. Real-life applications of wearable biosensors for measurements in tears have not yet been accomplished due to a number of technical challenges. The main problems for real-life applications of non-invasive wearable sensors are lack of correlation with *in vivo* concentrations of target analytes, time lags in response with respect to circulating blood levels, inhomogeneity and variability of the samples due to absence of the standard sampling procedure and problems related with the sampling. For contact lens-based systems, these difficulties are accompanied with volume and area restrictions, biocompatibility issues and transparency / appearance requirements. All these challenges should be considered or/and addressed in newly reported wearable systems for tear analysis, if they are to have a real impact on human health rather than just being speculations on a popular topic.

The analysis of saliva has a long history dating back to the 1960s (Graf and Mühlemann 1966). Recently, Wang's group reported an example of a wearable electrochemical biocatalytic sensor for continuous lactate monitoring in saliva (Kim et al. 2014). The mouthguard biosensor was fabricated by screen-printing of a three-electrode system on a flexible plastic substrate that was subsequently attached by double-side adhesive to the mouthguard body. The electrochemical reader was connected to the biosensor externally. LOx was immobilised on the working electrode printed with PB containing graphite ink by entrapment in electropolymerised polymer. Hydrogen peroxide generated by the enzymatic reaction was monitored amperometrically at 0.042 V *vs* a printed Ag reference. Further development of the system is needed for integration of electronics and a wireless transmitter onto the mouthguard. A dental graphene-modified silk tattoo affinity biosensor for continuous wireless monitoring of bacteria was reported by Mannoor and co-workers (Mannoor et al. 2012). This strategy can be used for the development of biocatalytic wearable sensors. Due to the remaining technical complications, real-life application

of a salivary wearable sensor has again, not yet been reported. Besides the problems mentioned above with reliable sample collection, common for all non-invasively available body fluids, saliva is constantly subjected to contamination from food, which not only influences the results of analysis, but also represents additional source of operational instability of the biorecognition element. In addition, the rates of salivation can vary considerably in individuals and “dry mouth syndrome” can be a particular problem in elderly people. If the challenges can be overcome, analysis of saliva can have practical use for lactate, salivary amylase and the detection of some other protein markers. However, salivary biosensors for glucose monitoring seem to be of limited use, since glucose concentrations in blood and saliva show poor correlation. Despite this latter limitation, it has been suggested that the method may still be sufficient for deprived communities.

Wearable biosensors for sweat analysis can be divided into two main groups: textile/plastic based devices and epidermal-based sensors (Bandodkar and Wang 2014). Epidermal electronics has an advantage over textile-based biosensors as it facilitates long-lived, robust contact with the skin, thus, possessing 'wear-and-forget' functionality (Kim et al. 2011; Windmiller and Wang 2013). The first example of tattoo-like epidermal electronics, based on flexible lithographic structures, was reported by Rogers’s group (Kim et al. 2011). The authors demonstrated the use of these wearable devices for real-time, non-invasive monitoring of physical body parameters. The first attempt to incorporate a biocatalytic recognition element into a printed tattoo-like electrochemical device (Windmiller et al. 2012) was undertaken by Wang’s group (Jia et al. 2013a). The electrodes were screen-printed on temporary transfer tattoo paper using carbon and Ag/AgCl inks with additions of chopped carbon fibres to increase the tensile strength of the electrodes. The working electrode was modified with carbon nanotubes/tetrathiafulvalene-mediated LOx covered by a chitosan membrane. Amperometric detection of lactate was performed at +0.05 V *vs* a printed Ag/AgCl electrode. The biosensor was applied for real-time continuous monitoring of lactate in sweat of a volunteer during cycling exercise (Fig. 3B).

Further effort is needed to incorporate an electronic interface, data processing and wireless transmission of results for a truly wearable device. The developed platform could be used for incorporation of other bioelectrochemical systems for detection of various analytes of interest in health applications. Heikenfeld and co-workers recently published a very interesting report on an epidermal digital health device in the form of an adhesive patch (Rose et al. 2015). A commercial radio-frequency identification chip was adapted for potentiometric sensing of solutes in sweat with the possibility of powering the device and reading results wirelessly using an Android™ smartphone app. The sensing electrodes can be folded over to be in direct contact with skin or additional microfluidics can be incorporated to bring sweat from the skin to the sensor. The platform could allow further integration of biocatalytic components on the electrode for biosensing. In general, sweat analysis represent a promising approach for non-invasive evaluation of blood lactate concentrations (Sakharov et al. 2010), essential for monitoring oxygen deficit in clinical medicine and sports physiology. However, sweat glucose shows a poor correlation with blood glucose, despite being the target analyte for a number of sweat-based biosensors reported in the literature.

Interstitial skin fluid (ISF) is one more example of a body fluid available for non-invasive analysis and capable of providing vital information on patient health status. Most of the research in this field are focused on non-invasive glucose monitoring for diabetes management. The GlucoWatch Biographer™ (Cygnus, Inc.) is a famous example of a once commercial device based on ISF monitoring. The device used reverse iontophoresis to obtain a sample for glucose monitoring using an enzyme electrode. However, due to problems with accuracy, patient skin irritations from wearing the device and the need for frequent calibration of the device using pinpricks, it was withdrawn from the market. In spite of the many technical challenges related with continuous non-invasive or minimally invasive glucose monitoring in ISF, that seem to be difficult to overcome, researches are still working in this direction. Wang's group recently reported an analog of the GlucoWatch™ device in the form of a tattoo-based sensor (Bandodkar

et al. 2015b). The device utilised enzymatic electrochemical sensing of ISF glucose in a sample extracted by reverse iontophoresis. The fabrication of the sensor was similar to those described earlier for lactate monitoring in sweat (Jia et al. 2013a). The working electrode was screen-printed using PB ink and was functionalised with a GOx containing chitosan membrane. Hydrogen peroxide produced in the enzymatic reaction was monitored at -0.1 V *vs* a printed Ag/AgCl electrode (Jia et al. 2013a).

Wearable sensors can be used not only for healthcare applications, but also for a wearer's local environment monitoring. Wang's group reported an example of enzyme immobilisation on a textile for amperometric monitoring of phenolic contaminants in seawater (Malzahn et al. 2011). Electrodes were screen-printed on the sleeve of a neoprene wetsuit using carbon and Ag/AgCl inks. An enzyme that recognises phenols, tyrosine, was mixed into the carbon ink and printed on the top of the working electrode and subsequently covered by a Nafion solution. Reduction of the quinone product formed by the enzymatic reaction was monitored amperometrically at -0.3 V vs Ag/AgCl. The biosensor was coupled with an encapsulated miniaturised potentiostat and LED, which gave a signal when the phenol concentration exceeded a threshold value.

2.3.2. Self-powered biocatalytic sensors

A main obstacle for realisation of fully wearable biosensors is finding a suitable power source for the sensor and electronic circuit elements required for data recording and transmission. Biofuel cells (BFCs) based on enzymes and microbes are potentially able to harness energy from the wearer's body and are considered a promising power supply for wearable biosensors. There are some examples in the literature on using BFCs for wearable devices powering. Shleev's group demonstrated integration of a biofuel cell into a contact lens as a power source for a biosensing device operating in tears (Falk et al. 2012). The reported membrane-less direct electron transfer based enzymatic BFC (EBFC) consisted of gold microwires covered with gold nanoparticles, which were functionalised by cellobiose dehydrogenase and bilirubin oxidase as cathodic and anodic counterparts, respectively. Oxidation of glucose from human tears by

cellobiose dehydrogenase on the anode, with subsequent reduction of oxygen to water by bilirubin oxidase on the cathode in the presence of protons liberated in the anodic reaction, resulted in the generation of 1 μW cm^{-2} maximum power density. The EBFC showed more than 20 h of operational half-life. In another example, Wang and co-workers described an epidermal biofuel cell generating power by harvesting lactate from sweat (Jia et al. 2013b). The BFC was formed by two tattoo-based carbon screen-printed electrodes. The anode was modified first with carboxy-functionalised carbon nanotubes and tetrathiafulvalene as an enzyme mediator, then a mixture of LOx and albumin was drop-cast onto the surface of the anode, followed by chitosan solution. The cathode was modified with platinum black and covered by a Nafion membrane. Mediated enzymatic oxidation of lactate at the anode and reduction of oxygen at the cathode, provided maximum current densities in the range of 5-70 μW cm^{-2}, depending on the lactate levels in the sweat of different individuals.

In the aforementioned examples, BFCs were used only as a power source for the biosensing device. However, BFCs can act not only as an energy supply, but also as an analytical device in so-called 'self-powered biosensors.' A self-powered biosensor is a type of BFC with power output, open circuit potential or a generated current proportional to the concentration of the analyte/fuel. In such a configuration, the sensor itself provides the power for the sensing device. The main advantages of self-powered biosensors are their plain design of just two electrodes, since no external voltage needs to be applied to the electrodes, and conjugation of energy generation and electroanalysis in one simple device, which makes them particularly attractive for applications in battery-free, low cost, integrated printed electronics and in wearable biosensing systems. Although BFCs have been known for a long time, with early work dating back to 1911 and 1931, and researched in detail since 1964 (Yahiro et al. 1964), self-powered biosensors recently received revived attention as a new energy conversion technology, when Willner and co-workers published a membraneless enzymatic BFC operating as an analytical device (Katz et al. 2001; Katz et al. 1999).

Work in a similar direction has been published recently by many groups. Sode's group reported a direct electron transfer based membraneless EBFC, with an open circuit voltage proportional to the glucose concentration (Kakehi et al. 2007). The anode of the BFC was based on carbon paste modified with covalently attached glucose dehydrogenase catalysing mediatorless oxidation of glucose. A Pt wire was used as a cathode for oxygen reduction. This self-powered biosensor was combined with a wireless transmitter system for simple and miniaturised continuous glucose monitoring. Feldman and co-workers from Abbott Diabetes Care, demonstrated a BFC based on an osmium redox polymer GOx anode and a Pt/carbon mixture cathode separated by a load resistor operating in amperometric mode, by measuring the current flowing through the resistor (Liu et al. 2012). The system had good operational stability over more than 60 days with a current output in the order of 12 nA. These examples described self-powered biosensors where the bioanode was coupled with a noble metal, thus representing the case of an 'uncompleted' cell. Additional difficulties appear when the cell consists of two bioelectrodes based on different bioelectrocatalytic systems having different optimum conditions for operation, which should be combined in a common sample with a specific pH, temperature and ionic concentration. As already mentioned, Willner and co-workers first reported two bioelectrocatalytic systems operating in a single solution (Katz et al. 1999). In their device, the anode and the cathode of the cell were modified with monolayers of mediated GOx and a cytochrome *c*/cytochrome oxidase couple, respectively. The BFC was operated in 1 mM glucose containing air-saturated buffer at pH 7 at 25°C, providing a maximum power density of 5 μW cm^{-2}. They further demonstrated application of the system for self-powered detection of glucose, where the open-circuit potential of the developed BFC was proportional to the analyte concentration (Katz et al. 2001). Ramanavicius's group recently reported a self-powered biosensor with the cathode and anode based on the same enzyme, GOx (Krikstolaityte et al. 2013). Addition of glucose to the cell led to its mediated oxidation at the anode and its oxidation with subsequent reduction of the hydrogen peroxide formed by HRP at the cathode, with generation of a maximum power density

of 3 μW cm^{-2}. The power output of the BFC was proportional to the concentration of glucose. Although the anode and the cathode of the EBFC were powered by the same substrate, the system still required two enzymes for operation. In our group, we introduced the concept of a single enzyme based self-powered biosensor (Sekretaryova et al. 2014a) (Fig. 3C). As an example, we demonstrated the application of the system for self-powered detection of free cholesterol in whole plasma. The anode and the cathode were both based on cholesterol oxidase immobilised into a sol-gel matrix on carbon cloth electrodes. Mediated biocatalytic cholesterol oxidation was used as the anodic reaction and electrocatalytic reduction of the generated hydrogen peroxide on PB as the cathodic reaction. The open circuit potential, power density and generated current were proportional to the concentration of the analyte, but the generated current was used as the analytical signal for the detection of cholesterol in the plasma, since it provided better analytical performances. The maximum power density generated by the cell was 11 μW cm^{-2}.

Another strategy for biosensing with BFCs, is the detection of enzyme inhibitors by their influence on the output of the BFC. In 2010, Dong's group developed the first self-powered biosensor based on inhibition of EBFCs and demonstrated its application for cyanide detection (Deng et al. 2010). The BFC consisted of a glucose dehydrogenase modified bioanode and a laccase based biocathode. Laccase catalyses the reduction of O_2 to water and has copper in its active site. Cyanide affects the active site of laccase and inhibits O_2 consumption, but does not affect the glucose dehydrogenase activity. The decrease of O_2 reduction on the biocathode resulted in a decrease of the power output, which is then a measure of the cyanide concentration. The same group further reported self-powered biosensors for detection of mercury ions (Wen et al. 2011) (Fig. S2A) and acetaldehyde (Zhang et al. 2012) using the same approach. The main disadvantage of such type of self-powered biosensors is their inability for continuous monitoring due to a decrease in power generation with increasing concentration of the analyte. Minteer and co-workers introduced another way of using the inhibition effect in self-powered biosensors by

measuring the recovery of the power output of the BFC induced by molecules that can decouple the inhibition effect and thus detect these molecules (Germain et al. 2008; Meredith and Minteer 2011). In 2008, they demonstrated the use of intact and viable mitochondria for application in biocatalytic sensors that was also a first attempt at self-powered biosensing based on inhibition (Germain et al. 2008). The system consisted of two electrodes, a cathode for reduction of oxygen and a mitochondria-based bioanode for oxidation of the fuel (pyruvate). Oligomycin was used as an inhibitor of ATP synthase in the mitochondria. In the presence of only pyruvate, the cell generated low power density due to inhibition of the mitochondria activity by oligomycin. Addition of nitroaromatic explosives led to decoupling of the inhibitor from the mitochondria and generation of significant power density. The system was used only for qualitative sensing of the presence or absence of nitroaromatic compounds. Later Minteer's group described an enzymatic BFC using the same principle (Meredith and Minteer 2011). The BFC consisted of a GOx-based bioanode and a Pt cathode and was used for sensing of ethylenediaminetetraacetic acid (EDTA). The GOx based anode was inhibited before sensing by using Cu^{2+} that binds to the active site of the enzyme. EDTA was shown to reverse the binding of Cu^{2+} to the active site and recover the current output of the BFC. The generated current density was proportional to the concentration of EDTA. Since different molecules can inhibit enzymes or decouple the inhibition effect, the essential drawback of self-powered biosensors based on the inhibition effect is their lack of selectivity.

Self-powered, logic-gate biocatalytic sensing systems, represent one of the latest trends in biosensing technology. BFC-based logic-gate biosensing devices combine biocomputing elements with the sensing process for the creation of potentialy 'smart' electroanalytical systems, which are able to detect multiple analytes in a complex matrix using a single transducer, by applying Boolean logic operations (Zhou and Wang 2012). The first logic gate made of a self-powered enzymatic biosensor was reported by Dong's group in 2011. The system consisted of a GOx-based anode and a bilirubin oxidase-based cathode. Both enzymes were co-immobilised

with mediators in a biopolymer, chitosan. The ON state (high power output) for the system was achieved in air-saturated solutions, too high or too low levels of O_2 led to an OFF state (low power output). Such an O_2 controlled switchable BFC could be used as a diagnostic system to monitor the O_2 content in body fluids and make a logical decision whether the level of dissolved O_2 in the body fluid is within acceptable limits or not. The main disadvantage of most logic-gate, self-powered biosensors reported to date, is that they are activated by signals that are not relevant for diagnostic applications (due to the nature of the signal or to non-physiological concentrations).

In all examples mentionedabove, an external read-out system was used to obtain quantitative information about the analyte concentration. There are some reports in the literature describing attempts to integrate self-powered biosensor with read-out systems, allowing naked-eye detection and thus being completely autonomous. Sode's group reported an integrated, wireless, self-powered system referred to as a 'BioCapacitor', which was based on an EBFC combined with a capacitor functioning as a transducer and a LED (Hanashi et al. 2009) (Fig. S2B). As a model system, a BFC composed of glucose dehydrogenase as the anodic biocatalytic system for the oxidation of glucose, and Pt/C as the cathodic catalyst for oxygen reduction, was used. The power generated during oxidation of glucose charged the capacitor connected to the LED via a charge pump circuit. The charging rate of the capacitor was proportional to the concentration of the analyte. The charged capacitor was discharged through the LED, making it blink with a frequency indicative of the fuel concentration. In addition to being an integrated system available for naked-eye, autonomous detection, the BioCapacitor overcomes the problem of the low power densities of BFCs, which are often not sufficient to power conventional electronics. This problem may be alleviated in the future as low voltage electronics are developed to overcome the heat generation problems in super computers, but for now it remains a real problem. Miyake *et al.* reported similar devices for glucose (Miyake et al. 2011a) and fructose sensing (Miyake et al. 2011b). Jönsson-Niedziolka and co-workers demonstrated a different approach for the

construction of an integrated self-powered biosensor (Fig. 3D) (Zloczewska et al. 2014). The BFC consisted of an indium tin oxide anode modified with carbon nanomaterials, vertically aligned carbon nanotubes or carbon nanoparticles, for oxidation of ascorbic acid and a bilirubin oxidase-based air-breathing cathode for oxygen reduction. The EBFC was connected to a PB electrochromic display functioning similar to those reported by Crooks and co-workers for an integrated paper-based device, as described above (Liu and Crooks 2012). The oxidation of ascorbic acid on the anode led to reduction of the PB in the display and a change of colour from blue to transparent with increasing analyte concentration. The display was regenerated by connecting it to the biocathode, which returned the PB to its oxidised blue form. The operation of the device for monitoring of glucose in orange juice was demonstrated.

2.3.3. Challenges and future outlook for wearable sensors

In spite of considerable progress in the development of wearable sensors, there are several challenges to overcome in order to construct functioning, real-time, integrated monitoring devices. The first obstacle is retention of the biocatalytic activity of the biorecognition system during the time it is worn. To solve this problem further efforts in the engineering of stable biorecognition elements and an improvement of immobilisation techniques are needed. Another challenge arises when combining flexible bioelectronics, which are essential for the construction of comfortable 'wear-and-forget' devices, with biological sensing elements (Chuang et al. 2010). There is a lack of fundamental understanding of the behavior of bioelectrocatalytic systems and their analytical performance on flexible materials. The next point, which was rarely addressed in early works on wearable biosensors, but has recently attracted more attention, is the integration of electrochemical sensors, electronics, a power source and wireless transmission system into a miniaturised device for continuous use. The combined endeavour of researches from different areas, such as electrical engineering, materials science, bioelectrochemistry, product design etc., will hopefully provide success in this direction. The last serious problem in wearable sensors for

medical diagnostics is finding a correlation between the concentrations of various analytes in body liquids available for non-invasive analysis and patient health status (Turner 2013).

Further development of wearable sensors towards fully autonomous, easy-to-wear devices requires improvements in the field of printed and flexible electronics, such as integration of chemical sensors in body sensor networks (Diamond et al. 2008) and electronic skin (Hammock et al. 2013), which are already established for physical sensors. Expansion of power sources capable of power harvesting directly from biological fluids on the wearer's body, such as BFC-based self-powered biosensors and logic-gate self-powered biosensors, seems to be a promising approach toward fully integrated wearable biosensors. New non-invasive electrochemical sensors for additional important analytes are also highly desirable for building personalised health monitoring systems, including both physical sensors and biosensors for various analytes. Furthermore, integration of sensing devices with wireless systems capable of data transmission directly to mobile phones, tablets or area networks, is an emerging field of biosensor technology targeting mobile healthcare, which will allow a significant reduction in healthcare costs via the future integration with "big data" to power expert systems to assist decision making.

3. Electron transfer in bioelectrocatalytic systems

An essential element of bioelectrocatalytic systems is the electron transfer (ET) between the biorecognition element and the electrode surface. The ET provides the grounds for bioelectronics and biosensing devices and influences such important parameters in bioelectroanalysis as response time, sensitivity, detection limit and partially, stability, and hence should be carefully considered when designing electroanalytical systems.

The electrode and the active centre of a biocatalyst can be considered as a donor-acceptor pair and thus Marcus theory can be applied to describe the electron transfer rate between them (Marcus and Sutin 1985). Since a protein layer surrounds the active centre of an enzyme, the ET distance between the enzyme and the electrode is rather long. Due to an energy barrier for direct electron communication, ET occurs via a tunneling mechanism (Armstrong et al. 1985). This

makes direct electron coupling possible only for a limited number of biocatalysts having their redox active centre close to the periphery. However, several methods to link biocatalyst electrically with the electrode have been developed for the construction of bioelectrocatalytic systems. In general, ET is classified by two main mechanisms (Fig. 4): mediated electron transfer (MET) and direct electron transfer (DET). Problems related with establishment of efficient electron transfer and recent developments in biocatalytic systems based on these two mechanisms will be discussed in this section.

3.1.Mediated electron transfer

Since DET between the electrode and the enzyme is impeded for most redox enzymes, an intermediate electron carrier is necessary to transport the electrons (Fig. 4A) between the enzyme and the electrode. In the first description of an "enzyme electrode" by Clark and Lyons in 1962 (Clark and Lyons 1962), GOx was immobilised into a semipermeable membrane on top of an oxygen sensitive electrode and consumption of O_2, the co-substrate of the enzymatic reaction, was measured. In this case, O_2 acted as an indirect electron carrier between the enzyme and the electrode. In the same paper, detection of the product of the catalytic reaction, hydrogen peroxide, was described, and this again acted as an indirect carrier. Such types of biosensors, where changes in concentration of the biocatalyst (enzyme or whole cell) co-substrate or co-product of the biocatalytic reaction are monitored are often referred to as indirect or 'first generation biosensors'.

In many applications the dependence of the first generation biosensor response on co-substrate, usually oxygen, concentration and the high potentials required for operation, limit the overall performance of the sensors and restrict their applicability. So in the next step of bioelectrocatalysis development, the natural co-substrate was replaced by artificial redox compounds known as "mediators" which are able to transfer electrons between the biocatalyst and an electrode. Biosensors based on this principle are commonly referred to as 'second generation biosensors'. Despite some attempts to use ferricyanide, quiones, azines and metal

complexes as mediators in the 1970s (Kulys and Švirmickas 1979; Schläpfer et al. 1974), the wider application of mediated biosensors started with the discovery of immobilised enzyme electrodes using the ferricinium ion (oxidized ferrocene) as a mediator for GOx, reported in 1984 (Cass et al. 1984). This formed the basis for a new generation of commercial home blood glucose sensors that still dominate the market today.

3.1.1. Natural and artificial electron carriers

First generation biosensors

In spite of the already mentioned disadvantages of natural electron carriers for ET between the biocatalyst and the electrode, such a configuration remains relevant for construction of oxidase-based biosensors. The overall enzymatic reaction for the first generation of biosensors based on oxidases can be represented as follows:

$$\text{Substrate} + \text{Enzyme}_{ox} \rightarrow \text{Enzyme}_{red} + \text{product}$$

$$\text{Enzyme}_{red} + O_2 \rightarrow \text{Enzyme}_{ox} + H_2O_2$$

Here, the increasing H_2O_2 or the decreasing O_2 concentrations can be electrochemically detected in order to monitor the analyte concentration. Monitoring of the H_2O_2 concentration is typically used in biosensing applications today. Oxidation of hydrogen peroxide at an electrode surface at high anodic potentials has been applied in many recent works for biosensing in microchannels (Itoh et al. 2012; Rattanarat et al. 2015; Weltin et al. 2014), in paper based devices (Määttänen et al. 2013; Ruecha et al. 2014) and in wearable biosensors (Kakehi et al. 2007; Liu et al. 2012; Yao et al. 2011). However, high anodic potentials can introduce significant errors to analysis in complex matrices due to oxidation of easily oxidised interference compounds. In order to reduce the applied potential and accordingly the influence of interferences, various catalysts are used. Horseradish peroxidase, a natural biocatalyst, catalysing the reduction of hydrogen peroxide at potentials close to zero, can directly transfer electrons via its active center to the electrode (Yaropolov et al. 1979) and thus, in combination with another oxidases or by itself, be used to measure diverse substrates in complex matrices (Krikstolaityte et al. 2013; Liu and Crooks 2012;

Matharu et al. 2013). Utilisation of horseradish peroxidase (HRP) for hydrogen reduction has some disadvantages such as the relatively low stability of the biocatalyst and the need for proper orientation of the enzyme on the electrode surface for establishment of DET. The use of Prussian Blue (PB), also called 'artificial peroxidase', overcomes these disadvantages (Karyakin et al. 1994). In neutral media PB catalyses oxidation of hydrogen peroxide with a high rate constant (0.01 cm s^{-1}) according to the following mechanism (Karyakin et al. 1999):

$$H_2O_2 + 2e^- \xrightarrow{k_{cat}} 2OH^-$$

PB is widely applied in the literature for the construction of oxidase-based biosensors. It can be directly introduced in carbon ink and printed on paper (Dungchai et al. 2011; Jia et al. 2013a) or plastic-based substrates (Kim et al. 2014; Noiphung et al. 2013), or deposited on the electrode surface after electrode fabrication (Beni et al. 2015; Sekretaryova et al. 2014a).

Nanomaterials, such as metallic and metal oxide nanoparticles and carbon-based materials, possessing catalytic properties towards hydrogen peroxide oxidation are currently heavily used for biosensor construction, since they allow lowering of the applied potential as well as increasing the electrode surface area, and thus significantly increasing the sensitivity. There are many reviews published recently on the application of nanomaterials in biocatalytic systems (Shi et al. 2014; Wang and Dai 2015; Yang et al. 2015) and, therefore, they will not be discussed here.

Second generation biosensors

Utilisation of artificial redox compounds that can replace natural substrates (usually oxygen) in the biocatalytic reaction in second-generation biosensors avoids the oxygen dependence of the biosensor response and simultaneously lowers the working potential. In a catalytic reaction, the mediator first reacts with the enzyme active site and then participates in rapid ET with the electrode, where it is cycled between its oxidised and reduced forms (Chaubey and Malhotra 2002). In order to compete with natural electron carriers in the ET pathway, an artificial mediator should provide fast electron transfer kinetics. The redox potential of the mediator should be

chosen relative to the redox potential of the enzyme active site in such a way that an unforced flow of electrons between the biocatalyst's active site and the electrode is created. So in the case of oxidative biocatalysis, the redox potential of the mediator should be more positive than the potential of the active site, and in the case of reductive biocatalysis, more negative. Moreover, the potential should be low enough to reduce the influence of interfering compounds. Therefore, the range of appropriate mediators is limited.

Ferrocene and its derivatives, ferri/ferrocyanide, complexes of transition metals such as osmium and ruthenium, as well as redox organic dyes (mainly azines) are widely used redox mediators in biosensors (Chaubey and Malhotra 2002). Ferri/ferrocyanide are some of the most commonly used and most efficient soluble inorganic mediators (Dubinin et al. 1991; Ramsay and Turner 1988). However due to its small size and high solubility, it easily diffuses away from the electrode surface into the bulk solution, which reduces the long-term operational stability of the device. The less soluble ferrocene derivatives provide a partial solution to this problem and have achieved considerable success in commercial home-use devices, but the ferrocinium ion is still soluble (Cass et al. 1984). Tertrathiafulvalene (TTF) has been proposed as one alternative to ferrocene derivatives as an insoluble mediator for amperometric biosensors (Turner et al. 1987a). In spite of their high efficiency in mediating the ET of oxidases, osmium and ruthenium complexes (Motonaka et al. 1994; Ohara et al. 1993) have essential drawbacks due to their high toxicity.

In the first, simple configurations of biosensors, mediators were just added in solution with the enzyme and used as diffusional electron shuttles in homogeneous reactions (Kulys and Švirmickas 1979; Schläpfer et al. 1974). This configuration is still used in modern biosensing technology in order to characterise the effectiveness of new mediator-enzyme combinations in terms of electron transfer rates (Sekretaryova et al. 2014b; Wegerich et al. 2011) applying the well-developed methodology suggested by Nicholson and Shain in 1965 (Nicholson and Shain 1965) and further adapted for enzyme electrodes by Davis (Davis 1985). It is also often practiced

in paper-based microfluidics, where the enzyme and the mediator are stored in dry form in a special chamber and react homogeneously in solution when analyte is added (Chen et al. 2013; Liu and Crooks 2012; Nie et al. 2010a). Soluble mediators are now also widely used in commercial glucose sensors where they are applied as a dried layer, which is dissolved on entry of the sample to a capillary-fill chamber. In a more complex configuration, the enzyme is immobilised on the electrode surface and the mediator is added in solution and acts as a diffusional electron shuttle in a heterogeneous reaction (Parlak et al. 2015; Ramsay and Turner 1988; Zanini et al. 2011). This configuration is usually used when it is too difficult to immobilise both mediator and enzyme. Although the freely diffusing mediator allows efficient MET, systems with the mediator in solution have limited practical applications outside of "one-shot" disposable devices. Therefore, in modern bioelectrocatalytic systems for continuous sensing applications, the biocatalyst and the mediator are usually co-immobilised on the electrode surface.

3.1.2 Biocatalyst and mediator co-immobilisation

If all components needed for the electroanalytical reaction are immobilised on the electrode surface a 'reagentless biosensor' can be constructed. There are four main methods to immobilise the biocatalyst on the electrode surface: physical adsorption on the electrode surface, covalent bonding to the electrode, cross-linking of the biocatalyst and entrapment of the biocatalyst in membranes or polymeric matrixes (Sassolas et al. 2012). However, the immobilisation technique for a reagentless mediated biosensor should provide possibilities not only for biomolecule immobilisation, but also suitable biding sites for mediators and routes for efficient electrical communication between the biocatalyst and the mediator. In most of the cases, mediation is the rate-determining step of the overall ET process, limiting the bioelectrocatalytic functions of bioelectronic devices and thus, MET pathways should be carefully designed when assembling bioelectrocatalytic systems.

Systems with a diffusional mediator

Incorporation of the mediator in the conductive ink used for printing the working electrode is an effective, cheap and fast method for mediator integration (Cass et al. 1984; (Crew et al. 2011; Gilbert et al. 2011; Henao-Escobar et al. 2013; Pemberton et al. 2009). The biocatalyst can be immobilised on top of the printed, insoluble mediator using one of the above mentioned immobilisation techniques (Crew et al. 2011; Gilbert et al. 2011; Henao-Escobar et al. 2013) or mixed into the ink (Hart et al. 1996; Malzahn et al. 2011; Pemberton et al. 2009). The biorecognition layer can also be printed independently using different printing techniques, such as gravure printing (Pavinatto et al. 2015) and inkjet printing (Hart et al. 1996; Newman et al. 1992; Zhang et al. 2014). The ET mechanism in systems with a printed mediator and a biocatalyst immobilised on top is still not clear. It has been suggested that the mediator or mediating ion can slowly diffuse from the top layer into the solution forming a steady-state concentration near the electrode surface (Habermüller et al. 2000). This leads to a low long-term stability of such systems and to possible contamination of the biosensor enviroment by leaked mediator.

The use of hydrophobic compounds as mediators in hydrophilic media allows the mediator to be retained on the electrode surface. However, establishment of an effective electron communication between the hydrophobic mediator and the hydrophilic biorecognition element is challenging. Feroccene (Cass et al. 1984) and TTF (Turner et al. 1987a) are probably the most well known examples of hydrophobic mediators used for biosensor construction. Reduced forms of these mediators are hydrophobic, while oxidised forms formed in the enzymatic reactions are hydrophilic. It was shown that such mediators can be efficiently immobilised in polyelectrolyte membranes (Nafion) (Dong et al. 1992). The hydrophilic and hydrophobic domains in the Nafion structure allow both the hydrophilic and the hydrophobic forms of the mediator to be retained, as well as the biomolecule in the membrane, and to support the ET. This improves the stability of the biosensor significantly. Moreover, since Nafion is a cation conductor it works as an

additional barier for anionic interferencies. In most reports, the formation of membranes is carried out in aqueous media. Such conditions are, however, not optimal for the formation of uniform membranes, since polymer suspensions rather than real polymer solutions are used. In our work, we suggested co-immobilisation of a new water-insoluble mediator, unsubstituted phenothiazine, and a biocatalyst in the same sol-gel membrane, by carrying out immobilisation from water-organic media with a high content of organic solvent (Sekretaryova et al. 2014b; Sekretaryova et al. 2012). The concept of immobilisation from media with a high content of organic solvent was based on previously reported data of high enzyme stability in water-organic mixturers. Such a medium is favorable for membrane formation and leads to a stable film containing both hydrophobic and hydrophilic areas that allows efficient retention of the hydrophobic mediator and the hydrophilic biocatalyst in the same membrane. We suggested that the mediator has diffusional ability in such a membrane and thus can provide efficient electrical communication between the active site of the enzyme and the electrode. However, the complete mechanism of ET in membranes with hydrophilic and hydrophobic domains is still not clear and further investigations of such systems are underway. To further improve communication between a hydrophobic mediator and a biocatalyst, various conducting nanomaterials can be used. It has been shown that carbon nanotubes (CNTs) can form charge transfer complexes with TTF, a strong electron donor, through π-π interactions (Wu et al. 2003). In biocatalytic systems based on CNT-TTF complexes (Jia et al. 2013a; Jia et al. 2013b; Wu et al. 2003), CNTs serve as efficient electron conductors by forming a system of reticulated nanowires around the biocatalyst. The combination of CNTs with feroccene as an electron transfer system for biosensors has also been reported (Erden et al. 2015; Haddad et al. 2015). Graphene oxide and graphene flakes, possesing high electron conductivity and being rather new materials in biosensing technology, have recently been applied to enhance electrical communication in electrode-mediator-biocatalyst systems (Dey and Raj 2013; Peng et al. 2014).

Systems with covalently attached mediator

In all the examples mentioned, the mediator retained diffusional activity providing efficient electron transfer. The main disadvantage of such systems is the long-term risk of mediator leakage out of the membrane, which can lead to insufficient operational stability of the system for continious monitoring. Covalent attachment of the mediator to the electrode, biocatalyst or to the membrane, helps to overcome this issue. However, this raises the serious problem of providing efficient electron communication between the mediator and the biocatalyst. There are two main mechanisms suggested to have efficient ET with covalently attached enzyme. One is based on sequences of electron hopping reactions between the redox mediator molecules covalently attached to the matrix, providing so-called 'wires' for ET between the biomolecule and the electrode. A second mechanism suggests relative mediator mobility by its binding via long and flexible spacer chains either to the electrode surface, the membrane or the enzyme (Habermüller et al. 2000).

Polymers including redox-active sites and referred to as 'redox polymers' are arguably the most succsesful examples of enzyme 'wiring' for efficient electrical communication using covalently attached mediators. Redox polymers for biocatalyst immobilisation in biosensor construction were first employed by Heller's group (Degani and Heller 1989; Gregg and Heller 1990). GOx was modified by covalently attached 'electron relays' (Heller 1990). However, this configuration was found to be inefficient for ET, since the electron transfer was dependent on the orientation of the biomolecule. In further work (Degani and Heller 1989; Gregg and Heller 1990), Os-based polycationic redox polymers utilising an electron hopping mechanism were used to establish electrical communication between the enzyme active site and the electrode. Various Os- (Antiochia and Gorton 2014; Hasan et al. 2013; Liu et al. 2012; Zafar et al. 2012) and Ru-based (Deng et al. 2014) redox polymers are still widely used in modern biosensing systems for biocatalyst and enzyme co-immobilisation in order to establish an efficient electron transfer. The high toxicity of Os and Ru complexes has, however, restricted the application of conducting

polymers modified with redox mediators for biocatalyst immobilisation. Covalent functionalisation of conducting polymers can be achieved by synthesis of monomers functionalised with mediators, such as ferrocene and ferrocene derivatives (Dicks et al. 1988; Hendry et al. 1993; Şenel 2011; Soganci et al. 2014). Due to sterical factors, homopolymerisation of mediator modified monomers is usualy not possible and the formation of polymer films is achieved via co-polymerisation with unmodified monomers. Since the effectiveness of the electron transfer is controlled by the number of electron mediator units the co-polymerisation reaction should be controlled relative to the number of mediator-modified units. The mediator can also be added in the polymerisation mixture, containing monomer and biocatalyst, as a polymer dopant (Pesántez et al. 2012; Şenel 2011). Although this is a simple strategy for biocatalyst and mediator co-immobilisation, this methodology again raises the question of mediator stability inside the polymeric film, since the mediator is not attached covalently. Conducting polymers reperesent a promising matrix for biocatalyst and mediator immobilisation, since they stabilise the biomolecules and enhance effective communication between the biocatalyst and the electrode via an electron hopping mechanism. Moreover, conducting polymers are particulary suitable for immobilisation of biomolecules on miniaturised micro-sized interfaces (Dicks et al. 1988; Lupu et al. 2013; Pesántez et al. 2012; Ross and Cammann 1994; Xu et al. 2008).

Mediated electron transfer efficiency

It should be noted that for characterisation of MET efficiency of a new mediator-enzyme combination, measurements in solution using the theory for homogeneous reactions (Davis 1985; Nicholson and Shain 1965) are usually performed. The calculated MET rate constants can therefore be compared and the best mediator-enzyme combination can be chosen. However, significant differences exist between homogeneous and heterogeneous catalysis, that lead to different kinetics of reactions involving free and immobilised enzymes. Despite the large amount of research published on redox mediators, there is no common methodology in the literature to

estimate the efficiency of MET when the enzmye and mediator are immobilised on the surface. We have suggested using double-step chronoamperometry, which allows the electron transfer process for co-immobilized enzyme and mediator to be investigated (Sekretaryova et al. 2014b).

3.2. Direct electron transfer

Direct electron transfer is based on electron transfer from the electrode to the substrate directly via the active centre of the enzyme or *vica versa*. So the electrode acts as a second substrate for the enzymatic reaction (Fig. 4B). The hypothesis of DET between an enzyme and an electrode was deduced from Marcus theory (Marcus and Sutin 1985) as mentioned above. In the 60s to early 70s of the last century, the first reports of redox activity of some enzymes appeared (Betso et al. 1972; Guilbault 1966). In the late 70s, the first enzyme electrode based on direct bioelectrocatalysis was reported (Berezin et al. 1978). In this work, laccase was immobilised on a carbon black electrode and DET from molecular oxygen reduction by the enzyme was observed at 1.2 V. Biosensors based on DET are often referred to as 'third generation biosensors' (the definition is also valid for whole cell biosensors). A strictly limited number of enzymes possess DET properties. Amoung them are enzymes containing copper in their active sites (laccases, tyrosinase, billirubin oxidase), heme (cytochrome C peroxidase, fructose dehydrogenase, cellobiose dehydrogenase), pyrroloquinoline quinone (PQQ) (some dehydrogenases) (Karyakin 2012). However, even for these enzymes, proper orientation of the biomolecule on the electrode surface is required to observe DET since, according to Marcus theory, the rate of electron transfer between the enzyme active site and the electrode decreases exponentially with increasing donor-acceptor distance.In the case of proteins the electron transfer rate drops by a factor of about 10^4 when the donor-acceptor distance is increased from 8 to 17 Å (Gray and Winkler 1996). This means that when an enzyme is immobilised on the electrode surface, only the first layer of biomolecules can undergo DET, leading to low sensitivity and stability of such biosensors compared to MET based systems. Thus, special attention in the construction of DET biocatalytic systems must be paid to the design of suitable

surfaces for biocatalyst immobilisation by using new materials and various techniques of surface functionalisation that will be discussed further in this section.

3.2.1. Surface functionalisation of electrodes

DET between a biocatalyst and an electrode can be facilitated by functionalisation of the electrode surface for oriented immobilisation of the biocatalyst. Self-assembled monolayers (SAMs) represent one of the most commonly used ways of surface functionalisation, since they promote proper biocatalyst immobilisation and prevent denaturation of the proteins at the surface. Following the pioneering work by Hill and Taniguchi with co-workers on the modification of gold electrodes with a SAM for DET of cytochrome c (Taniguchi et al. 1982), further extensive investigations of SAM promotion of the direct electrochemistry of enzymes have been performed. For example, Patil *et al.* demonstrated direct electrochemistry of ascorbate oxidase, an enzyme containing copper in its active site, immobilised via chemisorptive interactions on a L-cysteine SAM-modified gold electrode (Patil et al. 2014). The authors investigated the thermodynamics and kinetics of biocatalyst adsorption, DET based on enzyme intramolecular electron transfer, the electrocatalytic activity towards ascorbic acid oxidation under an oxygen and nitrogen atmosphere and oxygen reduction. Suarez and co-workers suggested a methodology of cytochrome c chemical modification with short-chain thiol derivatives via lysine residues on the protein shell (Suárez et al. 2013). The modified enzyme adsorbs readily on the bare gold electrode forming a biological SAM exhibiting fast DET, with an electron transfer rate of 1600 s^{-1}. No ET was observed in the case of the unmodified enzyme, confirming facilitation of ET by efficient orientation of the enzyme active site via the SAM. The elaborated system was used for non-invasive detection of endogenous hydrogen peroxide released by unicellular aquatic microorganisms upon cadmium exposure. The SAM is a 2D immobilisation method, meaning that only biocatalyst in the first layer can communicate directly with the electrode surface. This leads to low biocatalyst loading and thus, to a low sensitivity of third generation biosensors. To increase the active surface area available for the enzyme

immobilisation, various 2D materials, such as nanotube/nanowire/nanorod arrays (Xu et al. 2010) and ordered porous materials (Zhu et al. 2009) have been used. Glennon and Razeeb with co-workers demonstrated facilitation of HRP DET on a SAM-modified nanowire array electrode (Xu et al. 2010). A vertically aligned nanowire electrode was fabricated by electrodeposition of gold into an anodic aluminum oxide membrane. HRP was immobilised covalently on the SAM. DET and electrocatalysis on the nanowire arrays with different lengths were investigated. The arrays with the longest nanowires showed better performance and the highest electron transfer rate (2.22 s^{-1}). The biosensor was used for detection of hydrogen peroxide via its catalytic reduction.

Entrapment or encapsulation of enzymes in a conducting material represents a 3D immobilisation technique, which provides a possibility for high loading of the electrode with the biocatalyst active in DET. This is especially important in miniaturised sensors with a small electrode surface in order to increase the biosensor sensitivity and stability due to the dramatic increase in the amount of biocatalyst. Sol-gel composites with nanomaterials or conducting polymers are the most commonly used matrix for 3D immobilisation. Shim and co-workers demonstrated DET of HRP covalently immobilised on a conducting polymer film of 5,2′:5′,2″-terthiophene-3′-carboxylic acid (Kong et al. 2003). Voltammetric studies proved the presence of DET between HRP and the electrode with an electron transfer rate of 1.03 s^{-1}. The sensor possessed electrocatalytic activity towards hydrogen peroxide reduction at low potentials. Li's group demonstrated the application of a polymer/room-temperature ionic liquid composite material as a matrix for haemoglobin immobilisation, facilitating DET (Lu et al. 2006). Haemoglobin was immobilised in a chitosan matrix with 1-butyl- 3-methyl-imidazolium tetrafluoroborate ionic liquid, which served as a conductor for ET between hemoglobin and the electrode. Well-defined redox peaks were observed in cyclic voltammograms due to haemoglobin DET. The electrocatalytic properties of the functionalised electrode towards oxygen and trichloroacetic acid were demonstrated. Many works have been published recently

on the use of nanomaterials for DET facilitation in 3D immobilisation of biocatalysts and therefore it will be considered separately in the next section.

3.2.2. Nanomaterial-based electrodes

Application of nanomaterial-based or functionalised electrodes to facilitate direct electrochemistry of enzymes has been extensively investigated over the last decades. It has been suggested that nanomaterials, having similar dimensions as biomolecules, can form better contact with enzyme molecules by reducing the distance between the redox centre of the protein and the electrode surface thus increasing the rate of DET (Liu et al. 2013). Various nanomaterials, such as carbon nanotubes (Ding et al. 2010; Tasca et al. 2011), nanoparticles (Guo et al. 2011; Murata et al. 2009) and more recently graphene-derived materials have been used for the construction of DET-based biosensors.

Carbon nanotubes

Gorton's group reported efficient DET between cellobiose dehydrogenase and surface modified single-wall carbon nanotubes (SWCNTs) (Tasca et al. 2011). The SWCNTs were modified with aryl diazonium salts to form carboxylic or amino groups on the surface of the nanotubes, having negative or positive charges, respectively, in acidic pH. The enzyme was immobilised on the surface of the modified SWCNTs via adsorbtion. The modified electrodes having positive amino groups showed fast DET with current densities of 500 μA cm^{-2} at 200 mV vs NHE in a 5 mM lactose solution, which is close to the values reported for MET-based cellobiose dehydrogenase-modified electrodes. In another example, haemoglobin, a haeme containing protein, and SWCNTs were co-electrospun to form SWCNT-haemoglobin microbelts (Ding et al. 2010). FT-IR spectroscopy was performed to demonstrate that electrospinning and incorporation of SWCNTs into the microbelts did not destroy the native structure of haemoglobin. The microbelts showed fast haemoglobin DET kinetics towards catalytic reduction of hydrogen peroxide, trichloroacetic acid and nitrite. This biosensor was applied for analysis of tap water spiked with

the aforementioned analytes. The work demonstrated the first example of incorporating carbon nanotubes into electrospun protein microbelts.

There are plenty of papers published recently claiming DET of GOx immobilised on CNTs. Two different mechanisms of ET are suggested. According to the first one, a CNT penetrates into a GOx molecule, the second hypothesis suggests that the enzyme molecule partially unfolds on the CNT providing electrical contact between its active site and the CNT (Wooten et al. 2014). The possibility of GOx based DET has raised much debate in the scientific literature, since it was shown that GOx is a structurally rigid glycoprotein with a hydrodynamic radius of 43 Å, consisting of two subunits, each containing active sites and facing each other (Wilson and Turner 1992). Having in mind the rapid decay in electron transfer with increasing distance, this structure dictates the inability of GOx to undergo DET in its native catalytically active conformation. Gorski and co-workers conducted very careful and persuasive experiments on DET between CNTs and GOx (Wooten et al. 2014), where GOx and CNTs were encapsulated in a chitosan matrix. The voltametric investigations showed a pair of surface-confined current peaks, which are usually used in literature as an indication of DET for GOx. However, the system did not show any catalytic response (an increase of the anodic peak during cyclic voltammetry) on additions of glucose into an oxygen free solution. The observed voltammetric peaks were due to the redox activity of an enzyme cofactor, flavin adenine dinucliotide, which had dissociated from the active centre of the enzyme. This result strongly indicates the absence of DET between enzymatically active GOx and the electrode, not only in the demonstrated example, but arguably in all types of GOx-CNT-based sensors. In the opinion of the authors, this might be the case not only for carbon nanotubes, but also for the DET reported for GOx and other nanomaterials and hence, such works will not be considered in this review.

Nanoparticles

Metal and metal oxide nanostructures are widely applied for the construction of DET-based biosensors to facilitate electron transfer between enzymes and the electrode (Guo et al. 2011;

Murata et al. 2009; Palanisamy et al. 2012). Li and co-workers reported immobilisation of HRP on negatively charged gold nanoparticle-coated polyaniline nanowires, for DET-based *in situ* detection of hydrogen peroxide released extracellularly from ishemic cells (Guo et al. 2011). FT-IR measurements showed no denaturation of HRP due to immobilisation. Voltammetric studies were performed to investigate the DET. The electron transfer rate constant was calculated to be 3.52 s^{-1}. In another work, a three-dimensional AuNP electrode without additional modification with a thiol self-assembled monolayer, was used to achieve bilirubin oxidase DET for oxygen reduction (Murata et al. 2009). The electrode showed DET current densities up to 6 mA cm^{-2} in O_2 saturated buffer solution, which is even higher than that shown for MET. The combination of carbon nanotubes and metal-based nanostructures for haemoglobin DET in hydrogen peroxide reduction has also been reported (Palanisamy et al. 2012). ZnO sponges of 500 nm – 1 μm were grown on multiwalled CNTs and used as a matrix for haemoglobin immobilisation using the electrostatic interaction between the positively charged ZnO/CNTs composite and the negatively charged enzyme. The CNTs provided interconnection of individual ZnO sponges and served as a template for the initial growth of the ZnO structures. UV-VIS spectroscopic analysis confirmed the absence of enzyme denaturation due to the immobilisation. DET was investigated using cyclic voltammetry, which showed the highest peak current densities for the final composite compared to all other combinations of the materials used for the composite fabrication. The electron transfer rate constant was calculated to be 1.26 s^{-1}. It should be noted, that when a combination of various nanomaterials is used for DET-based biosensor construction, the influence of all the components on the DET rate should be investigated separately in order to draw conclusions on the advantageous properties of the final composite for DET. Such investigations, unfortunately, are rarely performed in published reports.

Graphene-related materials

Graphene-related structures, being relatively new materials for biosensor construction, attract considerable attention (Huang et al. 2011; Lu et al. 2010a; Wu et al. 2010; Zuo et al. 2010). The

high conductivity of graphene in the lateral direction makes it an alternative to the previously widely used CNTs for promotion of DET between the active site and the electrode (Zuo et al. 2010). Wu *et al.* reported the application of chitosan-dispersed graphene nano-flakes prepared by chemical reduction of graphene oxide, as a platform for cytochrome c adsorption (Wu et al. 2010). Voltammetric studies demonstrated the promotion of DET by the graphene flakes. The calculated electron transfer rate was 1.95 s^{-1}, which was higher than the value reported for a similar system using CNTs instead of graphene flakes. The biosensor was used for nitric oxide sensing via its DET-based catalytic oxidation by cytochrome C. In another paper, HRP direct electrochemistry on single-layer graphene nanoplateletes was investigated (Lu et al. 2010a). The platelets were functionalised with tetrasodium 1,3,6,8-pyrenetetrasulfonic acid in order to avoid aggregation of individual single layer graphene sheets. Voltammetric studies demonstrated an enhancement of DET between the enzyme and the electrode surface when using graphene nanoplatelets. UV-VIS spectroscopy indicated that the enzyme in the composite film retained its secondary structure in a form similar to the native state. The biosensor showed a fast DET-based response towards hydrogen peroxide. Hybrid structures using nanoparticles and graphene-based materials for DET facilitation are also reported as in the case of CNTs.

Huang and co-workers reported one example of DET of catalase on a nanocomposite material, consisting of graphene functionalised with amino groups and AuNPs. Voltammetry revealed promotion of DET between the enzyme and the electrode by the composite and the synergistic effect of graphene and AuNPs (Huang et al. 2011). The electron transfer rate was calculated to be 2.34 s^{-1}. The biosensor possessed catalytic properties towards hydrogen peroxide.

Our comment on the investigation of electron transfer properties of hybrid materials consisting of several nanomaterials is also valid for graphene-based composites. In all mentioned examples, the number of layers in the graphene-based materials used in the work is not controlled. Since graphene flakes have a tendency to aggregate and to form graphite structures, it is questionable to refer to this material as graphene. According to the IUPAC definition, graphene is a *'single*

carbon layer of the graphite structure...The term graphene should be used ***only*** *when the reactions, structural relations or other properties of* ***individual layers*** *are discussed.'* Another term should be used when the number of layers in graphene-based materials is not controlled to avoid misunderstanding of the ET properties of real graphene. To the best of our knowledge, DET properties of single layer graphene has not been investigated yet.

We would like to emphasise that changes in the electrode capacitive properties should be taken into consideration when making conclusions on DET facilitation between a biocatalyst and an electrode modified with nanomaterials. Immobilisation of nanomaterials leads to a significant increase in the electrode capacitance that can be observed, for example, from voltammetric studies as a 'thickening' of the voltammogram. Therefore, higher peak currents attributed to DET between an electrode and an enzyme in the case of nanomaterials do not always mean more effective electron transfer. In order, to make a solid conclusion on facilitating DET when using nanomaterials, a background subtracted voltammogram (with deduction of the voltammogram under similar conditions, but without biocatalyst) should be presented. Another important issue is the necessity to perform experiments on DET transfer under conditions when natural electron shuttling (very often via oxygen) is avoided, in order to ensure that direct electrochemistry between the enzyme and the electrode is occurring and to exclude the possibility of electron transfer by other mechanisms. Unfortunately, these conditions are very seldom met in published works.

4. Concluding remarks and overall outlook

Biocatalytic systems continue to play an important role in health applications, such as personal diagnostics, pharmacology, food safety and environmental monitoring. Herein, we have reviewed some of the most important trends in biosensing technology in our view. We believe that the increasing demand for personal analysis, combined with the recent progress in information and communication technology, will stimulate development of new inexpensive, portable and integrated biosensing systems suitable for point of care testing. The rise of mobile health applications available on smartphone platforms dictates a key direction for future biosensor development towards systems that can interface directly with modern telecommunications. All-printed and paper-based, pocket-size devices and wearable biosensors, integrated with wireless communication technologies, are likely to play significant role in aforementioned applications.

Despite the extensive research published on the development of new bioelectrocatalytic systems for health applications, enzyme-based blood glucose biosensors continue to dominate the world market for biosensors in terms of dollar sales. They also still feature heavily in the academic literature, at least partly because they provide a good model "gold standard" for comparison of techniques. One note of caution in interpretation this data, however, is that dollar sales are not necessarily the most important indicator of success. Such data are heavily influenced by the large number of sensors used by any one individual with Type 1 diabetes, and this masks the success of many other commercial bioelectrocatalytic systems meeting very real clinical and other needs. However, as pointed out in this review, a number of issues need to be addressed before a new bioelectrocatalytic systems can make an impact in real-life applications. For all aspects of the future development of biosensing systems, further optimisation of the sensor architectures in terms of stability, reproducibility and sensitivity is still required. Therefore, methods of biocatalyst immobilisation that provide for both their stabilisation and for facilitating electron transfer have been considered in the review. Further research to understand electron transfer

mechanisms, for both direct and mediated pathways, and to develop new and more efficient ways to modulate electron transfer in biocatalytic systems are required for the future further commercial success of novel biosensing devices and to realise the now, often quoted, vision of wearable sensors connected to the internet of things.

Figure legends

Figure 1. Miniaturised systems for biosensing. (A) Layouts of screen-printed microband single and multilayer arrays and optical microscopy image of graphite layers appearing as five dark interfacial layers. Reprinted with permission from (Vagin et al. 2014). Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) Flexible Au IDE inkjet-printed on plastic and optical microscopy image of electrode modification with gravure-printed enzyme-containing layer. Reprinted with permission from (Pavinatto et al. 2015). Copyright © 2014 Elsevier B.V. (C) Microdevice for fish freshness monitoring. Reprinted with permission from (Itoh et al. 2012). Copyright © 2012 Elsevier. (D) Sensing chip containing micropatterned gold MEA modified with HRP and with hepatocytes grown on a chitosan membrane as schematically illustrated. Reprinted with permission from (Matharu et al. 2013). Copyright © 2013, American Chemical Society.

Figure 2. Paper-based biosensing devices. (A) Dumbbell shaped μPAD with integrated plasma isolation for glucose determination in whole blood placed on a carbon SPE. Reprinted with permission from (Noiphung et al. 2013). Copyright © 2013 Elsevier B.V. (B) Integration of a carbon paste microelectrode into an EμPAD. Reprinted with permission from (Santhiago et al. 2013). Copyright © 2013, American Chemical Society. (C) EμPADs integrated with a commercially available glucometer for analysis of glucose, cholesterol and L-lactate. Adapted from (Nie et al. 2010a) with the permission from The Royal Society of Chemistry. (D) Hy-PE paper-based integrated device. Reproduced with permission from (Beni et al. 2015). Copyright 2015, The Electrochemical Society.

Figure 3. Wearable sensing systems and self-powered biosensors. (A) Contact lens biosensor for glucose monitoring Reprinted from (Yao et al. 2011) with permission from Elsevier. (B) Tattoo based epidermal biosensor for real-time continuous monitoring of lactate in sweat. Adapted with

permission from (Jia et al. 2013a). Copyright © 2013, American Chemical Society. (C) Single enzyme based self-powered biosensor generating a current proportional to the free cholesterol concentration. Reprinted with permission from (Sekretaryova et al. 2014a). Copyright © 2014, American Chemical Society. (D) Self-powered biosensor integrated with a PB display for visual electrochemical detection of ascorbic acid (AA). Reprinted with permission from (Zloczewska et al. 2014). Copyright © 2013 Elsevier B.V.

Figure 4. Schematic illustrations of electron transfer mechanisms utilised for biosensing. (A) Mediated electron transfer. (B) Direct electron transfer.

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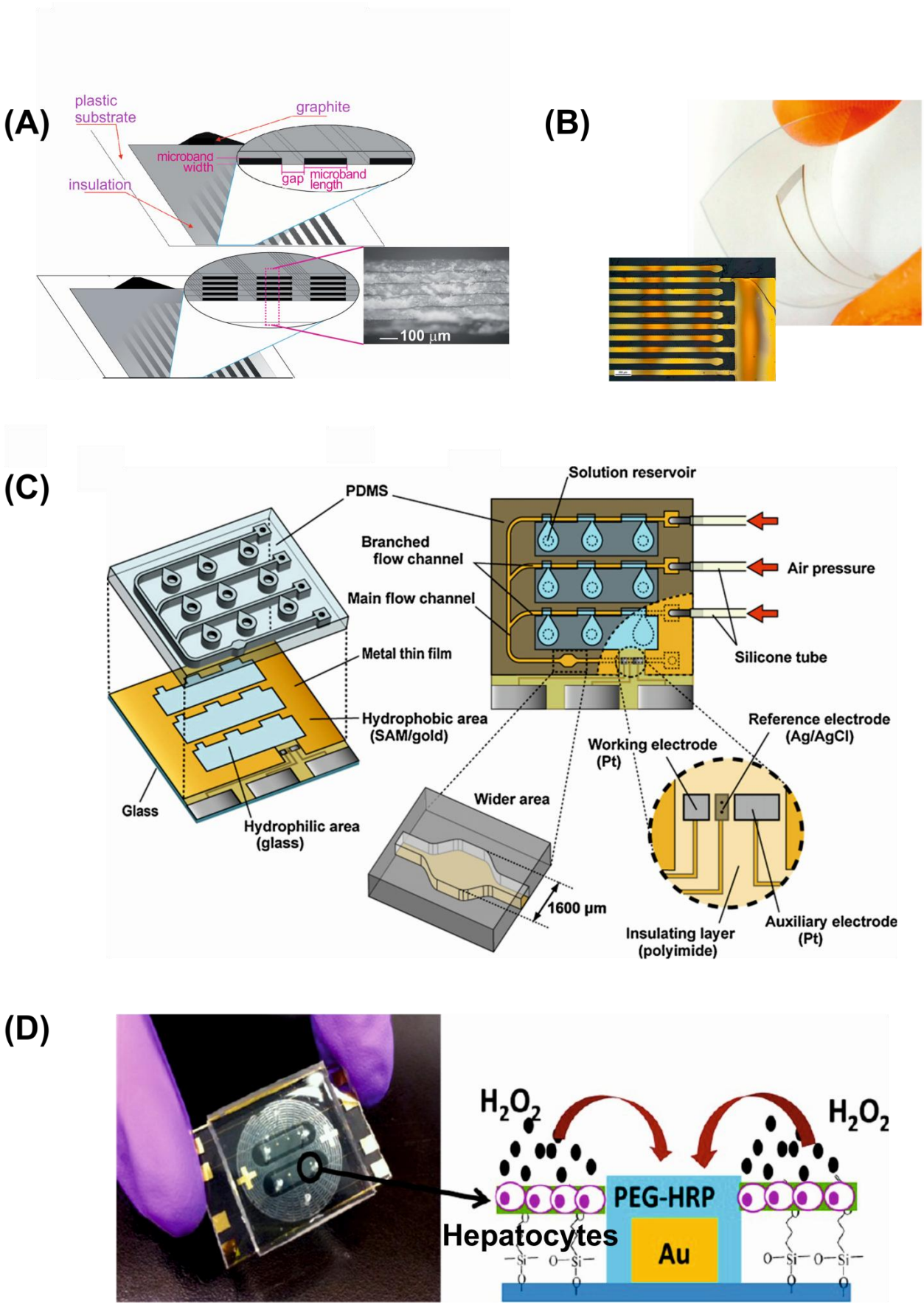

Fig. 1

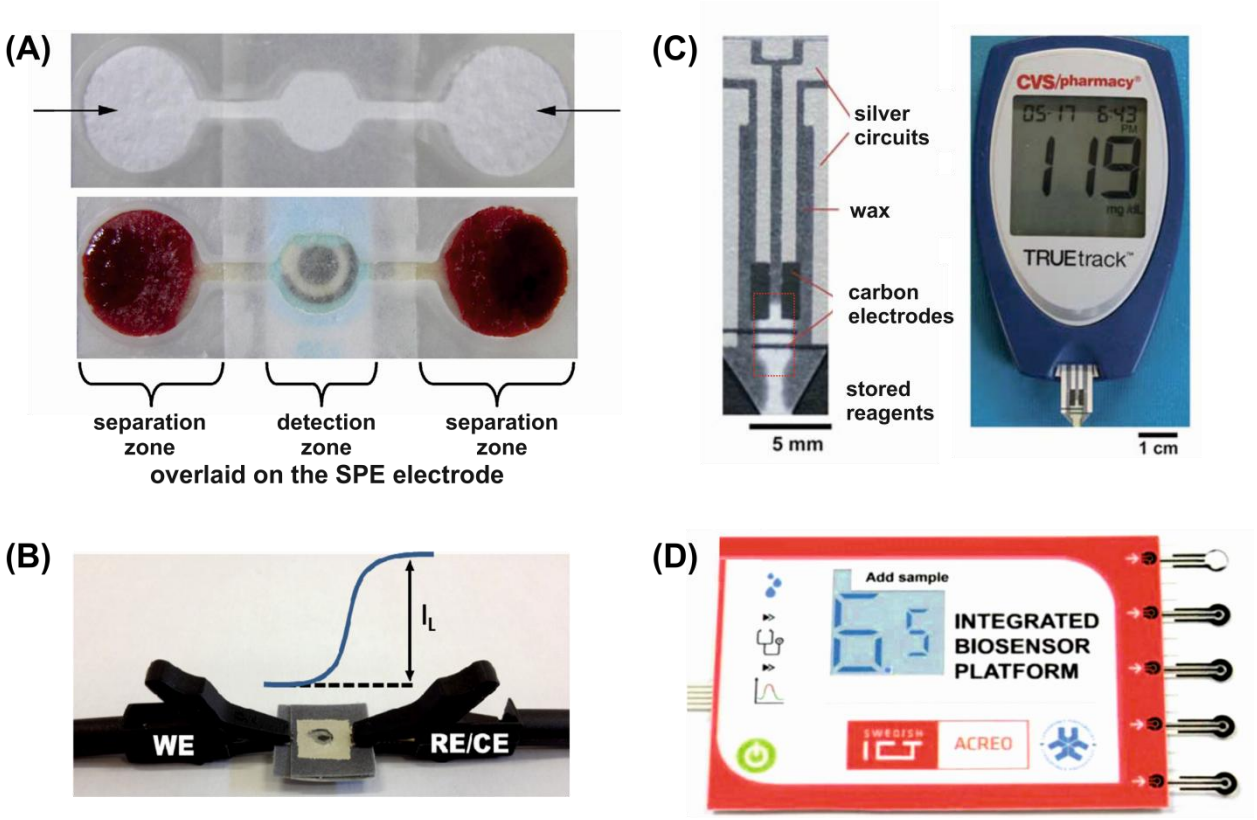

Fig. 2

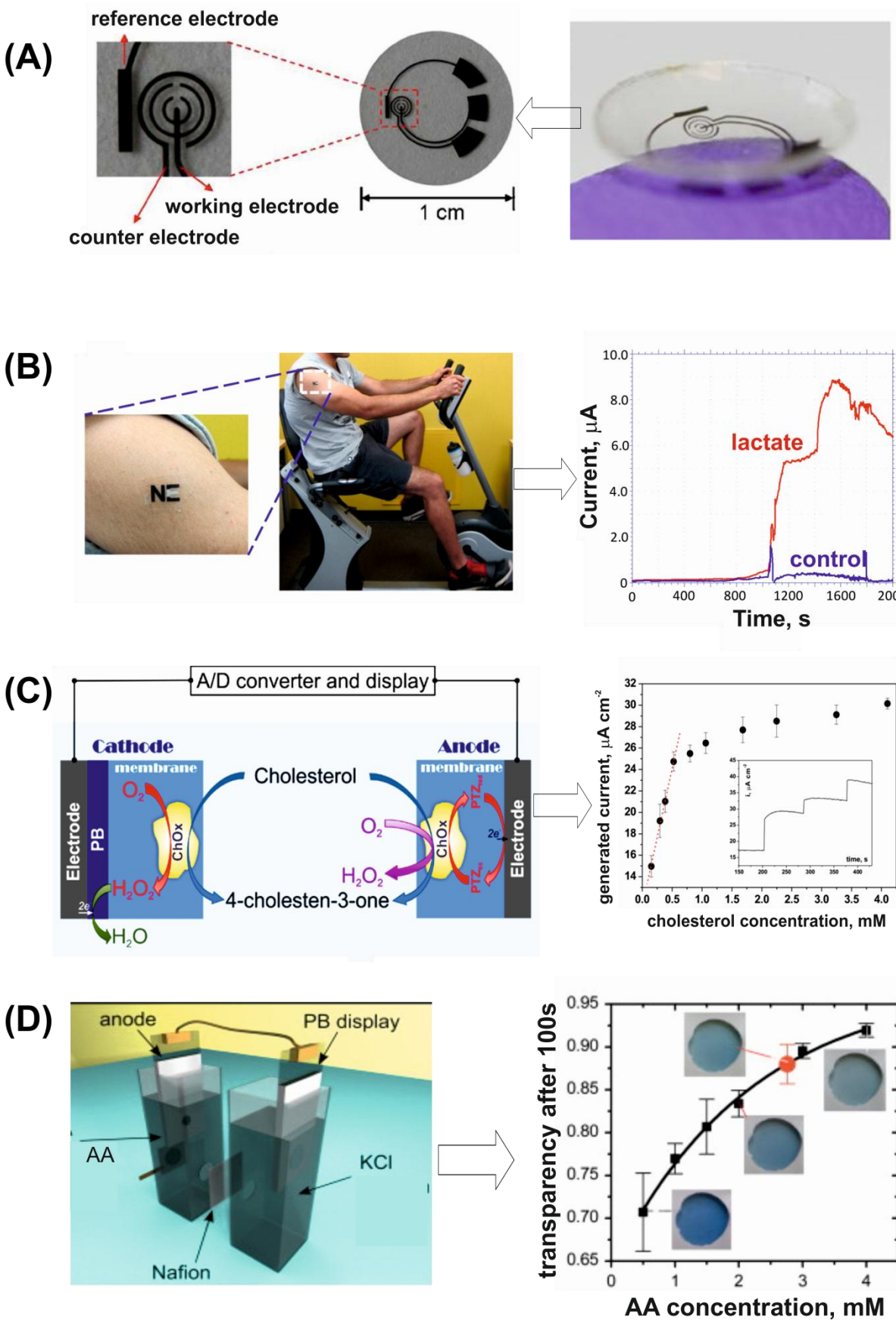
(A)
reference electrode
working electrode
counter electrode
1 cm
(B)
Current, μA
lactate
control
Time, s
(C)
A/D converter and display
Cathode
membrane
Anode
membrane
Electrode
PB
ChOx
Cholesterol
4-cholesten-3-one
H2O
generated current, μA cm-2
cholesterol concentration, mM
(D)
anode
PB display
AA
KCl
Nafion
transparency after 100s
AA concentration, mM

Fig. 3

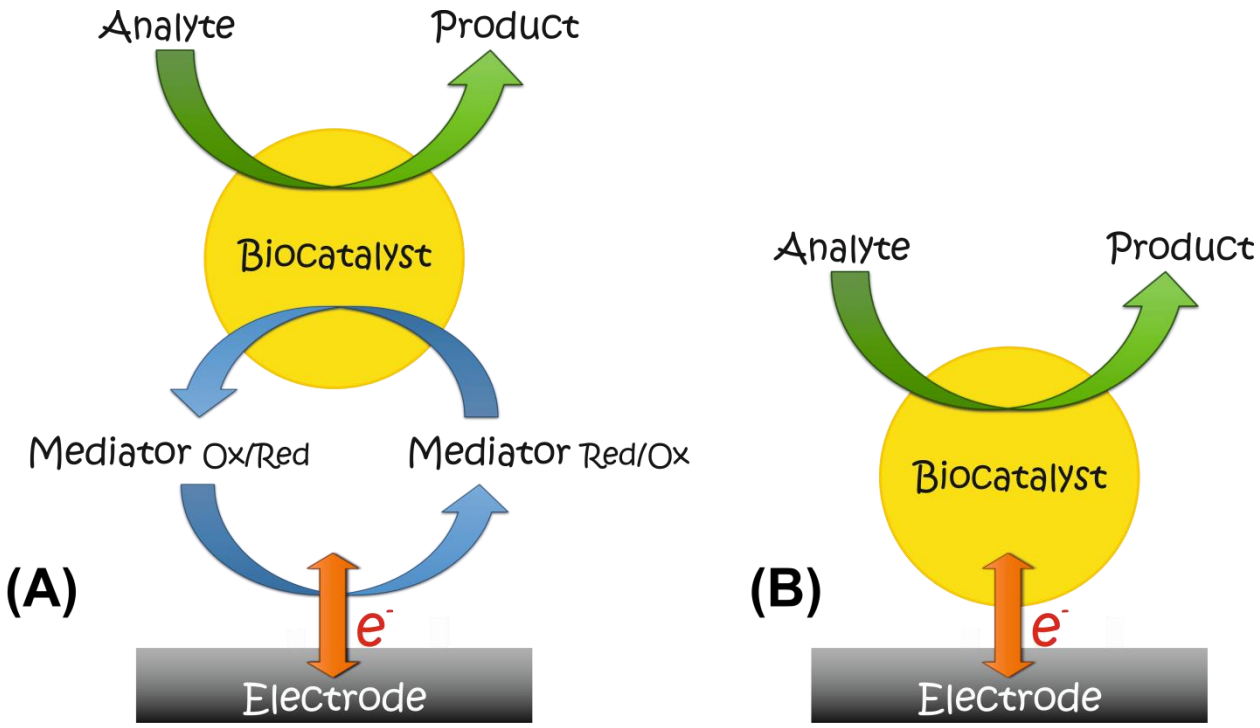

Fig. 4