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MINI-REVIEW

Strategies to extend the lifetime of bioelectrochemical enzyme electrodes for biosensing and biofuel cell applications

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Abstract Enzymes are powerful catalysts for biosensor and biofuel cell electrodes due to their unique substrate specificity. This specificity is defined by the amino acid chain's complex three-dimensional structure based on non-covalent forces, being also responsible for the very limited enzyme lifetime of days to weeks. Many electrochemical applications, however, would benefit from lifetimes over months to years. This mini-review provides a critical overview of strategies and ideas dealing with the problem of short enzyme lifetime, which limits the overall lifetime of bioelectrochemical electrodes. The most common approaches aim to stabilize the enzyme itself. Various immobilization techniques have been used to reduce flexibility of the amino acid chain by introducing covalent or non-covalent binding forces to external molecules. The enzyme can also be stabilized using genetic engineering methods to increase the binding forces within the protein or by optimizing the environment in order to reduce destabilizing interactions. In contrast, renewing the inactivated catalyst decouples overall system lifetime from the limited enzyme lifetime and thereby promises theoretically unlimited electrode lifetimes. Active catalyst can be supplied by exchanging the electrolyte repeatedly. Alternatively, integrated microorganisms can display the enzymes on their surface or secrete them to the electrolyte, allowing unattended power supply for long-term applications.

Keywords Enzyme inactivation · Biofuel cell · Biosensor · Amino acid replacement · Immobilization · Self-regeneration

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Introduction

Redox enzymes catalyze electron transfer reactions with unique selectivity. In contrast to traditional abiotic catalysts such as platinum, they are mostly unaffected by the simultaneous presence of fuel and oxidant and typically do not get passivated by impurities (Moehlenbrock and Minteer 2008). This and their availability for a wide variety of fuels and working conditions makes them particularly suitable biocatalysts for many electrochemical applications in the field of sensing and energy generation. Biosensors for the direct analysis of untreated samples or for monitoring of environmental parameters depend on the catalysts selectivity. The same applies for energy harvesting biofuel cells, which can efficiently convert ambient fuels in order to supply energy-autonomous distributed and decentralized systems (Woias et al. 2005). Such systems cannot be provided with purified fuels like conventional fuel cells but have to make use of a crude mixture of fuel, oxidant, and various kinds of impurities present in their environment.

The redox enzyme's outstanding specificity derives from the protein shell around the active center. It acts as selective barrier and allows only one or few substrates to enter the catalytic site and thereby to be converted. The protein shell, however, also creates a barrier for the electrons being transferred between active center and electrode (Barton

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et al. 2004). Only a few enzymes have active centers close enough to the surface or intramolecular electron transfer mechanisms to directly interact with the electrode (direct electron transfer). Most enzymes require mediators to shuttle the electrons between active enter and electrode (mediated electron transfer).

The complex structure of the protein shell is also responsible for the very limited lifetimes of enzymatically catalyzed bioelectrochemical electrodes, which are typically in the range of days to few weeks (Minteer et al. 2007), while many applications would benefit from lifetimes over months to years. In most cases, damage of the fragile three-dimensional conformation is responsible for the inactivation of the enzymes' catalytic activity. The primary structure as a linear sequence of several hundred amino acids only provides the basis for a complicated secondary and tertiary structure. Numerous interactions of backbone and side chain atoms cause a three-dimensional coiled arrangement of the polypeptide chain(s), in larger proteins organized within several independent folding domains. In proteins containing more than one amino acid chain, further interactions define quaternary structures, the spatial arrangement of the subunits (Voet et al. 1999).

Correct folding of the entire protein is necessary to achieve catalytic activity: Reactions are catalyzed at the active center, usually placed in the center of the protein and consisting of amino acids from different sites of the polypeptide chain, quite often arranged around metal ions or a coenzyme. For instance, in glucose oxidase, an enzyme frequently used in biofuel cell anodes and biosensors, the active center includes the six amino acids Y68, E412, F414, W426, H516, H559, and the coenzyme flavin adenine dinucleotide (FAD) as shown in Fig. 1 (Meyer et al. 1998).

Protein conformation is stabilized by a variety of interactions based on hydrophobic effects, electrostatic interactions, coordinative complexes, and sometimes even covalent disulfide bonds. Under physiological conditions, however, there is a low difference in a protein's free energy of only about 40 kJ/mol between native (folded) and denatured (unfolded) protein (Voet et al. 1999). Several influences may reduce an enzyme's lifetime by subunit dissociation or by disturbing the balance of weak non-covalent forces that maintain the native conformation (Voet et al. 1999; Igarashi and Sode 2003): Protein structures contain flexible regions, allowing vibration. Thus, even at room temperature, the kinetic energy of some parts of an enzyme molecule can be high enough to rearrange weak non-covalent interactions and thereby inactivate the enzyme. Chemical modifications can also lead to changes within the conformation. The solvent water for instance may react with proteins, resulting in deamidation of glutamine and asparagine residues, whereas oxidation modifies thiol groups (Manning et al. 1989).

This review summarizes state-of-the-art strategies and concepts to overcome the lifetime limitation of bioelectrochemical electrodes caused by enzyme inactivation: The first section presents techniques to stabilize the enzyme itself by genetically optimizing the enzyme structure, immobilizing the enzyme, or reducing its interactions to water. The second section presents concepts to decouple electrode lifetime from the limited enzyme lifetime by renewal of the catalyst. This renewal can be realized by repeated manual exchange of the enzyme-containing electrolyte or by integration of microorganisms that continuously secrete active enzyme into the electrolyte or display it at their surface.

Electrodes with stabilized enzymes

Stabilizing the enzyme itself is one approach to extend the lifetime of enzymatically catalyzed electrodes. The following section summarizes different ways to stabilize enzymes. Introduction of additional covalent or non-covalent bonds within the protein structure or to external matrices, as well as the removal of unfavorable sterical effects, can increase the energy required to denature the enzyme. Also, destabilizing interactions with the solvents can be reduced. All

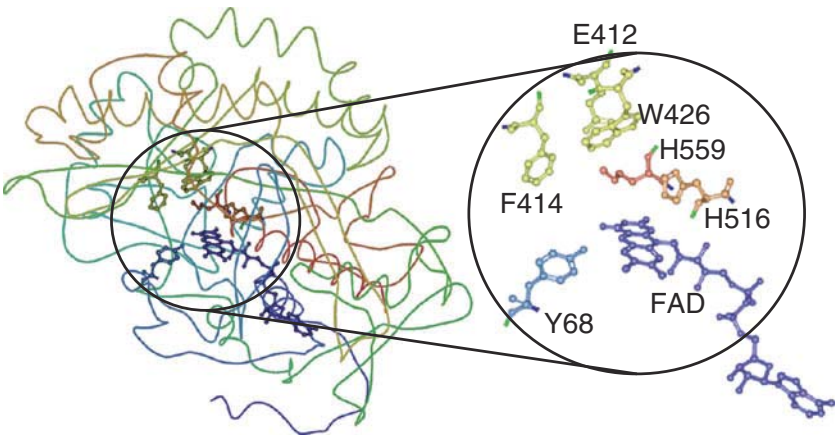

Fig. 1 Structure of glucose oxidase from *Aspergillus niger*. The active center consists of six amino acids located at different sites of the primary structure and the FAD coenzyme. All of them have to be correctly arranged to ensure enzymatic activity (Meyer et al. 1998). Enzyme structure 1CF3 (Wohlfahrt et al. 1999) was depicted with PDB ProteinWorkshop (Moreland et al. 2005)

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theses approaches lead to enzymatic catalysts with an extended but still limited lifetime (Wilkins et al. 1995).

Stabilization by altering the enzyme's amino acid sequence

Genetic engineering methods allow for changing the enzyme's amino acid sequence in order to introduce further/stronger interactions, remove unfavorable steric effects, or replace potential sites for chemical degradation (Manning et al. 1989). Introduction of amino acid replacements by *rational protein design* requires knowledge of the target protein structure and a substantial understanding of the structure–function relationship (Katchalskikatzir 1993). In contrast, *directed protein evolution* uses cycles of generating mutant libraries and screening for improved protein variants. Structural knowledge is not required, but the method is more time consuming, and its success depends on a careful selection of screening conditions (Güven et al. 2010).

For example, Koji Sode et al. were able to significantly improve the stability of water-soluble pyrrolquinoline quinone glucose dehydrogenase (PQQGDH) from *Acinetobacter calcoaceticus*, a homodimeric enzyme. For the stabilization of the quaternary structure, they used site-directed mutagenesis to replace a serine residue by a cysteine, adding a disulfide bond at the dimer interface. Decreased monomer dissociation of the mutant S415C increased the half-life at 55 °C more than 36-fold compared to the wild-type enzyme (Igarashi and Sode 2003). When tested as anode enzyme in a fuel cell with a bilirubin oxidase catalyzed cathode, the power output after 10 h of continuous operation was decreased by only 20% compared to 70% in case of the wild-type enzyme (Yuhashi et al. 2005).

To conclude, modifications of the protein structure by *rational protein design* or *directed protein evolution* require a broad knowledge and are very labor-intensive. However, they have successfully been applied to create stabilized enzymes without reducing their activity or changing the specificity. In addition, genetic engineering methods can also be used to further optimize the enzyme in terms of direct electron transfer (Okuda and Sode 2004), catalytic activity (Zhu et al. 2006), or substrate specificity (Sode et al. 2002).

Stabilization by interaction with an external matrix

Immobilization by binding of an enzyme to an external organic or inorganic matrix introduces additional covalent or non-covalent interactions. This decreases the enzymes' structural degrees of freedom and its liability for denaturation. This can also prevent leaching of enzyme from the electrode, enabling membrane-less biofuel cells. As immobilization is the most common enzyme stabilization method, it has already been reviewed for its ability to extend the lifetime of bioelectrodes (Moehlenbrock and Minteer 2008; Brito and Turner 2010).

Figure 2 illustrates the five different strategies to immobilize enzymes at electrode surfaces as categorized by Cooney et al. (2008): Physical adsorption as well as covalent binding forms an enzyme monolayer at the electrode surface and allows or even improves direct electron transfer. Both methods reduce or avoid enzyme leaching, but binding to the planar surface can lead to decreased stability or even protein denaturation. Cross-linking of enzymes usually increases their stability at the expense of a decreased activity. Microencapsulation into micelles or micellar polymers offers the highest potential to significantly increase enzyme lifetime and stop enzyme leaching, although mass transfer problems may occur. Entrapment by mixing enzymes into a polymer mostly only decreases their leaching. Nonetheless, this technique is widely used in biosensors and biofuel cells as it allows co-immobilizing electron transfer mediators.

Ye et al. (2005) reported the entrapment of glucose oxidase from *Aspergillus niger* into electropolymerized poly-*o*-aminophenol on a phthalocyanine-functionalized carbon nanotube electrode. The sensor fabricated with this electrode used the indirect detection principle of iron-phthalocyanine catalyzing the oxidation of hydrogen peroxide, a byproduct of glucose oxidase. At 25 °C, the sensor's sensitivity remained constant over 15 days and decreased to 77.2% after 90 days.

Kerr and Minteer (2006) even gave a conference presentation on a lipogenase bioanode stable for over 1 year without significant power degradation using microencapsulation into modified Nafion.

In general, immobilization techniques represent the most popular strategy to enhance the lifetime of enzymatic electrodes. They can be easily adapted from one enzyme-electrode system to another and are able to significantly stabilize the polypeptide structure. As shown above, in some cases, even lifetimes within the relevant range of months to years have been reported. In other cases, however, also the polymer itself can be a source of instability (Binyamin et al. 2001), diffusion limitations may occur, and quite often, reproducibility and a poor spatially controlled deposition are a problem (Cosnier 1999).

Stabilization by reduced interactions with the solvent

Many enzymes can be stored over extended time periods as lyophilized preparations. These are more resistant against inactivation processes due to reduced interactions with water (Hageman 1988). Multi-pulse electrode activation

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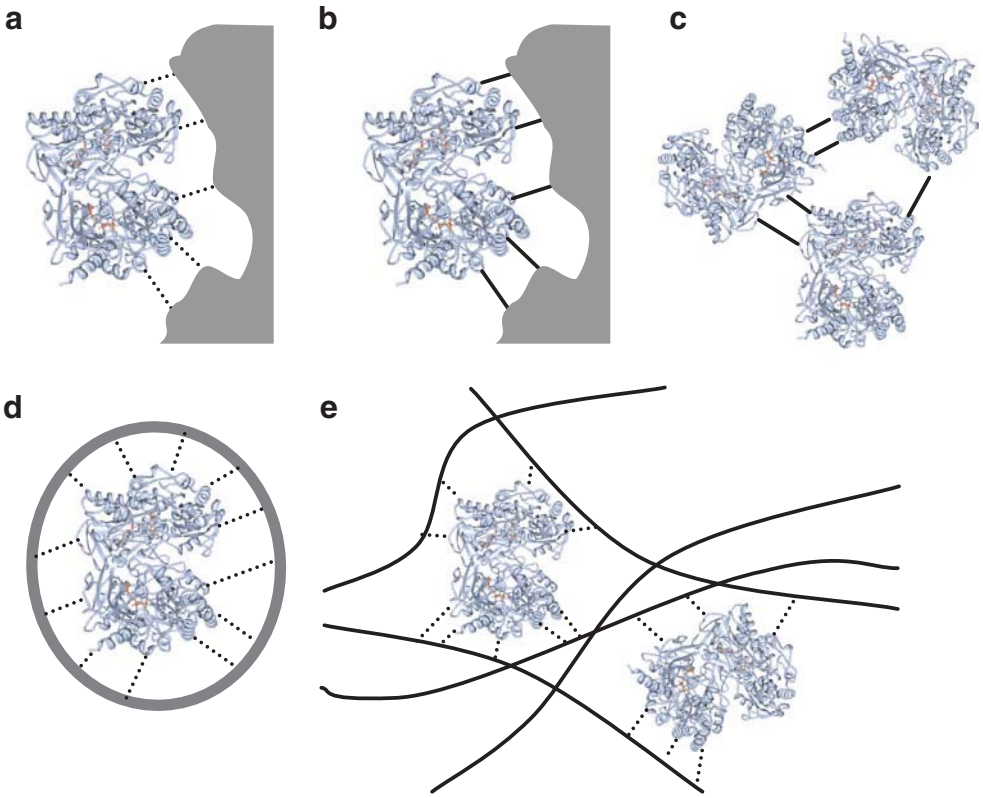

Fig. 2 Enzyme stabilization by immobilization introduces additional covalent and non-covalent forces to an external matrix. **a** Non-covalent physical adsorption of an enzyme to the electrode, **b** covalent binding of an enzyme to the electrode, **c** covalent cross-linking of enzymes, **d** microencapsulation of an enzyme by a micelle or a micellar polymer, and **e** entrapment of an enzyme into a polymer

uses this principle in bioelectrochemical systems: As shown in Fig. 3, an array of several fuel cells or electrodes is connected in parallel, each containing dried enzymes being shielded from activation by removable covers. Opening of these covers allows electrolyte to hydrate the enzyme and thereby activates the electrodes stepwisely so that their lifetimes are added.

Oike et al. (2008) published fuel cells with multi-pulse activated anodes with lyophilized glucose dehydrogenase pre-stored in microfabricated anode compartments. They used starch paste as degradable glue to fix polymer covers, allowing opening of the covers after a defined time of exposition to the electrolyte. Additionally, they introduced magnetic forces to help in peeling off the covers of the anodes. Covers with different mass ratios of magnetic particles opened after 1 to 7 h of exposure to the electrolyte. Their results show that it is a major challenge to develop glue/cover combinations that open reproducibly after a defined time. The opening time is influenced by material, molecular mass, composition, and thickness of the degradable material and in their case also the strength of the magnetic forces.

Multi-pulse electrode activation demands for exact timing of the opening and adjustment to the lifetime of

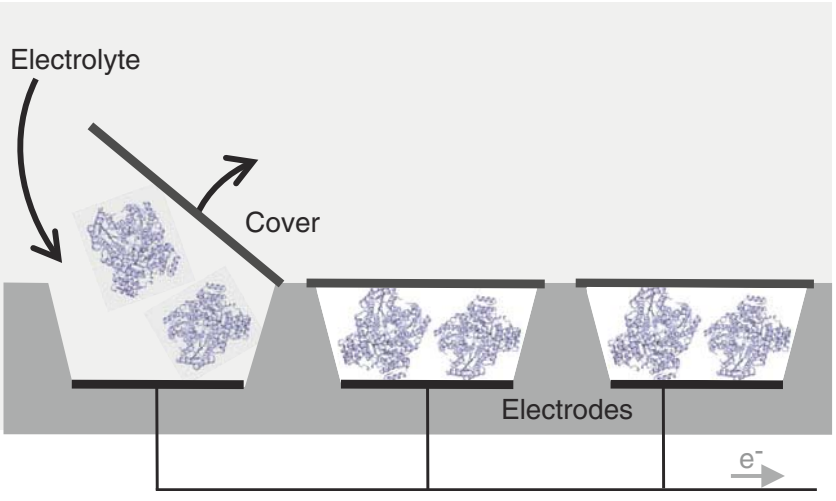

Fig. 3 Bioanode with multi-pulse activation of enzymes. The anode enzymes are stored as a more stable dried preparation and are consecutively activated by opening of the removable cover, allowing the electrolyte to hydrate the enzymes (Togo et al. 2008)

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the enzyme to achieve enhancement of electrodes' lifetime instead of power density accumulation. Also, a longer lifetime can only be achieved on the expense of the electrodes' power density. Controlled-release systems as used for sustainable drug delivery (Fu et al. 2000) may be an alternative approach with the advantages of better timing properties and less surface requirement. Such systems have not been reported yet for being applied in bioelectrochemical systems. In any case, maximum system lifetime is limited by the stability of the stored enzyme.

Electrodes with renewal of enzymes

Biological cells face the enzyme stability problem by continuously producing fresh enzyme replacing the inactivated ones. Following this principle, the bio-inspired approaches presented in this section aim to decouple overall system lifetime from enzyme lifetime to achieve theoretically unlimited electrode lifetimes. Active enzyme is either supplied manually by the repeated exchange of enzyme-containing electrolyte or in situ produced by microorganisms secreting the enzymes or displaying them on their surface.

Discontinuous renewal by periodic manual exchange

The most straightforward approach to implement regeneration of the electrode is the repeated manual exchange of the electrolyte containing externally produced enzymes. This requires enzymes that are dissolved in the electrolyte rather than immobilized to the electrode.

Atanasov et al. presented an implantable glucose biosensor with exchange of immobilized glucose oxidase (Fig. 4). The indirect detection of consumed oxygen (Atanasov and Wilkins 1994) or produced hydrogen peroxide (Xie and Wilkins 1991) by a platinum electrode eliminates the need for a mediator or direct contact between enzyme and electrode. This allows immobilizing glucose oxidase from *A. niger* on carbon particles to improve enzyme stability (Xie and Wilkins 1991) and handling properties when exchanging the enzyme with syringes through septa. Sensor lifetime without catalyst renewal was 2 weeks in buffer (Wilkins et al. 1995) and serum (Gamburzev et al. 1996) with a slight decrease in the detected current. Exchanging the enzyme-loaded particles recharged the sensor and completely restored the detected current. They showed continuous sensor operation in phosphate-buffered solution for 4 months (Atanasov and Wilkins 1994; Yang et al. 1997), and implanted in dogs for 25 days (Atanasov et al. 1997), achieving complete restoration of the sensor after each exchange procedure. Immobilizing the enzyme catalyst on exchangeable carbon particles also solves the problem of device sterilization prior to implantation, as the system can be autoclaved to guarantee sterility and loaded with the thermolabile enzyme afterwards (Atanasov et al. 1997). The manual exchange procedure may be combined with refilling an insulin pump (Atanasov et al. 1997), which could be associated with the sensor in an artificial mechanical pancreas (Xie and Wilkins 1991).

Despite the remarkable results of Atanasov et al., a sensor with periodic regeneration of the catalyst can hardly give a stable signal, as the detected current decreases within each regeneration cycle before being restored again (Wilkins et al. 1995; Atanasov et al. 1996). For enzymatically catalyzed biofuel cells, however, indirect current generation principles are not yet reported. To avoid mediators as additional source of instability, Rubenwolf et al. (2009) presented a direct electron transfer type cathode with laccase from *Trametes versicolor* adsorbed to graphite felt. Regular exchange of the catholyte provided a sufficient concentration of fresh enzyme in the solution, so that desorption of inactivated enzymes allows adsorption of active ones. In a first experiment at continuous load conditions for 2 weeks, they were able to reduce cathode potential decay by 74%. Graphite felt electrodes, however, show poor performance. High surface area nanomaterials increase the amount of adsorbed enzyme to enhance the power density of direct electron transfer type fuel cell electrodes (Rubenwolf et al. 2010), but will also alter adsorption and desorption behavior and thereby renewal of inactivated enzyme.

In summary, periodic renewal of the inactivated catalyst is a straightforward approach to achieve theoretically unlimited lifetime if the system allows regular attendance. In indirect or mediated bioelectrochemical systems, the enzymes can additionally be immobilized to improve handling properties. Systems with direct electron transfer, however, require a direct but reversible contact of enzyme and electrode and can only be realized by adsorption of the enzyme to the electrode.

Continuous renewal by in situ microbial surface display

Microorganisms can be genetically modified to display enzymatic catalysts on the outer surface layer of the cell membrane or cell wall. At these sites, the enzyme is exposed to the electrolyte and can be involved as catalyst in indirect or mediated electrode reactions as depicted in Fig. 5. Proliferation of the cells and continuous expression ensure a constant high level of active enzyme within the electrode compartment.

Biosensor and biofuel cell electrodes with microorganisms displaying the redox enzyme itself or as fusion protein are described in a patent application of Canon Kabushiki Kaisha (Kubo and Nomoto 2008). The authors describe the

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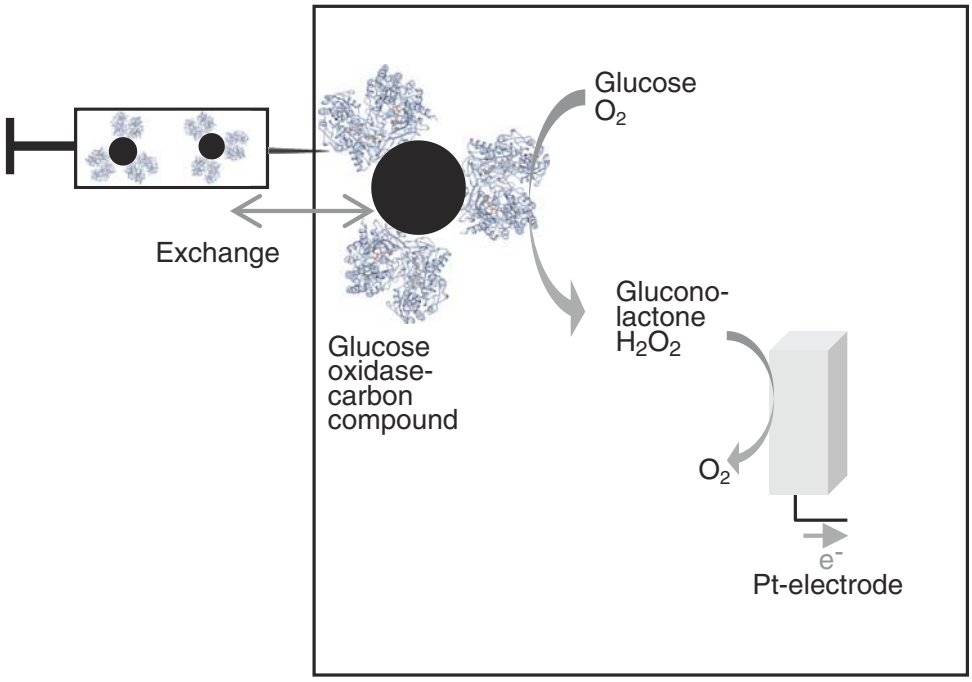

Fig. 4 Indirect glucose sensor electrode with external supply of fresh enzyme by repeated manual exchange of the catalyst. Glucose oxidase is immobilized to carbon particles for better handling properties and exchanged every 2 weeks with the help of syringes. The indirect sensor uses a platinum electrode to detect hydrogen peroxide produced by the enzyme upon oxidation of glucose (Xie and Wilkins 1991)

possible application of the principle in (a) a mediated glucose fuel cell where the glucose oxidase displaying yeast *Saccharomyces cerevisiae* is adhered to alumina particles on a glass substrate and (b) a metal complex polymer-mediated fuel cell or sensor where the same organism is immobilized by a dialysis membrane.

Fishilevich et al. (2009) present a first fuel cell based on this principle, using a recombinant *S. cerevisiae* strain displaying glucose oxidase from *A. niger* upon induction. With methylene blue as mediator, they observe addition of enzymatic and microbial electrode activity. Each of the two following inductions of enzyme expression within 15 days led to complete regeneration of the open circuit voltage. A next step may include usage of a more stable integrative vector and constitutive expression of the glucose oxidase gene to avoid manual induction.

In general, continuous renewal of the inactivated catalyst by surface display can result in bioelectrochemical systems with theoretically unlimited lifetime without the risk of accumulating inactive enzyme. The catalyst is immobilized on the microbial surface, which avoids catalyst leakage but also inhibits direct electron transfer. Therefore, bioelectrochemical systems using this principle necessitate mediated or indirect current generation or detection principles. Furthermore, the used genetically modified microorganisms cannot be implanted and may be hazardous to the environment.

Continuous renewal by in situ microbial secretion of enzymes

Microorganisms can not only be modified to display enzymatic catalysts on their surface layer but also to secrete

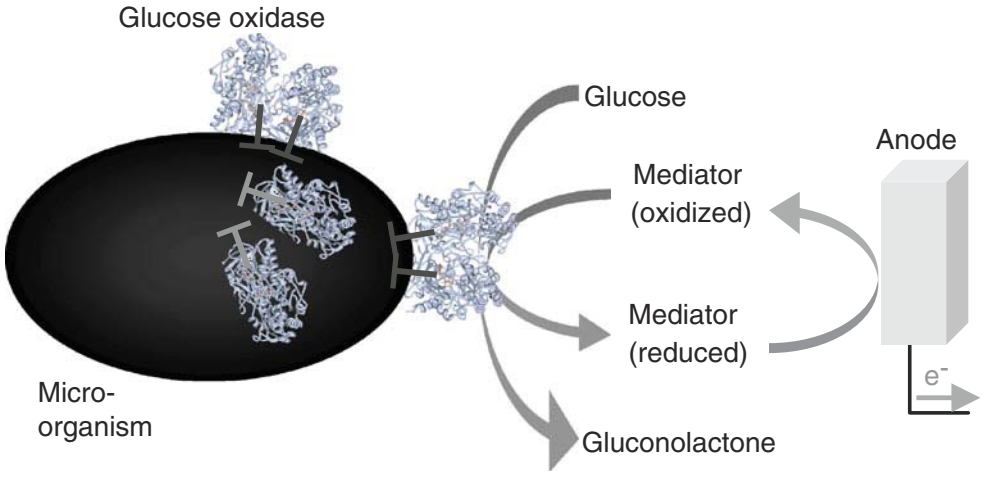

Fig. 5 Self-regenerative biofuel cell anode with continuous in situ catalyst renewal by microbial surface display. Genetically modified microorganisms display glucose oxidase on their surface being connected to the electrode by a mediator (Kubo and Nomoto 2008)

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them into the medium. Some microorganisms as wood-degrading fungi even secrete redox enzymes to the medium without modification. If these types of microorganism are incorporated into a biofuel cell, they can continuously produce fresh enzymes to replace inactivated ones.

Yamaguchi et al. proposed an indirect glucose sensor, the principle depicted in Fig. 6. The modified yeast *Pichia pastoris* secreted glucose oxidase from *A. niger*. As preliminary study, a stationary yeast culture maintained glucose oxidase activity in the medium within the tested time range of 2 weeks (Yamaguchi et al. 2007, 2008). Further steps may include a more stable genomic integration of the gene, adaptation of culture conditions to be sensor compatible, and finally, yeast cultivation within a sensor compartment.

Continuous renewal of the inactivated catalyst by microbial secretion is a promising approach to achieve theoretically unlimited lifetime in autonomous systems. Additionally, it is also applicable in biofuel cells with direct electron transfer if a reversible direct contact to the electrode can be established. However, biosensors and biofuel cells based on this principle can not be implanted, and genetically modified microorganisms may be hazardous to the environment.

Conclusions

To overcome the lifetime limitations of enzymatically catalyzed biofuel cell and biosensor electrodes, a wide variety of strategies and ideas have been presented in literature, each being suitable for specific applications.

Most experience and remarkable results have been gained in stabilizing the enzyme with the help of immobilization techniques, being also comparably easy to transfer from one enzyme-electrode system to the other. Immobilization of the enzyme is quite often also necessary to prevent enzyme leakage in open systems or co-immobilize the mediator. However, the combination with continuous enzyme renewal strategies to further increase system lifetime is not possible.

Genetic engineering methods may be applied to stabilize the enzyme when immobilization is undesired. They require more knowledge and are more labor-intensive but provide the possibility to also alter other catalyst properties such as specificity, turnover rates, or direct electron transfer. The stabilized enzymes can also be immobilized or used in systems including renewal of the catalyst, combining the electrode stabilization effects of both approaches.

Manual renewal of the inactivated enzymes by repeated exchange of the electrolyte does not allow unattended power generation and needs external production and purification of the enzyme. This can be acceptable for implanted drug delivery systems, where catalyst renewal may be combined with refilling of drug reservoirs. In contrast to the exchange of batteries in implanted devices, refilling of enzymatic catalysts can be realized by injections without any surgical procedure.

Bio-inspired continuous in situ production of active enzyme within the biofuel cell electrode compartment is the only way to achieve unattended systems with constant power or signal generation over a theoretically unlimited lifetime. This strategy, however, is the most recently reported one, and so far, no experiments exceeding 2 weeks

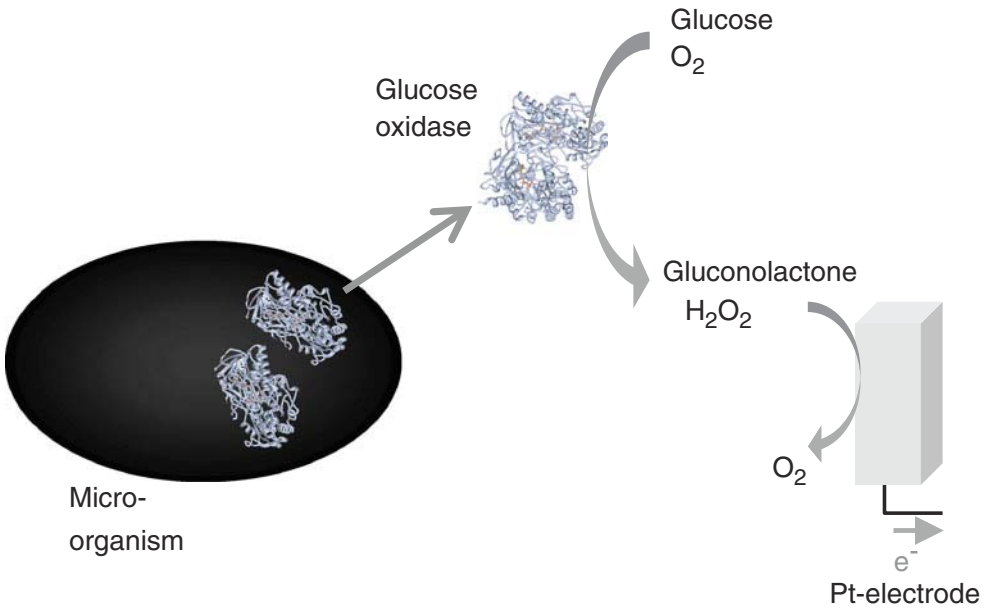

Fig. 6 Glucose sensor electrode with continuous in situ production of soluble active enzyme as suggested by Yamaguchi et al. (2008). Genetically modified yeast secretes glucose oxidase into the electrolyte. The indirect sensor uses a platinum electrode to detect hydrogen peroxide produced by the enzyme upon oxidation of glucose

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have been shown. Environmental conditions and available substrates need to fit the requirements of continuous microbial cultures, and great care has to be taken to prevent contaminations from overgrowing the redox enzyme-producing cultures. In general, approaches for the in situ production of fresh enzyme within an electrochemical device promise to combine the high selectivity and power output of enzyme electrodes with the self-regeneration capability of microbial systems.

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