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(54) Benævnelse: **Elektrokemisk sensor med covalent bundet enzym**

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Description

The present application concerns an electrochemical sensor, a method for its production and a method for determining an analyte in a fluid medium using the electrochemical sensor.

Measuring systems for biochemical analysis are important components of clinically relevant analytical methods. This primarily concerns the measurement of analytes which can be directly or indirectly determined with the aid of enzymes. Biosensors i.e. measuring systems equipped with biological components, which allow a repeated measurement of the analyte either continuously or discontinuously and which can be used *ex vivo* as well as *in vivo* have proven to be particularly suitable for the measurement of analytes. *Ex vivo* biosensors are typically used in flow-through cells whereas *in vivo* biosensors are preferably implanted into subcutaneous fat tissue. In this connection, a distinction is drawn between transcutaneous implants which are only introduced into the tissue for a short period and are in direct contact with a measuring device located on the skin, and full implants which are inserted surgically into the tissue together with a measuring device.

Electrochemical biosensors allow the measurement of an analyte by means of two or more electrodes where at least one of the electrodes represents the working electrode on which the analyte to be determined is converted. Electrochemical biosensors which comprise an enzyme as a biological component contain the enzyme in or on the working electrode in which case for example the analyte can serve as a substrate for the enzyme and can be physicochemically altered (e.g. oxidised) by this enzyme. A redox mediator transfers the electrons released during the conversion of the analyte onto the conductive components of the working electrode, and the electrical measuring signal generated by the flow of electrons correlates with the concentration of the measured analyte.

Naturally occurring as well as synthetic redox pairs may be considered as redox mediators. Synthetic redox mediators such as for example those described by

Feldman *et al.* [Diabetes Technology & Therapeutics 5 (2003), 769-779] are, however, poorly suited to *in vivo* applications. This is due to the fact that a synthetic redox mediator can theoretically always produce an immune response by the body when the biosensor is introduced into the body. However, at least the toxicity of these substances must be considered and, if necessary, checked because redox mediators must always be able to freely diffuse through the electrode structure by which means they can also escape from the electrode and pass over into the surrounding organism. This point is not relevant for *ex vivo* applications provided it is ensured that it does not gain access to the body due to a potential return flow of the analyte.

Consequently, electrochemical sensors which use naturally occurring redox mediators are particularly suitable for *in vivo* applications. The redox pair oxygen/hydrogen peroxide proves to be particularly advantageous in this connection because the initial component (oxygen) is always present. The hydrogen peroxide generated in the enzymatic conversion of an analyte by means of an oxidase in the presence of oxygen is reoxidised on the working electrode of the electrochemical biosensor, an electrical signal being generated by the release of electrons and the redox mediator being converted back into its oxidised form. The kinetics of this enzymatic reaction follows a "ping-pong" mechanism [Leskovac *et al.*, The International Journal of Biochemistry and Cell Biology 37 (2005), 731-750].

A not insignificant problem when measuring analytes with the aid of enzymes which require oxygen as a co-substrate is, however, that temporary reductions of the oxygen concentration compared to the initial situation can occur in tissues which can affect the function of conventional *in vivo* biosensors. Figure 1 shows the kinetics of the enzymatic oxidation of glucose to glucono-δ-lactone by means of glucose oxidase at various oxygen concentrations. The graph shows that in general the amount of analyte converted at a given oxygen concentration is reduced as the glucose concentration increases and, thus, the curve is in the nonlinear range in the physiologically relevant range despite the high binding constant of glucose oxidase for glucose (about 250 mM).

Furthermore, Figure 1 shows that at higher concentrations of the analyte, an approximately linear curve is not obtained until an oxygen concentration of about 1 mM. The *in vivo* concentration of dissolved oxygen in aqueous systems and in particular in the interstitial fluid of subcutaneous fat tissue is, however, considerably lower. Whereas water has an approximate oxygen concentration of 0.21 mM at 37°C, the expected oxygen concentration in subcutaneous fat tissue is only 0.1 mM or even less which is why the curves are in each case curved at physiological glucose concentrations. This deviation from a linear course results in undesired transient function characteristics in *in vivo* biosensors.

Thus, the limited availability of oxygen in the tissue is a limiting factor for the linearity of the function curve of the electrochemical sensor in numerous enzymatic biosensors which require oxygen as a co-substrate. The linearity of the function curve can in principle be improved by using working electrodes with a cover membrane which inhibits the diffusion of the analyte more strongly than the diffusion of the co-substrate. Figure 3 shows among others the function curve of an enzymatic biosensor with a cover membrane consisting of polyurethane which promotes the diffusion of oxygen more strongly than that of glucose (measured values indicated by squares). This shows that the measuring signal of the sensor can be kept approximately linear up to a glucose concentration of about 10 mM by using a suitable cover membrane. The curve becomes increasingly curved at higher concentrations.

However, the use of cover membranes in electrochemical sensors is associated with certain problems. For instance, electrochemical sensors which are used to determine different analytes must usually also contain different cover membranes in order to provide a different diffusion of the substrate and co-substrate. At the same time it must be ensured that the cover membranes are highly biocompatible for *in vivo* applications which involves considerable technical requirements and ultimately leads to increased production costs.

In order to lower the polarisation voltage of the working electrode of an electrochemical biosensor relative to a reference electrode and thus to reduce the effect of interfering substances on the measuring signal of the working electrode, some electrochemical biosensors additionally utilize an electrocatalyst which promotes the transfer of electrons from the redox mediator onto the conductive components of the working electrode. An example of such an electrocatalyst is cobalt phthalocyanine which catalyses the oxidation of hydrogen peroxide to oxygen [Crouch *et al.*, Biosensors and Bioelectronics 21 (2005), 712-718]. The cobalt (II) cation of the cobalt phthalocyanine complex is here firstly reduced by hydrogen peroxide to cobalt(I) before the latter is converted back into its original divalent state with release of an electron at the anode.

Another example of an electrocatalyst known from the literature is manganese dioxide in the form of pyrolusite [Cui *et al.*, Nanomedicine: Nanotechnology, Biology and Medicine 1 (2005), 130-135; Luo-*et al.*, Biosensors and Bioelectronics 19 (2004), 1295-1300]. Although the mechanism of the catalytic oxidation of hydrogen peroxide on manganese dioxide is not understood in detail, the potential of a working electrode with manganese dioxide as the electrocatalyst is reduced by several 100 mV compared to a working electrode without manganese dioxide. Consequently, the effect of interfering substances such as ascorbate or urea on the measuring signal is considerably reduced.

Another reason for using electrocatalysts is the damage caused to enzymes by excess hydrogen peroxide. If this substance is not sufficiently rapidly decomposed at the working electrode, denaturation of the enzyme may occur. In order to counter this problem it was proposed in the literature to synthesise enzymes which are resistant to hydrogen peroxide, for example by mutation [US 2004/-0137547 A1]. However, it is extremely difficult to make such modifications to an enzyme without having an adverse effect on other properties of the enzyme such as for example its enzymatic specificity. Hence, the use of electrocatalysts for conversions in which hydrogen peroxide is generated appears to be considerably superior to the above method because electrocatalysts considerably increase the efficiency of the oxidation of hydrogen peroxide and in this manner

prevent excess peroxide from occurring in the electrode matrix or in its environment.

An additional problem which is associated with the formation of hydrogen peroxide in an enzymatic determination of an analyte is that hydrogen peroxide can act as an inhibitor of the analyte or of the co-substrate oxygen. This competitive inhibition depends on the concentration of hydrogen peroxide and limits the conversion of the analyte. The use of an electrocatalyst which promotes the re-oxidation of hydrogen peroxide to oxygen consequently also has a positive effect with regard to the conversion of the analyte.

Various factors have to be taken into account when designing electrochemical biosensors. For instance, the biosensors have to have a sufficient amount of enzyme in the working electrode in order to prevent enzyme limitation of the measurement [Abel *et al.*, Journal of Molecular Catalysis B: Enzymatic 7 (1999), 93-100]. Furthermore, the enzyme molecules should be located in the structure of the working electrode over the complete measuring period of the biosensor i.e. the enzyme should not become detached or displaced in areas of the electrode which are reached by the measuring medium [Doretti *et al.*, Biosensors and Bioelectronics 11 (1996), 363-373]. Finally, the enzyme should also be stable in the working electrode of the biosensor. Factors which result in thermal deactivation of enzymes in electrochemical biosensors together with methods for their stabilisation have been investigated many times [Sarath Babu *et al.*, Biosensors and Bioelectronics 19 (2004), 1337-1341]. Enzyme degradation after manufacture of a biosensor ultimately leads to a limited shelf-life of the sensor.

In order to take the above factors into consideration, attempts were made to stabilize the enzyme by immobilizing it in the electrode matrix of the working electrode which has led to an intensive search for suitable immobilisation methods for enzymes in electrochemical biosensors. Both adsorptive and chemical immobilisation are used in practice. However, adsorptive immobilisation is disadvantageous for various reasons. On the one hand, it requires the working

electrode to be covered by a membrane which is impermeable to the enzyme which increases the work required to manufacture the biosensor and makes a wide variety of demands on the membrane. On the other hand, the aforementioned displacement of enzyme molecules within the electrode cannot be prevented in the case of adsorptive immobilisation which results in a change in the sensor function.

US 5,368,707 discloses biosensors which comprise working electrodes with an adsorptively bound enzyme and which are suitable for determining micromolar amounts of lead ions in liquids. In order to produce the biosensors, the surface of the working electrode consisting of a conductive material is coated with colloidal gold on the particles of which the appropriate enzyme is adsorbed which, in turn, can be covalently bound to a redox mediator.

Another disadvantage of electrodes provided with a cover membrane for supporting the adsorptive immobilisation of enzymes which should not be underestimated, especially for *in vivo* applications, is the necessity for noninvasive testing of the integrity of the cover membrane. Since even the smallest defects in the membrane are sufficient to result in bleeding of the enzyme from the electrode into the environment, extremely thorough testing is necessary especially in the case of *in vivo* biosensors. In view of the disadvantages of adsorptive immobilisation there is thus a concrete need to immobilize enzymes in electrochemical biosensors by covalent bonds to or in the electrode matrix.

JP 10-68651 describes sensors for detecting analytes such as glucose which comprise electrodes with a covalently bound enzyme. For this purpose, the surface of the electrodes coated with SnO_2 as a conductive material is activated with a strong acid, functionalised with a coupling reagent and finally brought into contact with the enzyme.

EP 0 247 850 A1 discloses biosensors for the amperometric detection of an analyte. These sensors contain electrodes with immobilised enzymes which are immobilised or adsorbed onto the surface of an electrically conductive support

where the support consists of a platinised porous layer of resin-bound carbon or graphite particles or contains such a layer. For this purpose, electrodes made of platinised graphite and a polymeric binder are firstly prepared and these are subsequently brought into contact with the enzyme. In this case, the enzyme is immobilised either by adsorption to the electrode surface or by coupling to the polymeric binder using suitable reagents.

Amperometric biosensors with electrodes comprising an enzyme immobilised or adsorbed onto or into an electrically conductive, porous electrode material are also described in EP 0 603 154 A2. In order to produce the enzyme electrodes, an oxide or oxide hydrate of a transition metal of the fourth period, such as for example manganese dioxide, acting as a catalyst is worked into a paste together with graphite and a nonconductive polymeric binder, and the porous electrode material obtained after drying the paste is brought into contact with the enzyme in a second step. The enzyme can be immobilised on or in the porous electrode material by crosslinking using glutardialdehyde.

US 2005/189240 describes an electrochemical sensor for determining the hydrogen peroxide concentration of a solution, which sensor comprises a mixed-valence metal oxide M_xO_y acting as catalyst.

A major disadvantage of the electrochemical biosensors described in JP 10-68651, EP 0 247 850 A1 and EP 0 603 154 A2 is that the enzyme is first immobilised on the electrode which has been prefabricated without enzyme. As a consequence, there is the problem that the enzyme cannot be coupled to the electrode components in a controlled manner. Accordingly, when glutardialdehyde is used as the crosslinking reagent, the enzyme not only binds in an uncontrolled manner to any reactive components of the electrode material, but also crosslinks to itself. Furthermore, this procedure contaminates the electrode with the reagents which are used and, hence, the electrode has to again be thoroughly cleaned especially before use in an *in vivo* biosensor which increases the complexity of production and thus costs.

US 4,938,860 discloses a suitable electrode for electrochemical sensors comprising a platinum coated anode formed as a film and an enzyme layer which is bound to the anode. The enzyme layer is bound to the platinised anode preferably by using an aminosilane and a suitable crosslinking agent such as for example glutardialdehyde. However, a disadvantage of the electrode described in US 4,938,860 is that due to the construction of the anode as a film only a small surface is provided for the enzymatic conversion of the analyte and platinum is a relatively expensive material to use as a catalyst.

Hence, the object of the invention was to provide an enzymatic electrochemical sensor for determining an analyte in which the disadvantages of the prior art are at least partially eliminated. In particular, the sensor should ensure specific and durable immobilisation of the enzyme, have high efficiency and thus achieve a high signal yield. Furthermore, it should be possible to produce the sensor in a simple and low-cost manner.

This object was achieved according to the invention by means of an electrochemical biosensor for determining an analyte in a fluid medium, comprising at least one working electrode and at least one reference electrode, at least the working electrode comprising particles of an electrocatalyst in an electrode matrix, wherein an enzyme which is suitable for determining the analyte is covalently bound to the particles of the electrocatalyst and has no covalent bonds to the other components of the electrode matrix, and wherein the electrocatalyst is MnO_2.

The metal oxide MnO_2 acts as the electrocatalyst which is present in the electrode matrix of at least the working electrode. The metal oxide MnO_2 has the ability to catalyse the conversion of a redox mediator used to determine the analyte.

The electrocatalyst is provided according to the invention in particulate form, wherein the particle size can be varied depending on the respective requirements. Within the scope of the present invention, 90% of the electrocatalyst par-

ticles usually have a diameter of 0.1 µm to 20 µm, a diameter of 0.5 µm to 5 µm having proven to be particularly preferred. In any case, the particle size of the electrocatalyst should always be less than the layer thickness of the working electrode which is in the range of 1 µm to 50 µm, preferably in the range of 5 µm to 20 µm.

The ability to control the effective surface area of the electrocatalyst by means of particle size is of crucial importance especially for its functionalisation with enzyme. Accordingly, a higher effective surface area of the electrocatalyst can also increase its loading with enzyme and thus result in a higher enzyme activity stated in units per milligram electrocatalyst which in general is determined by the amount of electrocatalyst in the working electrode as well as its porosity and area. The term "unit" as used within the scope of the present application represents the amount of enzyme which is required to convert 1 µmol substrate per minute under standard conditions. The enzyme-coated electrocatalyst particles used for the purposes of the present invention usually have an enzyme activity of about 0.01 U/mg to about 10 U/mg, with an enzyme activity of about 0.1 U/mg to about 10 U/mg having proven to be particularly advantageous.

In the electrochemical sensor according to the invention, the enzyme is selectively covalently bound to the particles of the electrocatalyst, with the enzyme particularly preferably having no covalent bonds to the other components of the electrode matrix. The covalent binding of the enzyme to the electrocatalyst has the advantage that the diffusion path of the redox mediator to catalytically active sites of the electrode can be kept small which results in a high efficiency of the working electrode and thus a high signal yield of the electrochemical sensor.

Furthermore, after regeneration by the electrocatalyst, the redox mediator is also adsorptively bound to the electrocatalyst as a result of which, for example in the case of an oxygen/hydrogen peroxide system, a locally high oxygen activity is generated in the region of the surface of the electrocatalyst which decreases towards the surrounding measuring medium. On the other hand, the covalent binding of the enzyme to the electrocatalyst results in a high local activity of re-

generated redox mediator on the enzyme, which is reflected by greater linearity and stability of the generated measuring signal in relation to the concentration of analyte as shown for example in Figure 3 (measured values shown as triangles). In this case, even a transient reduction in the concentration of the redox mediator in the environment for example due to an impaired blood perfusion in the tissue, does not lead to a transient change of the measuring signal.

Finally, the covalent coupling of the enzyme to the electrocatalyst ensures the constancy of the function because a detachment of enzyme molecules can be ruled out under typical measurement conditions (physiological electrolyte concentration, physiological pH, body temperature). Thus, the electrochemical sensor according to the invention remains operational over an extended time period and operates virtually free of drift.

In order to covalently bind the enzyme to the particles of the electrocatalyst, the present invention envisages in a preferred embodiment that the electrocatalyst particles have a functionalised surface and in particular a surface functionalised with amino groups and/or carboxyl groups to which the enzyme is bound. The surface can for example be functionalised by coating the electrocatalyst particles with a suitable reagent to form functional groups on the surface of the electrocatalyst particles by means of which the enzyme can be covalently bound to the electrocatalyst particles.

Coating reagents which are used within the scope of the present invention are substances which, on the one hand, undergo a covalent binding with the electrocatalyst e.g. with hydroxy groups of the electrocatalyst and, on the other hand, contain at least one functional group which serves to covalently bind the enzyme. This means that the coating reagents are at least difunctional i.e. comprise at least two functional groups. The functional groups of the coating reagent which are used for covalent binding to the electrocatalyst and for covalent binding to the enzyme can be identical or different but are preferably different. Preferred coating reagents are silanes which carry at least one suitable func-

tional group by means of which the enzyme is covalently bound to the coating reagent.

The surface of the electrocatalyst particles is more preferably functionalised with an aminosilane which binds to the surface of the electrocatalyst particles while forming silicon-oxygen bonds and at the same time provides free amino groups for a covalent binding of the enzyme to the electrocatalyst particles. Suitable aminosilanes comprise for Example 3-aminopropyltrimethoxysilane and 3-aminopropyltriethoxysilane, with 3-aminopropyltriethoxysilane being particularly preferred.

Alternatively, the surface of the electrocatalyst particles can be functionalised with a carboxysilane which binds to the surface of the electrocatalyst particles while forming silicon-oxygen bonds and, optionally after hydrolysis, provides free carboxyl groups for covalent binding of the enzyme to the electrocatalyst particles. In this connection, 3-(triethoxysilyl)propyl succinic acid anhydride which is commercially available as Geniosil® GF 20 (Wacker) has proven to be a particularly suitable silane.

The enzyme can be covalently bound to the functionalised surface of the electrocatalyst particles either directly or by means of crosslinking reagents. In a preferred embodiment, the enzyme is directly bound to the functionalised surface of the electrocatalyst particles. The enzyme can be coupled to the functionalised surface of the electrocatalyst particles in any desired manner and can comprise prior activation of functional groups on the functionalised surface of the electrocatalyst particles and/or of the enzyme. Functional groups can for example be activated by reacting the functionalised electrocatalyst and/or the enzyme with a suitable activation reagent. Preferred activation reagents comprise carbodiimides such as for example dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as well as combinations of carbodiimides and succinimides. A particularly suitable activation reagent for the purposes of the present invention comprises a combi-

nation of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy-succinimide.

In another preferred embodiment, the enzyme is bound to the functionalised surface of the electrocatalyst particles by a crosslinking reagent, with the crosslinking reagent having reactive groups which can react covalently with the functional groups on the functionalised surface of the electrocatalyst particles as well as with the functional groups of the enzyme. Particularly preferably, reactive groups are present which enable crosslinking between the enzyme and the functional groups on the surface of the electrocatalyst particles. Any reagents which can perform the function described above may be considered as crosslinking reagents such as for example polyfunctional aldehydes, in particular dialdehydes such as glutardialdehyde, benzoquinones, bromocyanogen, hydrazines, succinimides, 2,4,6-trichloro-1,3,5-triazine or combinations thereof. A succinimide, more preferably a disuccinimide and most preferably disuccinimidyl suberate (DSS) is preferably used as a crosslinking reagent.

The electrode matrix of the working electrode can be generated by mixing the electrocatalyst particles covalently coated with enzyme with other components of the electrode matrix e.g. with a conductive electrode material and subsequently drying the resultant mixture, with the electrode matrix usually containing the electrocatalyst in an amount of about 1% by weight to about 50% by weight, preferably in an amount of about 5% by weight to about 20% by weight.

In another preferred embodiment, the electrode matrix is in a porous form. The porosity of the electrode matrix can among others be regulated by the particle size of the electrocatalyst and other components, where a high porosity is associated with a larger effective surface area of the electrode and thus a larger area of contact with the measuring medium. The conductive electrode material which can for example be provided in the form of a paste to produce the electrode matrix preferably comprises conductive solid particles such as graphite and/or fullerenes in combination with a nonconductive binder, in particular with

a nonconductive polymeric binder such as for example a perfluorinated polymer such as Nafion.

The enzyme immobilised on the electrocatalyst particles is preferably an oxidase and in particular alcohol oxidase (1.1.3.13), arylalcohol oxidase (EC 1.1.3.7), catechol oxidase (EC 1.1.3.14), cholesterol oxidase (EC 1.1.3.6), choline oxidase (1.1.3.17), galactose oxidase (EC 1.1.3.9), glucose oxidase (EC 1.1.3.4), glycerol-3-phosphate oxidase (EC 1.1.3.21), hexose oxidase (EC 1.1.3.5), malate oxidase (EC 1.1.3.3), pyranose oxidase (EC 1.1.3.10), pyridoxine-4-oxidase (EC 1.1.3.12) or thiamine oxidase (EC 1.1.3.23). The enzyme is particularly preferably glucose oxidase.

The reference electrode of the electrochemical sensor according to the invention serves to adjust the polarisation potential of the working electrode and can consist of any material which is suitable for the purposes of the present invention. A silver/silver chloride electrode is preferably used as the reference electrode.

Furthermore, the electrochemical sensor of the present invention can, in addition to the at least one working electrode and the at least one reference electrode, comprise at least one counter electrode which is preferably in the form of a noble metal electrode and in particular a gold electrode. A counter electrode in the form of a noble metal electrode is preferably coated with a suitable conductive material such as for example a paste containing conductive solid particles, in particular carbon paste.

According to the invention, the electrochemical sensor preferably contains two portions. The first portion which can be brought into contact with the fluid medium containing the analyte comprises the electrodes i.e. working electrode, reference electrode and optionally counter electrode. This portion is preferably provided with a biocompatible coating. The biocompatible coating allows the analyte to penetrate into the electrode matrix but is intended to prevent electrode components from escaping into the surrounding medium. In view of the

fact that due to the covalent binding of the enzyme to the electrocatalyst the enzyme does not bleed out of the working electrode or the electrochemical sensor, a biocompatible coating is not absolutely necessary for many applications. For instance, the electrochemical sensor according to the invention can also be used especially in *in vivo* biosensors when the biocompatible coating is not a barrier to enzymes. On the contrary, a biocompatible coating can be selected in this connection which provides optimal interaction with the surrounding tissue and/or blood or serum.

Biocompatible coatings can be generated in various ways. A preferred method is to use prefabricated membranes which are applied to the electrochemical sensor. The membrane can be immobilised on the sensor by various techniques, with adhesive bonding or laser welding being regarded as preferred. Prefabricated dialysis membranes have proven to be advantageous in this connection, and dialysis membranes made of polyether sulfone, such as those disclosed for example in EP 1 710 011 A1 which are commercially available under the trade name Ultrason® 6020 (BASF), are particularly suitable.

Alternatively, the biocompatible coating can be generated *in situ* by applying a solution of a suitable polymer onto the electrochemical sensor and subsequently drying it. The polymer is preferably applied to the biosensor by spraying, dip-coating or dispersing a dilute solution of the polymer but is not limited to these methods. An organic solvent is preferably used as the solvent and in particular an organic solvent with a boiling point of ≤ 100°C such as for example ethanol, the solvent containing an amount of about 0.1% by weight to about 30% by weight, preferably of about 0.5% by weight to about 15% by weight polymer. Polymers which are suitable for such purposes comprise in particular polymers which have a zwitterionic structure and mimic cell surfaces such as for Example 2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate (MPC-co-BMA). The resultant biocompatible coatings usually have a thickness of about 1 µm to about 100 µm, preferably of about 3 µm to about 25 µm.

The second portion of the electrochemical sensor lies in a region which is inaccessible to the fluid measuring medium and preferably comprises a measured-value acquisition unit. In a further preferred embodiment, the second portion additionally comprises a voltage source such as for example a primary or secondary battery and a unit which is selected from a wireless data transfer unit and a display for displaying the measured values. Alternatively, the second portion can comprise an interface for a measured-value acquisition unit which is separate from the electrochemical sensor.

The electrochemical sensor according to the invention is preferably designed for multiple measurements i.e. the sensor enables repeated measurement of the analyte to be determined. This is particularly desirable in applications in which a constant i.e. continuous or discontinuous monitoring of the presence and/or the amount of an analyte is to take place over an extended time period of e.g. one day or longer, in particular one week or longer such as for example in the case of dialysis patients. In a preferred embodiment, the invention consequently provides that the electrochemical sensor is designed as a flow-through cell through which a fluid containing the analyte is passed. Alternatively, the electrochemical sensor according to the invention can, however, also be designed as a fully or partially implantable device which for example can be implanted into fat tissue or into blood vessels.

The electrochemical sensor according to the invention can be used to determine an analyte in a fluid medium which can originate from any source. In a preferred embodiment, the electrochemical sensor is used to determine an analyte in a body fluid comprising but not limited to whole blood, plasma, serum, lymph fluid, bile fluid, cerebrospinal fluid, extracellular tissue fluid, urine as well as glandular secretions such as saliva or sweat, wherein whole blood, plasma, serum and extracellular tissue fluid are regarded as particularly preferred. The amount of sample required to carry out the analysis is usually from about 0.01 µl to about 100 µl, preferably from about 0.1 µl to about 2 µl.

The analyte to be determined qualitatively and/or quantitatively can be any biological or chemical substance which can be detected by means of a redox reaction. The analyte is preferably selected from the group consisting of malic acid, alcohol, ammonium, ascorbic acid, cholesterol, cysteine, glucose, glutathione, glycerol, urea, 3-hydroxybutyrate, lactic acid, 5'-nucleotidase, peptides, pyruvate, salicylate and triglycerides. In a particularly preferred embodiment, the analyte to be determined by means of the electrochemical sensor according to the invention is glucose.

In a further aspect, the present invention concerns a method for producing an electrochemical sensor according to the invention, comprising the steps:

(a) providing electrocatalyst particles,
(b) coating the electrocatalyst particles with an enzyme, wherein the enzyme is covalently bound to the electrocatalyst particles,
(c) mixing the electrocatalyst particles coated covalently with enzyme which are obtained in step (b) with a conductive electrode material and optionally further substances,
(d) processing the mixture obtained in step (c) to form an electrode, and
(e) combining the electrode obtained in step (d) with at least one further electrode.

In order to produce the electrochemical sensors according to the invention, particles of an electrocatalyst as defined above are preferably firstly reacted with a coating reagent by means of which the surface of the electrocatalyst particles is functionalised. By successively reacting the functionalised electrocatalyst particles with a crosslinking reagent and an enzyme, electrocatalyst particles covalently coated with enzyme are obtained which can be processed to form an electrode matrix by mixing them with other components as defined above.

The production method according to the invention does in fact prove to be particularly advantageous because the production of the electrocatalyst coated with enzyme can be carried out separately from the production of the electrode. Fur-

thermore, the electrocatalyst covalently coated with enzyme provides a defined starting material for preparation of the electrode paste, which starting material can be purified before introduction into the electrode paste thus obviating subsequent cleaning of the finished electrode.

In still another aspect, the present invention concerns an *in vitro* method for determining an analyte in a fluid medium, comprising the steps:

(a) contacting the fluid medium with an electrochemical sensor according to the invention, and
(b) determining the presence and/or the amount of analyte in the fluid medium by measuring a signal generated by the electrochemical sensor.

In order to determine the analyte, the electrochemical sensor can be designed in any manner which allows contact between the electrochemical sensor and the fluid medium. Accordingly, the sensor can for example be designed as a flow-through cell through which the medium containing the analyte flows. On the other hand, the sensor can also be designed as a diffusion sensor, wherein the contact between the sensor and medium takes place by diffusion.

A measurable signal is generated by the sensor depending on the presence and/or the amount of analyte. This signal is preferably an electrical signal such as for example electrical current, voltage, resistance etc. which is evaluated or read-out using suitable means. The electrochemical sensor is preferably an amperometric sensor.

The following figures and examples are intended to illustrate the invention in greater detail.

Figures

Figure 1 shows the conversion of glucose plotted against the glucose concentration [mM] when using glucose oxidase as the enzyme and oxygen/hydrogen peroxide as the redox mediator as a function of the oxygen concentration. K_m^{app} and V_{max}^{app} represent the enzyme kinetic constants for glucose according to Michaelis-Menten kinetics.

Figure 2 shows the measured signal [nA] of an electrochemical sensor according to the present invention plotted against time [sec] over the course of a 7 day measurement of the glucose concentration of a measuring solution in which the glucose concentration was periodically varied between 0 and 26 mM. An electrode without a cover membrane was used as the working electrode which contained manganese dioxide coated with glucose oxidase as the electrocatalyst and was prepared according to Example 6 of the present application.

Figure 3 shows the measuring signal [nA] of two electrochemical sensors with an immobilised enzyme plotted against the glucose concentration of a measuring solution [mM]. The measured values indicated by triangles show the function curve of an electrochemical sensor according to the present invention in which glucose oxidase was covalently bound to the electrocatalyst of the working electrode and no cover membrane was used. The measured values indicated by squares form the function curve of a sensor of identical dimensions in which the enzyme was immobilised in the working electrode by means of a cover membrane consisting of polyurethane and was not bound to the electrocatalyst by covalent binding.

Examples

Example 1: Preparation of carboxy-functionalised manganese dioxide

1.6 g manganese dioxide (Technipur) was suspended in 256 ml toluene, 84 g Geniosil® GF 20 (Wacker) was added to the resulting suspension and the reaction mixture was stirred for 24 hours at 50°C and 520 rpm under a nitrogen at-

mosphere in order to prepare carboxy-functionalised manganese dioxide. After cooling and sedimentation of the manganese dioxide, the toluene was decanted and the residue was washed twice with 250 ml toluene each time and then once with 250 ml acetone. 250 ml water was added to the functionalised manganese dioxide obtained in this manner and stirred for 24 h at room temperature. Subsequently, the water was centrifuged off and the residue was dried under a vacuum at 50°C over $CaCl_2$ to obtain about 1.5 mg of carboxy-functionalised manganese dioxide.

Example 2: Coupling of glucose oxidase to carboxy-functionalised manganese dioxide

500 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 400 mg N-hydroxysuccinimide and 70 mg glucose oxidase was added to 100 mg of the dried, carboxy-functionalised manganese dioxide from Example 1 and stirred for 24 h at room temperature in an aqueous solution. After sedimentation of the solid, the supernatant was removed and the solid was washed four times with potassium phosphate buffer pH 7.4. After drying the solid obtained in air, about 85 mg of enzyme-coated electrocatalyst was obtained which had an enzyme activity of 0.06 U/mg.

Example 3: Preparation of amino-functionalised manganese dioxide

8 ml 3-aminopropyltriethoxysilane (Sigma) was added to a well-stirred suspension of 200 mg manganese dioxide (Technipur) in 32 ml toluene which had been heated to 60°C and the mixture was stirred for a further 16 h at 60°C. After sedimentation of the solid, the clear supernatant was decanted and the solid was washed three times with 32 ml toluene each time. The remaining solid was dried in air to obtain about 182 mg of amino-functionalised manganese dioxide.

Example 4: Coupling of glucose oxidase to amino-functionalised manganese dioxide using glutardialdehyde as the crosslinking reagent

The dried solid from Example 3 was washed once with 32 ml 50 mM potassium phosphate buffer pH 7.4 and subsequently taken up in 16 ml 50 mM potassium phosphate buffer pH 7.4. 16 ml of a 10% glutardialdehyde solution (Sigma) was

added to this suspension while stirring. The reaction was terminated after 1.5 h at 25°C. The sedimented solid was washed three times with 32 ml 50 mM potassium phosphate buffer pH 7.4 each time, suspended in 16 ml of the same buffer while stirring and combined with 16 ml of a solution of 0.5 mg/ml glucose oxidase (Roche) in 50 mM potassium phosphate buffer pH 7.4. This mixture was stirred for 3 h at 25°C. After sedimentation of the solid, it was washed four times with 16 ml 50 mM potassium phosphate buffer pH 7.4 each time. After lyophilisation, about 200 mg of enzyme-coated electrocatalyst was obtained which had an enzyme activity of 0.12 U/mg.

Example 5: Coupling of glucose oxidase to amino-functionalised manganese dioxide using disuccinimidyl suberate as the crosslinking reagent

0.02 mg disuccinimidyl suberate in 20 µl dioxane and 0.008 mg glucose oxidase in 2 ml 0.1 M potassium phosphate buffer pH 8.5 were added to 20 mg of the dried, amino-functionalised manganese dioxide from Example 3 and stirred for 4 h at room temperature. After centrifuging the solid, the latter was washed twice with 5 ml 0.1 M potassium phosphate buffer pH 8.5 each time and subsequently taken up in 5 ml 0.1 M potassium phosphate buffer pH 8.5. After lyophilisation, about 18.8 mg of the enzyme-coated electrocatalyst was obtained which had an enzyme activity of 0.1 U/mg.

Example 6: Preparation of amperometric sensors

In order to prepare an electrochemical sensor with three electrodes (working electrode, reference electrode and counter electrode) which allows the determination of glucose in blood or subcutaneous fat tissue, a working electrode without a cover membrane was prepared in the first step. For this purpose, manganese dioxide functionalised with glucose oxidase according to Example 5 was mixed with carbon polymer paste PE 401 (Acheson) and diethylene glycol monobutyl ether, and the resultant mixture was applied by means of a dispensing technique onto the gold surface of a sensor strip made of polyester and dried at 25°C in a vacuum. The working electrode obtained in this manner was combined with a silver/silver chloride electrode as the reference electrode and with a gold electrode as the counter electrode. The conductor tracks were insulated.

Example 7: Determining the stability and linearity of the measured signal of amperometric sensors

The electrochemical sensor obtained according to Example 6 was immersed in a glucose solution located in a flow-through chamber and measured for 7 days during which the concentration of the glucose solution was continuously varied between 0 and 26 mM. Figures 2 and 3 show the results of this measurement.

Patentkrav

1. Elektrokemisk sensor til bestemmelse af en analyt i et flydende medium omfattende mindst en arbejdselektrode og mindst en referenceelektrode, hvorved i det mindste arbejdselektroden omfatter en elektrokatalysators partikler i en elektrodematrix,
kendetegnet ved,
at et til bestemmelsen af analytten velegnet enzym er bundet selektivt covalent til elektrokatalysatorens partikler og ikke har nogen covalente bindinger til elektrodematrixens øvrige bestanddele, og at elektrokatalysatoren er MnO_2.

2. Elektrokemisk sensor ifølge krav 1,
kendetegnet ved,
at 90% af elektrokatalysatorpartiklerne har en diameter på fra 0,01 µm til 20 µm, især fra 0,5 µm til 5 µm.

3. Elektrokemisk sensor ifølge krav 1 eller 2,
kendetegnet ved,
at det på elektrokatalysatorpartiklerne bundne enzym har en enzymaktivitet på fra 0,01 U/mg til 10 U/mg, især fra 0,1 U/mg til 10 U/mg.

4. Elektrokemisk sensor ifølge et af kravene 1 til 3,
kendetegnet ved,
at elektrokatalysatorpartiklerne har en funktionaliseret overflade, hvortil enzymet er bundet.

5. Elektrokemisk sensor ifølge krav 4,
kendetegnet ved,
at enzymet er bundet direkte til elektrokatalysatorpartiklernes funktionaliserede overflade.

6. Elektrokemisk sensor ifølge et af kravene 1 til 5,
kendetegnet ved,

at elektrodematrixen er porøs tildannet.

7. Elektrokemisk sensor ifølge et af kravene 1 til 6,
kendetegnet ved,
at elektrodematrixen endvidere omfatter et ledende elektrodemateriale.

8. Elektrokemisk sensor ifølge et af kravene 1 til 7,
kendetegnet ved,
at den mindst indeholder to afsnit, hvorved det første afsnit omfatter elektroderne, er forsynet med en biokompatibel belægning og kan bringes i kontakt med det flydende medium, som indeholder analytten, og hvorved det andet afsnit ligger i et område, som er utilgængeligt for det flydende medium.

9. Elektrokemisk sensor ifølge krav 8,
kendetegnet ved,
at den biokompatible belægning er tildannet ved påføring af en på forhånd fremstillet membran på sensoren eller ved påføring af en opløsning af en polymer på sensoren og efterfølgende tørring.

10. Elektrokemisk sensor ifølge krav 8 eller 9,
kendetegnet ved,
at den biokompatible belægning har en tykkelse på fra 1 µm til 100 µm, fortrinsvis fra 3 µm til 25 µm.

11. Elektrokemisk sensor ifølge krav 8,
kendetegnet ved,
at det andet afsnit har en måleværdiregistreringsenhed eller en interface til en måleværdiregistreringsenhed, som er separat i forhold til den elektrokemiske sensor.

12. Elektrokemisk sensor ifølge et af kravene 1 til 11,
kendetegnet ved,

at den er tildannet som hel eller delvis implanterbar indretning eller som gennemstrømningscelle.

13. Fremgangsmåde til fremstilling af en elektrokemisk sensor ifølge et af kravene 1 til 12, omfattende trinnene:

klargøring af elektrokatalysatorpartikler,
coatning af elektrokatalysatorpartikler med et enzym, hvorved enzymet bindes covalent med elektrokatalysatorpartiklerne,
blanding af de under trinnet (b) fremstillede elektrokatalysatorpartikler, som er belagt covalent med enzym, med et ledende elektrodemateriale og eventuelt yderligere substanser,
forarbejdning af den under trin (c) opnåede blanding til en elektrode, og
kombinere den under trinnet (d) opnåede elektrode med mindst en yderligere elektrode.

14. Fremgangsmåde ifølge krav 13,
kendetegnet ved,
at elektrokatalysatorpartiklerne under trin (b) først omsættes med et belægningsreagens, derpå med et tværbindingsreagens og til sidst med enzymet.

15. In-vitro-fremgangsmåde til bestemmelse af en analyt i et flydende medium, omfattende trinnene:

(a) bringning af det flydende medium i kontakt med en elektrokemisk sensor ifølge et af kravene 1 til 12, og
(b) bestemmelse af forekomsten og/eller mængden af analytten i det flydende medium ved måling af et signal, som fremstilles af den elektrokemiske sensor.

Figure 1

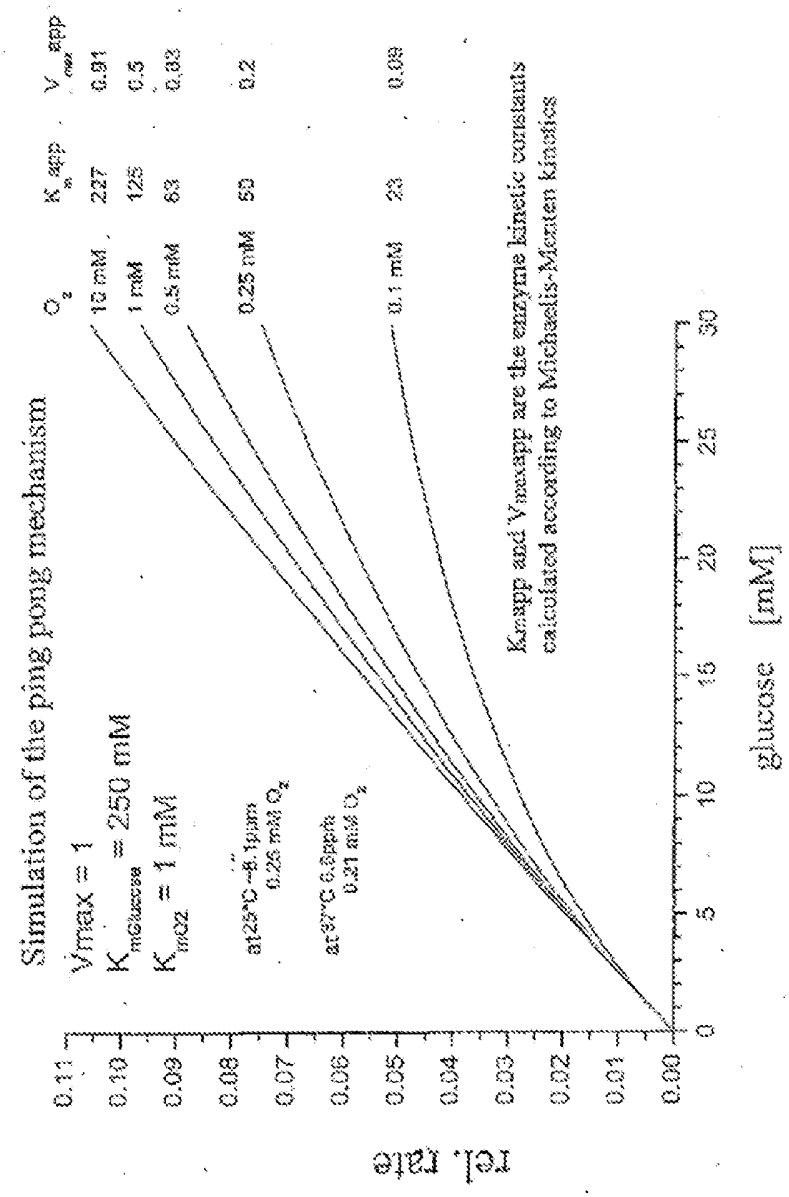

Simulation of the ping pong mechanism
Vmax = 1
K mGlucose = 250 mM
K mO2 = 1 mM
at 25°C ~8.1ppm
0.26 mM O2
at 37°C 6.0ppm
0.21 mM O2
O2
K m app
V max app
10 mM
237
0.91
1 mM
125
0.5
0.5 mM
63
0.32
0.25 mM
59
0.2
0.1 mM
23
0.09
Kmapp and Vmaxapp are the enzyme kinetic constants
calculated according to Michaelis-Menten kinetics
rel. rate
glucose [mM]
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
5
10
15
20
25
30

Figure 2

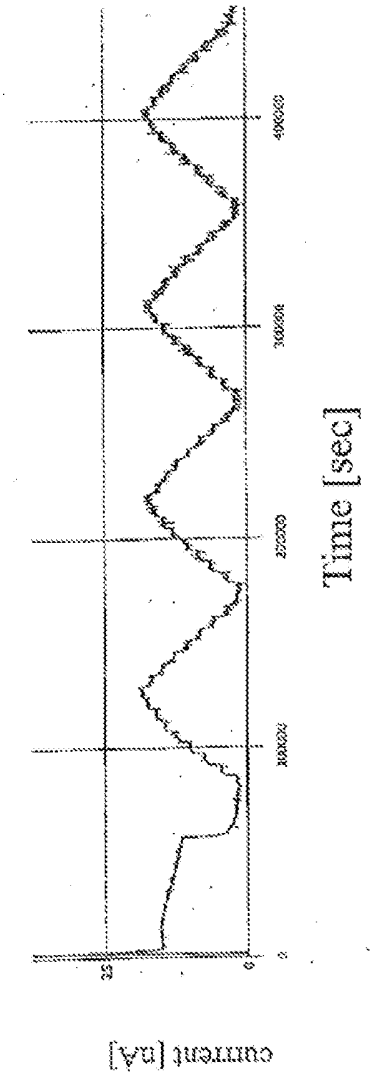
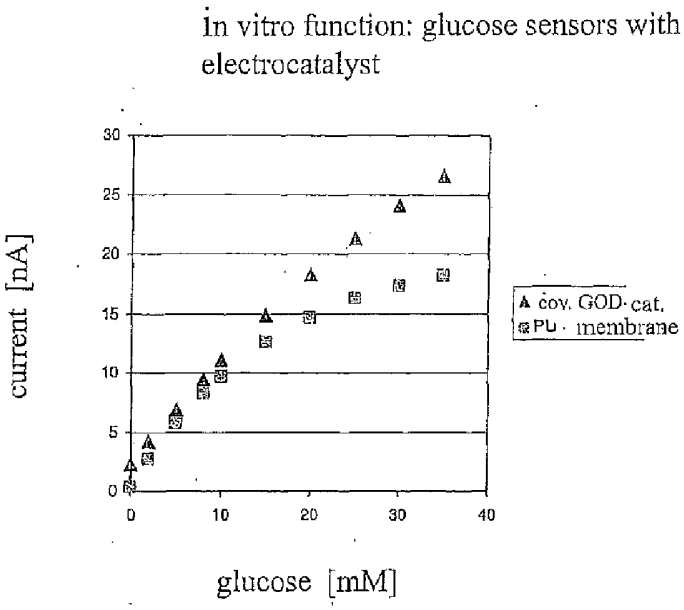


Figure 3


In vitro function: glucose sensors with electrocatalyst
current [nA]
glucose [mM]
0
5
10
15
20
25
30
0
10
20
30
40
cov. GOD-cat.
PU - membrane