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(54) **Process for the preparation of modified electrodes, electrodes prepared with said process, and enzymatic biosensors comprising said electrodes**

Verfahren zur Herstellung modifizierter Elektroden, danach hergestellte Elektroden und enzymatische Biosensoren mit diesen Elektroden

Processus de préparation d'électrodes modifiées, électrodes préparées avec ce processus, et biocapteurs enzymatiques comportant ces électrodes

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Description

Field of the invention

[0001] The present invention refers to the field of modified electrodes for the measurement of analytes in biological fluids, and in particular a new process for the preparation of electrodes modified with Prussian blue, modified electrodes prepared as described, biosensors comprising said electrodes and the method for the determination of analytes in biological fluids using said electrodes.

State of the art

[0002] The use of Prussian blue to modify amperometric enzymatic electrodes has been known for some time, and resulted from the discovery, going back to the 80s, that Prussian blue can be deposited in layers on electrodes of different materials such as platinum, vitreous carbon, SnO₂ and TiO₂, and has a catalytic effect in reduction of the hydrogen peroxide produced during the enzymatic oxidation of the analyte.

[0003] With said electrodes modified with Prussian blue, the concentration of hydrogen peroxide formed can be identified, thus permitting indirect measurement of the concentration of the oxidised analyte, which is directly proportional to the quantity of hydrogen peroxide produced.

[0004] These electrodes modified with Prussian blue have therefore been used for analytical applications, in particular in amperometric biosensors for the measurement of glucose levels in the blood. In addition to acting as an electrochemical mediator in reduction of the hydrogen peroxide, the layer of Prussian blue can also be used as a substrate for immobilisation of the oxidase enzyme.

[0005] The electrodes modified with Prussian blue can be prepared for example by means of electrochemical deposition of solutions of ferric ferrocyanide on an electrode consisting of one of the above-mentioned materials. The United States patent No. US 5,876,581, for example, describes an electrochemical deposition process in which a pair of electrodes are immersed in a solution containing the Iron (III) and hexacyanoferrate (III) ions; performing electrolysis with one of the two electrodes as cathode and the other as anode, a layer of insoluble ferric hexacyanoferrate (III), known as Prussian blue, deposits on the surface of the cathode.

[0006] The electrodes that can be used in this process according to US 5,876,581 require the presence of a cathode made of or coated in an inert metal such as platinum, rhodium, gold etc. or an oxide of a conductive metal or a semi-conductor.

[0007] More recently these traditional electrodes have been superseded by screen printed electrodes (SPEs), which have numerous advantages: they are inexpensive, easy to prepare, they are versatile and suitable for large-

scale industrial production.

[0008] As mentioned above, electrodes modified with Prussian blue have been used for some time now, depositing this product on the surface of the electrode by means of electrochemical techniques. Said techniques, however, are not suitable for the large-scale production of modified electrodes starting from screen printed electrodes for two main reasons: 1) the electrochemical procedures are generally long, and have to be performed electrode by electrode, with an enormous waste of time in the case of preparation of a large number of electrodes; and 2) the flat shape of the screen printed electrodes makes use of the electrochemical procedures difficult and laborious since the latter require immersion of the electrode in the solution containing the ionic species, and this can lead to the formation of a layer of Prussian blue also on the surface of the reference electrode, obstructing electrical conductivity and preventing use of the screen printed electrode for analytical purposes.

[0009] For these reasons, as far as the Applicant is aware, electrochemical procedures for deposition of Prussian blue on screen printed electrodes do not currently exist. A process for chemical deposition of Prussian blue on a screen printed electrode is described by Ricci et al. in Biosensors and Bioelectronics 18 (2003) 165-174. Said process comprises, prior to chemical deposition of the Prussian blue, electrochemical pre-treatment of the electrodes, which are electrochemically treated for 3 minutes at a potential of 1.7 V. According to this article, said procedure is essential for obtaining improved reproducibility and response of the electrode, but on the other hand makes production of the modified electrodes very laborious, requiring electrochemical pre-treatment for each of them and thus nullifying the advantages of the chemical deposition.

[0010] In the process described in the above article, furthermore, the Prussian blue is deposited on the electrode manually, by means of a complicated procedure which requires great caution in order to prevent an increase in the internal resistance of the system: a mixture must be prepared on the spot, adding a solution of potassium ferricyanide in HCl to a solution of ferric chloride in HCl, after which a drop of said mixture is deposited exclusively on the surface of the working electrode, trying to avoid the reference electrode and the counter-electrode.

[0011] Even with the above precautions, the electrodes modified in this way are limited in terms of reproducibility of the deposition and stability of the layer of Prussian blue; therefore, even though the screen printed electrodes are in themselves characterised by a high level of reproducibility of the electrode surface, responsible for the stability and reproducibility of the electric signal of the analyte, the poor reproducibility and lack of uniformity in preparation of the layer of Prussian blue creates serious problems in measurement of the quantity of analytes in the biological fluids, and in general in the applications for which the electrode is intended.

[0012] Given the advantages connected with the use of screen printed electrodes, it is evident that there is a great need in the sector for an easily scalable process, via which the surface of electrodes screen printed with Prussian blue can be modified, overcoming the drawbacks highlighted above for the known processes.

Summary of the invention

[0013] The Applicant has developed a new process, particularly inexpensive and simple to produce, which permits modification of screen printed electrodes with Prussian blue, obtaining electrodes with a high level of stability and reproducibility, useful in the production of planar biosensors for the quantitative determination of analytes in biological fluids.

[0014] The object of the present invention is therefore a process for the preparation of a screen printed electrode modified with Prussian Blue according to claim 1, comprising sequential deposition on the surface of said screen printed electrode of a solution comprising the Iron (III) or Iron (II) ion and at least one surface-active agent in an appropriate solvent and a solution comprising the ferrocyanide (II) or ferricyanide (III) ion and at least one surface-active agent in an appropriate solvent, said solutions having concentrations such as to obtain the formation of Prussian blue directly on the surface of the electrode.

[0015] The screen printed electrode modified with Prussian blue according to claim 16 prepared by means of the above-mentioned process, the enzymatic electrode according to claim 17 and the enzymatic biosensor according to claim 19 which comprise said electrode, and a method for determination of the quantity of an analyte in a biological sample according to claim 21 comprising contact between said sample and the above-mentioned enzymatic biosensor constitute a further subject of the invention.

[0016] Characteristics and advantages of the invention will be illustrated in detail in the following description.

Brief description of the figures

[0017]

Figure 1: Profile of the modified screen printed electrode prepared as described in Example 1.

Figure 2: Cyclic voltammogram recorded using the modified screen printed electrode as described in Example 1 (curve a), and cyclic voltammogram recorded with the same electrode in the presence of H_2O_2 (curve b), which shows the electrocatalytic activity of the Prussian blue.

Figure 3: Cyclic voltammogram recorded using the modified screen printed electrode as described in Example 3 for comparison (curve a), and cyclic voltammogram recorded with the same electrode in the presence of H_2O_2 (curve b), which shows the elec-

trocatalytic activity of the Prussian blue.

Figure 4: Comparison between cyclic voltammogram recorded using the modified electrode as described in Example 1 (curve a) and voltammogram recorded with the modified electrode as described in Example 3 for comparison (curve b). Note the greater deposition of Prussian blue obtained with the electrode of Example 1.

Figure 5: Comparison between the operative stability of a modified electrode according to Example 1 (Figure 5a) and a modified electrode according to Example 3 for comparison (Figure 5b), from which the greater operative stability of the electrode obtained with the procedure described in Example 1 can be noted.

Detailed description of the invention

[0018] The solution containing the Iron (III) or Iron (II) ion used in the process of the invention is for example an acid solution with pH between 0.5 and 6.0, preferably an acid solution obtained by addition of HCl, of a compound chosen from the group consisting of Iron (III) or Iron (II) salts of inorganic acids, for example ferric chloride, ferric sulphate and ferric nitrate; preferably the present solution is a solution of ferric chloride in aqueous HCl 0.01 M having pH 2.0.

[0019] The concentration of the Iron (III) or Iron (II) ion in the above-mentioned solution can be for example between 20 mM and 2 M, and is preferably 1 M.

[0020] The solution containing the hexacyanoferrate (III) ion according to the invention is for example an acid solution with pH between pH 0.5 and 6.0, obtained preferably by addition of HCl, of a salt containing the ferrocyanide $Fe(CN)_6^{4-}$ ion or ferricyanide $Fe(CN)_6^{3-}$ ion, for example sodium, potassium, ammonium or cobalt hexacyanoferrate; preferably the present solution is a solution of potassium ferricyanide $K_3Fe(CN)_6$ in aqueous HCl 0.01 M having pH 2.0.

[0021] The concentration of the Ferrocyanide (II) or Ferricyanide (III) ion in the above-mentioned solution can be for example between 20 mM and 2 M, and is preferably 1 M.

[0022] According to the invention, the present process comprises deposition of the solution containing the Iron (III) or Iron (II) ion on the surface of the working electrode and, immediately after, deposition of the solution containing the hexacyanoferrate (III) ion, in equal volume and concentration.

[0023] The volume of the two solutions with concentration between 20 mM and 2 mM is for example between 100 nL and 4 μ L for an electrode surface of between 0.314 mm² and 3.14 cm², preferably 3.14 mm².

[0024] Electrodes screen printed on inert material, for example polycarbonate, polyester, polyvinylchloride (PVC) or other plastic material, with suitable inks of conductive materials, for example graphite, silver, gold or platinum, are suitable for use in the present process for

preparing the modified electrodes of the invention; graphite ink is preferred for the working electrode.

[0025] If necessary, after mixing the two solutions, the mixture is left to rest for a period, for example, of between 2 minutes and 2 hours, and preferably 10 minutes; the working electrode surface is then washed with a washing solution consisting, for example, of an acid aqueous solution with pH between 0.5 and 6.0, and preferably an aqueous solution of HCl 0.01 M having pH 2.0.

[0026] Following this, the working electrode can be further washed with distilled water.

[0027] The electrodes thus modified are then left to dry in a stove for example at a temperature between 50°C and 200°C for a period of between 10 minutes and 3 hours. Preferably the electrode is placed in a stove at a temperature of 100°C for 1 hour 30 minutes.

[0028] The two solutions comprising the Iron (III) or Iron (II) ion and the ferrocyanide (II) or ferricyanide (III) ion according to the invention furthermore comprise at least one surface-active agent, chosen from cationic, anionic, amphoteric surface-active agents and their mixtures, in quantities for each solution, for example, of between 0.001 and 10% in weight with respect to the total volume of the solution. Preferably the surface-active agent is chosen from the group consisting of sodium lauryl sulphate and lauryl ethoxy sulphate, benzalkonium chloride, compounds belonging to the family of products known under the trade name Tween®, i.e. polyoxyethylene derivatives of esters of fatty acids with sorbitol, and their mixtures.

[0029] According to a particularly preferred embodiment of the invention, the surface-active agent polyoxyethylene (20) sorbitan monolaurate, sold under the name Tween® 20 is added to each of the two solutions comprising respectively the Iron (III) or Iron (II) ion and the ferrocyanide (II) or ferricyanide (III) ion, in quantities of 0.05% in weight with respect to the total volume of the solution.

[0030] With the present deposition procedure, a layer of Prussian blue can be obtained on the surface of the working electrode which is extremely reproducible and active from the electrochemical point of view. Furthermore, with the cyclic voltammetry technique, the quantity of Prussian blue present on the working electrode following the deposition has been determined, identifying values of between 10 and 200 nmol/cm² and preferably 100 nmol/cm².

[0031] With the present process, therefore, a massive deposition of Prussian blue can be obtained, never observed previously with either chemical or electrochemical processes; the high surface density of the Prussian blue present on the surface of the electrode allows extremely high operative stability to be obtained without affecting the catalytic activity of the Prussian blue vis-à-vis reduction of the H₂O₂.

[0032] The electrodes modified with Prussian blue prepared with the process of the invention can be used for the preparation of enzymatic biosensors with both two

and three electrodes, which are also the subject of the present invention. In said biosensors, the modified electrode is used as a support for immobilisation of a suitable enzyme, chosen on the basis of the type of analytical determination for which the biosensor is intended, i.e. such that the analyte to be detected functions as a substrate for the enzyme, generating a product that can be oxidised or reduced electrochemically on the modified electrode, varying the quantity of current detected in proportion to the quantity of analyte present in the fluid analysed.

[0033] The present biosensor comprises an enzymatic electrode, i.e. the modified electrode of the invention as described above, on which the enzyme has been immobilised by means of procedures commonly used and known to any expert in the sector, and a cell for receiving the biological fluid to be analysed, so that the latter can come into contact with the enzyme.

[0034] According to a preferred embodiment of the invention, the present biosensor comprises a modified electrode of the invention on which the glucose oxidase enzyme has been immobilised, and is used for determination of the glucose in biological fluids such as blood, serum or plasma; in this case the analyte is the glucose which, oxidised by the enzyme, produces H₂O₂ which is reduced on the modified electrode due to the potential applied between this electrode and the reference electrode, generating a current signal proportional to the quantity of H₂O₂ produced and therefore to the quantity of glucose present in the fluid contained in the cell.

[0035] A further subject of the invention is the method for determination of the quantity of an analyte in a biological fluid, comprising the application of an appropriate potential value between a modified electrode of the invention as described above and the reference electrode contained in the present biosensor, and reading of the generated current signal.

[0036] Preferably, the present method is used for determination of the quantity of glucose on biological fluids and the potential applied is low, for example between -250 mV and +200 mV, preferably -50 mV.

[0037] The present process for the preparation of electrodes modified with Prussian blue, as described above, has a high level of reproducibility of the deposition stage, which positively affects reproducibility of the measurement performed with the electrodes modified via this process; furthermore, it provides a modified electrode with greater operative stability than any electrode modified with Prussian blue prepared so far with the known processes. It has been observed that in the electrodes modified by means of the present process, the layer of Prussian blue is still active and presents a reduction in its electrochemical activity of only approximately 35%, after 150 hours of continuous use.

[0038] In addition to greater operative stability, the electrodes modified with Prussian blue prepared with the process of the invention also have greater long-term stability during storage.

[0039] These stability values, both operative and storage, and reproducibility values, in the case of biosensors with both two and three electrodes, are furthermore achieved by a process which does not require, contrary for example to the process described by Ricci et al. in *Biosensors and Bioelectronics* 18 (2003) 165-174, electrochemical pre-treatment of the electrode before deposition of the Prussian blue, and is therefore much less laborious and easy to automate, and therefore scalable at industrial level.

[0040] A further advantage of the modified electrodes of the invention, in the case of biosensors with two electrodes which can be used, for example, for determination of the glucose, is that of comprising a working electrode and a reference electrode, while they do not require the counter-electrode, contrary for example to the electrodes modified with Prussian blue described in the above-mentioned article by Ricci et al.

[0041] The following examples provide a non-limiting illustration of the invention.

Example 1

Preparation of the modified electrode

[0042] Following the known procedure, a screen printed electrode was prepared on a polyester sheet, comprising a circular-shaped working electrode with diameter of 2 mm prepared with a graphite ink and a silver reference electrode; the surface of the working electrode is delimited by an ink made of insulating material. Figure 1 shows the profile of the electrode obtained in this way.

[0043] The following two solutions were then prepared: 1) 1 M solution of potassium ferricyanide $K_3Fe(CN)_6$ in HCl 10 mM and 2) 1 M solution of ferric chloride in HCl 10 mM. Tween[®] 20 was added to each of the two solutions in a quantity of 0.05% in weight with respect to the total volume of the solution.

[0044] With an automatic dispensing machine, 1 μ l of the solution 1) was deposited on the surface of the working electrode and, immediately after, with the same automatic dispensing technique, 1 μ l of the solution 2) was deposited on the same surface of the working electrode, thus resulting in 2 μ l of a solution of ferric chloride and potassium hexacyanoferrate on said surface.

[0045] After 10 minutes, the electrodes thus modified were washed with a few millilitres of a 10 mM solution of HCl, then placed in a stove at 100°C for 1 hour.

[0046] The procedure described above resulted in deposition on the electrode of an extremely compact and stable layer of Prussian blue, with a high surface density of the Prussian blue on the working electrode.

[0047] The latter is confirmed by the results of the cyclic voltammetry shown in Figure 2, which permitted calculation of the quantity of Prussian blue on the surface of the working electrode: the surface density value measured is approximately 100 nmol/cm², much greater than reported in the literature so far using different deposition

techniques.

Example 2

5 Determination of the catalytic activity of Prussian blue for the electrode prepared in Example 1

[0048] The properties of the modified electrode prepared as described above in Example 1 were verified by means of cyclic voltammetry, in the potential range -0.4 to 0.4 V.

10 **[0049]** Figure 2 shows the voltammograms obtained for the screen printed electrode not yet modified and for the same electrode modified with Prussian blue, prepared as described above in Example 1. For the latter electrode, the increase in the cathodic wave to approximately 0.05 V in the presence of hydrogen peroxide can be seen in the voltammogram, showing the catalytic activity of the layer of Prussian blue deposited on the electrode.

Example 3 (comparison)

25 Preparation of the modified electrode as described by Ricci et al. in *Biosensors and Bioelectronics* 18 (2003) 165-174

[0050] Following the procedure described previously in the article by Ricci et al., *Biosensors and Bioelectronics* 18 (2003) 165-174, an electrode screen printed on a polyester sheet, comprising a circular shaped working electrode with diameter of 2 mm prepared with a graphite ink and a silver reference electrode, was modified; the surface of the working electrode is delimited by an ink made of insulating material.

35 **[0051]** The following two solutions were then prepared: 1) 0.1 M solution of potassium ferricyanide $K_3Fe(CN)_6$ in HCl 10 mM and 2) 0.1 M solution of ferric chloride in HCl 10 mM.

40 **[0052]** Before deposition of the two solutions by means of the electrochemical method, a procedure unsuitable for automation and much more laborious and longer than that of Example 1, the electrode was pre-treated by application of a constant potential equal to 1.7 V vs. Ag/AgCl for 3 minutes.

45 **[0053]** Using an automatic dispensing machine, 20 μ l of the solution 1) were deposited on the surface of the working electrode and, immediately after, using the same deposition technique, 20 μ l of the solution 2) were deposited on the same surface of the working electrode, thus obtaining 40 μ l of a mixture of ferric chloride and potassium hexacyanoferrate.

50 **[0054]** After 10 minutes, the electrodes thus modified were washed in a few millilitres of a 10 mM solution of HCl, then placed in a stove at 100°C for 1 hour. The electrocatalytic activity of the layer of Prussian blue thus deposited was assessed following the same procedure as the one described in Example 2. Figure 3 shows the

cyclic voltammograms obtained for the modified screen printed electrode as described above.

Example 4

Determination of the analytical performances of the modified electrodes according to the invention and according to the prior art

[0055] The analytical performances of the electrodes modified with Prussian blue prepared as described above in Examples 1 and 3 were tested considering the response to H_2O_2 , the operative and non-operative stability and the reproducibility. As regards reproducibility, the reproducibility values, calculated as Relative Standard Deviation percentage (abbreviated below to RSD%), for the modified electrode as described in Example 3 are approximately 20%, incompatible with any industrial type of application, whereas an RSD% of 5% was calculated for the electrode of the invention, modified as described in Example 1.

[0056] As regards the thickness of the Prussian blue deposited on the electrodes, with the procedure of the prior art described in Example 3, a layer of Prussian blue is obtained with a surface density of between 1 and 10 $nmol/cm^2$, therefore much lower than the one obtained for the electrode of the invention of Example 1 (approximately 100 $nmol/cm^2$) as shown by comparison of the cyclic voltammograms (Figure 4).

[0057] This results in greater operative stability of the Prussian blue, up to a maximum of 200 hours. Figure 5, for example, shows continuous monitoring with a flow technique, obtained with the two electrodes prepared following the procedures described in Examples 1 and 3. As can be clearly seen, with a 5 mM concentration of H_2O_2 , both the electrodes provide a reduction current due to the catalytic activity of the Prussian blue, but the electrode of Example 1 shows greater operative stability in the long term, with a reduction in the initial signal of only 20% after 200 hours.

[0058] In the case of the electrode of the prior art prepared in Example 3, a more marked reduction is observed equal to approximately 70% after 200 hours.

Example 5

Preparation of the biosensor

[0059] The modified electrode prepared as described above in Example 1 was used as a support for immobilisation of the glucose oxidase enzyme, for the purpose of obtaining a biosensor useful for continuous monitoring of glucose in the blood.

[0060] For this purpose 200 nL of a solution of glutaraldehyde (0.025% v/v in H_2O) were deposited on the surface of the working electrode, previously modified with Prussian blue as described above in Example 1. After approximately 25 minutes, 200 nL of a mixture obtained

by dissolving 10 mg of the glucose oxidase enzyme in 1 ml of an aqueous solution of Nafion® (0.1 %) were deposited on the same surface of the working electrode. Here again, it is left to rest for approximately 25 minutes so as to obtain complete drying of the solution deposited.

Claims

1. A process for the preparation of a screen printed electrode modified with Prussian blue, **characterised in that** it comprises sequential deposition on the surface of said screen printed electrode of a solution comprising the Iron (III) or Iron (II) ion and at least one surface-active agent in an appropriate solvent and a solution comprising the ferrocyanide (II) or ferricyanide (III) ion and at least one surface-active agent in an appropriate solvent, said solutions having concentrations such as to obtain the formation of Prussian blue directly on the surface of the electrode; wherein the solutions are deposited in equal volume and equal concentration; wherein the modified electrode is then left to dry in a stove at a temperature between 50°C and 200°C for a period of between 10 minutes and 3 hours; wherein said surface-active agent is chosen from the group consisting of sodium lauryl sulphate and lauryl ethoxy sulphate, benzalkonium chloride, polyoxyethylene derivatives of esters of fatty acids with sorbitol, and mixtures thereof.
2. The process as claimed in claim 1, wherein said screen printed electrode has not undergone pretreatment before said sequential deposition of the solutions.
3. The process as claimed in claim 1, wherein said solution comprising the Iron (III) or Iron (II) ion is an acid solution with pH between 0.5 and 6.0 of a compound chosen from the group consisting of Iron (III) or Iron (II) salts of inorganic acids.
4. The process as claimed in claim 3, wherein said Iron (III) or Iron (II) salt of inorganic acids is chosen from ferric chloride, ferric sulphate and ferric nitrate.
5. The process as claimed in claim 3, wherein said solution is a solution of ferric chloride in aqueous HCl 0.01 M having pH 2.0.
6. The process as claimed in claim 1, wherein said solution comprising the hexacyanoferrate (III) ion is an acid solution with pH between pH 0.5 and 6.0 of a salt containing the ferrocyanide (II) or ferricyanide (III) ion.
7. The process as claimed in claim 6, wherein said salt is chosen from sodium, potassium, ammonium and

cobalt hexacyanoferrate.

8. The process as claimed in claim 6, wherein said solution is a solution of potassium ferricyanide $K_3Fe(CN)_6$ in aqueous HCl 0.01 M having pH 2.0.
9. The process as claimed in claim 1, wherein said solutions have equal concentration of between 20 mM and 2 mM, and are deposited in equal volume of between 100 nL and 4 μ L for an electrode surface between 0.314 mm² and 3.14 cm², preferably 3.14 mm².
10. The process as claimed in claim 1, wherein said screen printed electrode is made of inert material screen printed with graphite ink.
11. The process as claimed in claim 1, furthermore comprising the stages of leaving the solutions deposited to rest for 10 minutes, washing the surface of the working electrode with a washing solution consisting of an aqueous acid solution with pH between 0.5 and 6.0, washing with distilled water if necessary and leaving to dry in a stove at a temperature between 50°C and 200°C, for a period of between 10 minutes and 3 hours.
12. The process as claimed in claim 11, wherein said washing solution is an aqueous solution of HCl 0.01 M having pH 2.0.
13. The process as claimed in claim 12, wherein the electrode is left to dry in a stove at a temperature of 100°C for a period of 1 hour 30 minutes.
14. The process as claimed in claim 1, wherein said surface-active agent is chosen from cationic, anionic, amphoteric surface-active agents and mixtures thereof, and added in quantities for each solution of between 0.001 and 10% in weight with respect to the total volume of the solution.
15. The process as claimed in claim 1, wherein said surface-active agent is polyoxyethylene (20) sorbitan monolaurate, in a quantity of 0.05% in weight with respect to the total volume of the solution.
16. The screen printed electrode modified with Prussian blue prepared with the process as defined in claims 1-15, wherein the quantity of Prussian blue is 100 nmol/cm².
17. An enzymatic electrode comprising the screen printed electrode modified as defined in claim 16 and a suitable enzyme immobilised on said electrode, chosen so that a product thereof can be oxidised or reduced electrochemically on said modified electrode.

18. The enzymatic electrode as claimed in claim 17, wherein said enzyme is the enzyme glucose oxidase.
19. An enzymatic biosensor comprising the enzymatic electrode as defined in claims 17-18 as the working electrode, a reference electrode, a counter-electrode if necessary and a cell for receiving the biological fluid to be analysed, so that the latter can come into contact with the enzyme immobilised on said enzymatic electrode.
20. The biosensor according to claim 19, comprising said screen printed electrode and said reference electrode as the sole electrodes.
21. A method for determination of the quantity of an analyte in a biological fluid comprising the application of an appropriate potential value between an enzymatic electrode as defined in claims 17-18 and a reference electrode included in the enzymatic biosensor as defined in claims 19-20, and reading of the current signal generated.
22. The method as claimed in claim 21, wherein said analyte is glucose and said potential applied is between -250 and +200 mV.
23. The method as claimed in claim 22, wherein said potential applied is equal to -50 mV.

Patentansprüche

1. Verfahren zur Herstellung einer Siebdruck-Elektrode, welche mit Preußischblau modifiziert ist, **dadurch gekennzeichnet, dass** dieses das sequenzielle Abscheiden einer Lösung, die Eisen (III)-Ionen oder Eisen (II)-Ionen und wenigstens ein oberflächenaktives Mittel in einem geeigneten Lösungsmittel enthält, sowie einer Lösung, die Ferrocyanid (II)-Ionen oder Ferricyanid (III)-Ionen sowie wenigstens ein oberflächenaktives Mittel in einem geeigneten Lösungsmittel enthält, auf der Oberfläche der Siebdruck-Elektrode umfasst, wobei die Lösung Konzentrationen aufweist, so dass die Bildung von Preußischblau direkt auf der Oberfläche der Elektroden erreicht wird, wobei die Lösungen in einem gleichen Volumen und in gleicher Konzentration abgeschieden werden, wobei die modifizierte Elektrode dann in einem Ofen bei einer Temperatur zwischen 50°C und 200°C für eine Zeitspanne zwischen 10 Minuten und 3 Stunden stehen gelassen wird, um zu trocknen, wobei das oberflächenaktive Mittel aus der Gruppe ausgewählt wird, welche aus Natriumlaurylsulfat und Laurylethoxysulfat, Benzalkoniumchlorid, Polyoxyethylenderivaten von Estern von Fettsäuren mit Sorbit und Mischungen hiervon besteht.

2. Verfahren nach Anspruch 1, wobei die Siebdruck-Elektrode vor dem sequenziellen Abscheiden der Lösungen keine Vorbehandlung unterlaufen hat.
3. Verfahren nach Anspruch 1, wobei die Lösung, welche die Eisen (III)-Ionen oder Eisen (II)-Ionen enthält, eine saure Lösung einer Verbindung ausgewählt aus der Gruppe bestehend aus Eisen (III)- oder Eisen (II)-Salzen von anorganischen Säuren mit einem pH-Wert zwischen 0,5 und 6,0 ist.
4. Verfahren nach Anspruch 3, wobei das Eisen (III)-Salz oder Eisen (II)-Salz von anorganischen Säuren aus Eisenchlorid, Eisensulfat und Eisennitrat ausgewählt wird.
5. Verfahren nach Anspruch 3, wobei die Lösung eine Lösung von Eisenchlorid in wässriger 0,01 M HCl mit einem pH-Wert von 2,0 ist.
6. Verfahren nach Anspruch 1, wobei die Lösung, welche die Hexacyanoferrat (III)-Ionen enthält, eine saure Lösung eines die Ferrocyanid (II)- oder Ferricyanid (III)-Ionen enthaltenden Salzes mit einem pH-Wert zwischen pH 0,5 und 6,0 ist.
7. Verfahren nach Anspruch 6, wobei das Salz aus Natrium-, Kalium-, Ammonium- und Kobalthexacyanoferrat ausgewählt wird.
8. Verfahren nach Anspruch 6, wobei die Lösung eine Lösung von Kaliumferricyanid $K_3Fe(CN)_6$ in wässriger 0,01 M HCl mit einem pH-Wert von 2,0 ist.
9. Verfahren nach Anspruch 1, wobei die Lösungen eine gleiche Konzentration zwischen 20 mM und 2 mM aufweisen und in einem gleichen Volumen von zwischen 100 nL und 4 μ L für eine Elektrodenoberfläche zwischen 0,314 mm² und 3,14 cm², vorzugsweise 3,14 mm², abgeschieden werden.
10. Verfahren nach Anspruch 1, wobei die Siebdruck-Elektrode aus einem inerten Siebdruckmaterial mit Graphittinte hergestellt worden ist.
11. Verfahren nach Anspruch 1, welches die Schritte des Stehenlassens der abgeschiedenen Lösungen für 10 Minuten, das Waschen der Oberfläche der Arbeitselektrode mit einer Waschlösung, die aus einer wässrigen Säurelösung mit einem pH-Wert zwischen 0,5 und 6,0 besteht, das Waschen mit destilliertem Wasser, falls erforderlich, sowie das trocknen lassen in einem Ofen bei einer Temperatur zwischen 50°C und 200°C für eine Zeitspanne zwischen 10 Minuten und 3 Stunden umfasst.
12. Verfahren nach Anspruch 11, wobei die Waschlösung eine wässrige Lösung von 0,01 M HCl mit einem pH-Wert von 2,0 ist.
13. Verfahren nach Anspruch 12, wobei die Elektroden in einem Ofen bei einer Temperatur 100°C für eine Zeitspanne von 1 Stunde 30 Minuten trocknen gelassen wird.
14. Verfahren nach Anspruch 1, wobei das oberflächenaktive Mittel aus kationischen, anionischen, amphoteren oberflächenaktiven Mitteln und Mischungen hiervon ausgewählt wird und in Mengen für jede Lösung zwischen 0,001 und 10 Gew.-% bezogen auf das Gesamtvolumen der Lösung zugegeben wird.
15. Verfahren nach Anspruch 1, wobei das oberflächenaktive Mittel Polyoxyethylen-(20)-sorbitanmonolaurat in einer Menge von 0,05 Gew.-% bezogen auf das Gesamtvolumen der Lösung ist.
16. Siebdruck-Elektrode, welche mit Preußischblau modifiziert, hergestellt nach einem Verfahren nach den Ansprüchen 1 bis 15, wobei die Menge an Preußischblau 100 nmol/cm² betrifft.
17. Enzymatische Elektrode, welche eine modifizierte Siebdruck-Elektrode nach Anspruch 16 und ein geeignetes Enzym enthält, welches auf der Elektrode immobilisiert ist und so ausgewählt ist, dass ein Produkt hiervon auf der modifizierten Elektrode elektrochemisch oxidiert oder reduziert werden kann.
18. Enzymatische Elektrode nach Anspruch 17, wobei das Enzym Glukoseoxidase ist.
19. Enzymatischer Biosensor enthaltend eine enzymatische Elektrode nach Anspruch 17 oder 18 als die Arbeitselektrode, eine Referenzelektrode, eine Gegenelektrode, falls erforderlich, und eine Zelle zum Empfang des zu analysierenden biologischen Fluids, so dass letzteres in Kontakt mit dem Enzym kommen kann, welches auf der enzymatischen Elektrode immobilisiert ist.
20. Biosensor nach Anspruch 19 umfassend die Siebdruck-Elektrode und die Referenzelektrode als einzige Elektroden.
21. Verfahren zum Bestimmen der Menge eines Analyten in einem biologischen Fluid umfassend das Aufbringen eines geeigneten Potentialwerts zwischen eine enzymatische Elektrode nach Anspruch 17 oder 18 und einer Referenzelektrode, welche in einem enzymatischen Biosensor nach Anspruch 19 oder 20 enthalten ist, und Auslesen des erzeugten Stromsignals.
22. Verfahren nach Anspruch 21, wobei der Analyt Glukose ist und das angelegte Potential zwischen -250

und +200 mV beträgt.

23. Verfahren nach Anspruch 22, wobei das angelegte Potential gleich -50 mV ist.

Revendications

1. Procédé de préparation d'une électrode sérigraphiée modifiée avec du bleu de Prusse, **caractérisé en ce qu'il** comprend le dépôt séquentiel sur la surface de ladite électrode sérigraphiée d'une solution comprenant l'ion de fer (III) ou de fer (II) et au moins un agent tensioactif dans un solvant approprié et d'une solution comprenant l'ion ferrocyanure (II) ou ferricyanure (III) et au moins un agent tensioactif dans un solvant approprié, lesdites solutions ayant des concentrations permettant d'obtenir la formation du bleu de Prusse directement sur la surface de l'électrode ; dans lequel les solutions sont déposées en volumes égaux et en concentrations égales ; dans lequel l'électrode modifiée est ensuite laissée à sécher dans une étuve à une température située entre 50 °C et 200 °C pendant une période de 10 minutes à 3 heures ; dans lequel ledit agent tensioactif est choisi dans le groupe constitué par le sulfate de lauryle et de sodium et l'éthoxysulfate de lauryle, le chlorure de benzalkonium, les dérivés polyoxyéthylénés d'esters d'acides gras avec le sorbitol, et leurs mélanges.
2. Procédé selon la revendication 1, dans lequel ladite électrode sérigraphiée n'a subi aucun prétraitement avant ledit dépôt séquentiel des solutions.
3. Procédé selon la revendication 1, dans lequel ladite solution comprenant l'ion de fer (III) ou de fer (II) est une solution acide ayant un pH situé entre 0,5 et 6,0 d'un composé choisi dans le groupe constitué par les sels de fer (III) ou de fer (II) d'acides inorganiques.
4. Procédé selon la revendication 3, dans lequel le sel de fer (III) ou de fer (II) d'acides inorganiques est choisi parmi le chlorure ferrique, le sulfate ferrique et le nitrate ferrique.
5. Procédé selon la revendication 3, dans lequel ladite solution est une solution de chlorure ferrique dans du HCl aqueux 0,01 M ayant un pH de 2,0.
6. Procédé selon la revendication 1, dans lequel ladite solution comprenant l'ion hexacyanoferrate (III) est une solution acide ayant un pH situé entre 0,5 et 6,0 d'un sel contenant l'ion ferrocyanure (II) ou ferricyanure (III).
7. Procédé selon la revendication 6, dans lequel ledit sel est choisi parmi l'hexacyanoferrate de sodium,

de potassium, d'ammonium et de cobalt.

8. Procédé selon la revendication 6, dans lequel ladite solution est une solution de ferricyanure de potassium, $K_3Fe(CN)_6$, dans du HCl aqueux 0,01 M ayant un pH de 2,0.
9. Procédé selon la revendication 1, dans lequel lesdites solutions ont des concentrations identiques situées entre 20 mM et 2 mM, et sont déposées en volumes égaux de 100 nl à 4 µl pour une surface d'électrode située entre 0,314 mm² et 3,14 cm², de préférence 3,14 mm².
10. Procédé selon la revendication 1, dans lequel ladite électrode sérigraphiée est faite d'un matériau inerte sérigraphié avec une encre de graphite.
11. Procédé selon la revendication 1, comprenant en outre les étapes suivantes : le fait de laisser au repos pendant 10 minutes les solutions déposées, le lavage de la surface de l'électrode de travail avec une solution de lavage constituée d'une solution acide aqueuse ayant un pH situé entre 0,5 et 6,0, un lavage avec de l'eau distillée si nécessaire et un séchage dans une étuve à une température située entre 50 °C et 200 °C pendant une période de 10 minutes à 3 heures.
12. Procédé selon la revendication 11, dans lequel ladite solution de lavage est une solution aqueuse de HCl 0,01 M ayant un pH de 2,0.
13. Procédé selon la revendication 12, dans lequel l'électrode est laissée à sécher dans une étuve à une température de 100 °C pendant une période de 1 heure 30 minutes.
14. Procédé selon la revendication 1, dans lequel ledit agent tensioactif est choisi parmi les agents tensioactifs cationiques, anioniques, amphotères et leurs mélanges, et est ajouté en des quantités pour chaque solution de 0,001 % à 10 % en poids par rapport au volume total de la solution.
15. Procédé selon la revendication 1, dans lequel ledit agent tensioactif est le monolaurate de polyoxyéthylène sorbitane (20), en une quantité de 0,05 % en poids par rapport au volume total de la solution.
16. Electrode sérigraphiée modifiée avec du bleu de Prusse préparée avec le procédé tel que défini dans les revendications 1 à 15, dans laquelle la quantité de bleu de Prusse est de 100 nmol/cm².
17. Electrode enzymatique, comprenant l'électrode sérigraphiée modifiée telle que définie dans la revendication 16 et une enzyme appropriée immobilisée

sur ladite électrode, choisie de façon qu'un produit de celles-ci puisse être oxydé ou réduit par voie électrochimique sur ladite électrode modifiée.

- 18.** Electrode enzymatique selon la revendication 17, dans laquelle ladite enzyme est l'enzyme glucose oxydase. 5
- 19.** Biocapteur enzymatique, comprenant l'électrode enzymatique telle que définie dans les revendications 17 et 18 en tant qu'électrode de travail, une électrode de référence, une contre-électrode si nécessaire et une cellule servant à recevoir le fluide biologique à analyser, de façon que ce dernier puisse entrer en contact avec l'enzyme immobilisée sur ladite électrode enzymatique. 10
15
- 20.** Biocapteur selon la revendication 19, comprenant ladite électrode sérigraphiée et ladite électrode de référence en tant que seules électrodes. 20
- 21.** Procédé de détermination de la quantité d'un analyte dans un fluide biologique, comprenant l'application d'une valeur de potentiel appropriée entre une électrode enzymatique telle que définie dans les revendications 17 et 18 et une électrode de référence incluse dans le biocapteur enzymatique tel que défini dans les revendications 19 et 20, et la lecture du signal de courant généré. 25
30
- 22.** Procédé selon la revendication 21, dans lequel ledit analyte est le glucose et ledit potentiel appliqué est situé entre -250 mV et +200 mV.
- 23.** Procédé selon la revendication 22, dans lequel ledit potentiel appliqué est égal à -50 mV. 35

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Figure 1

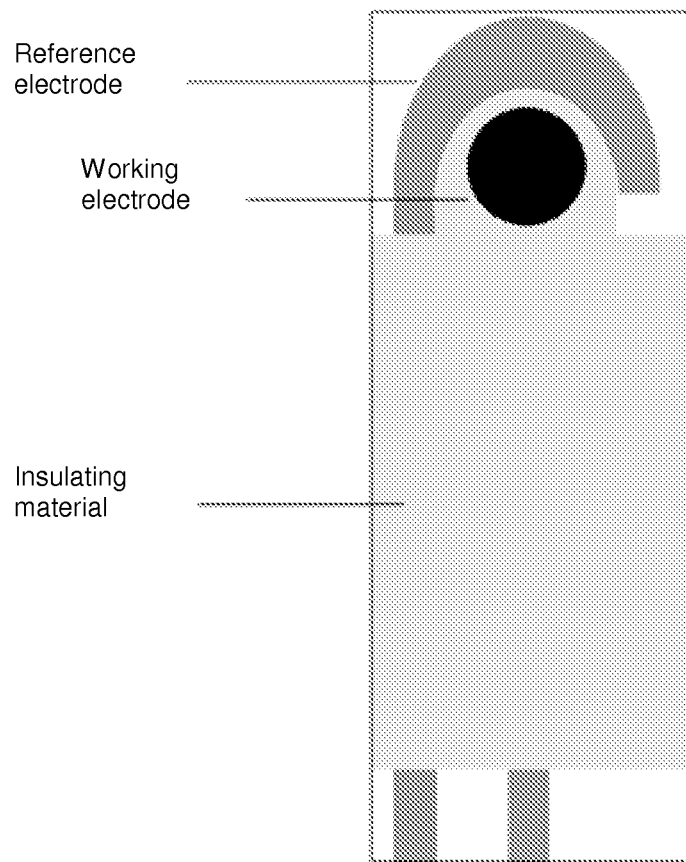


Figure 2

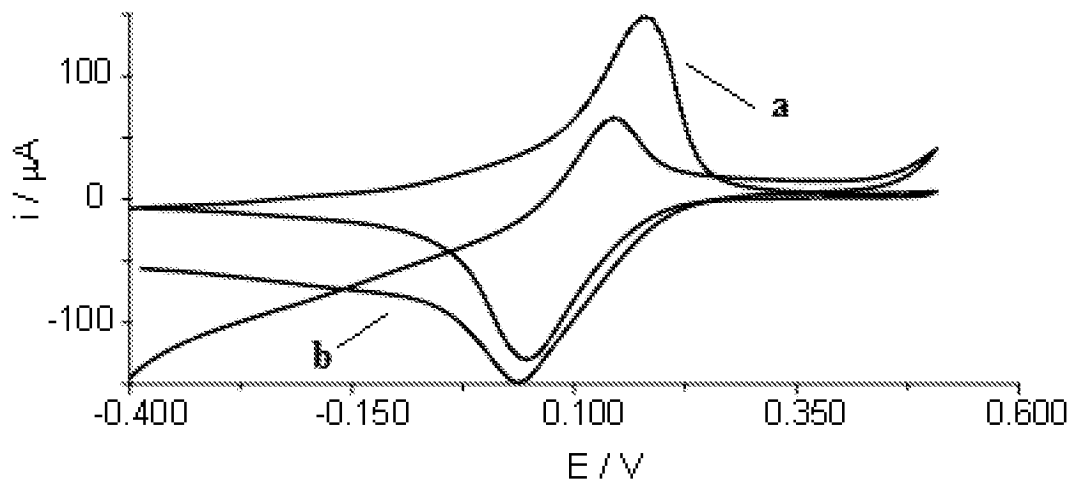


Figure 3

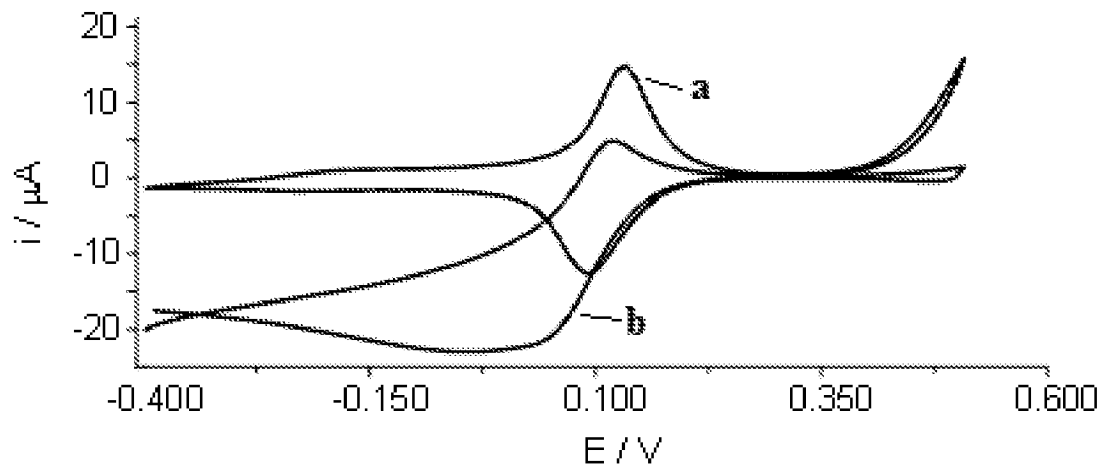


Figure 4

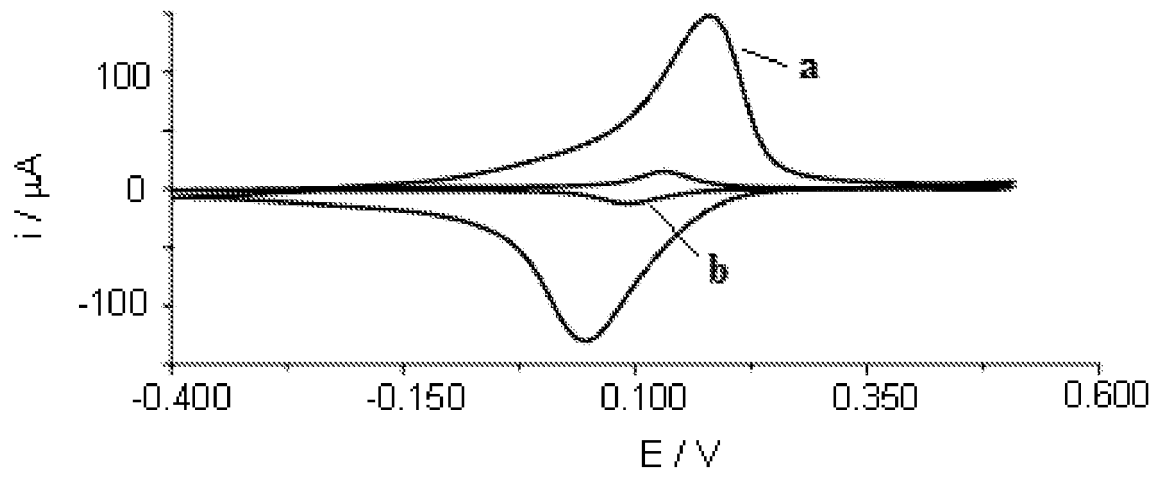


Figure 5a

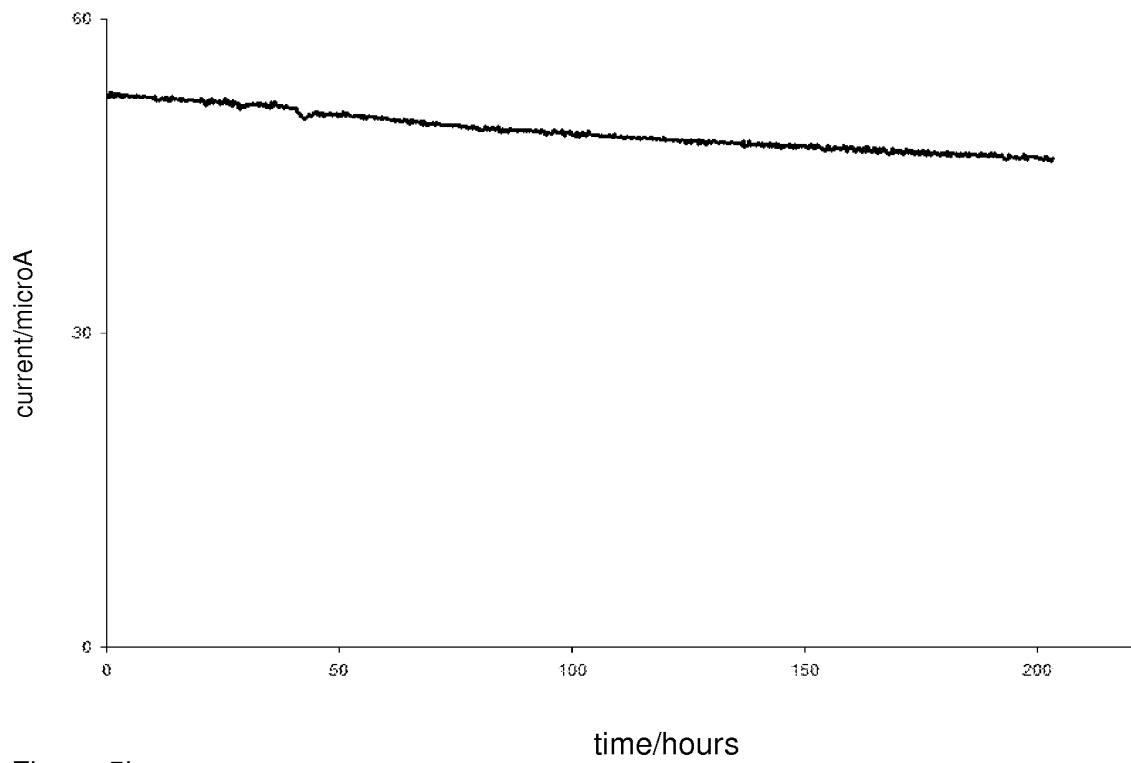
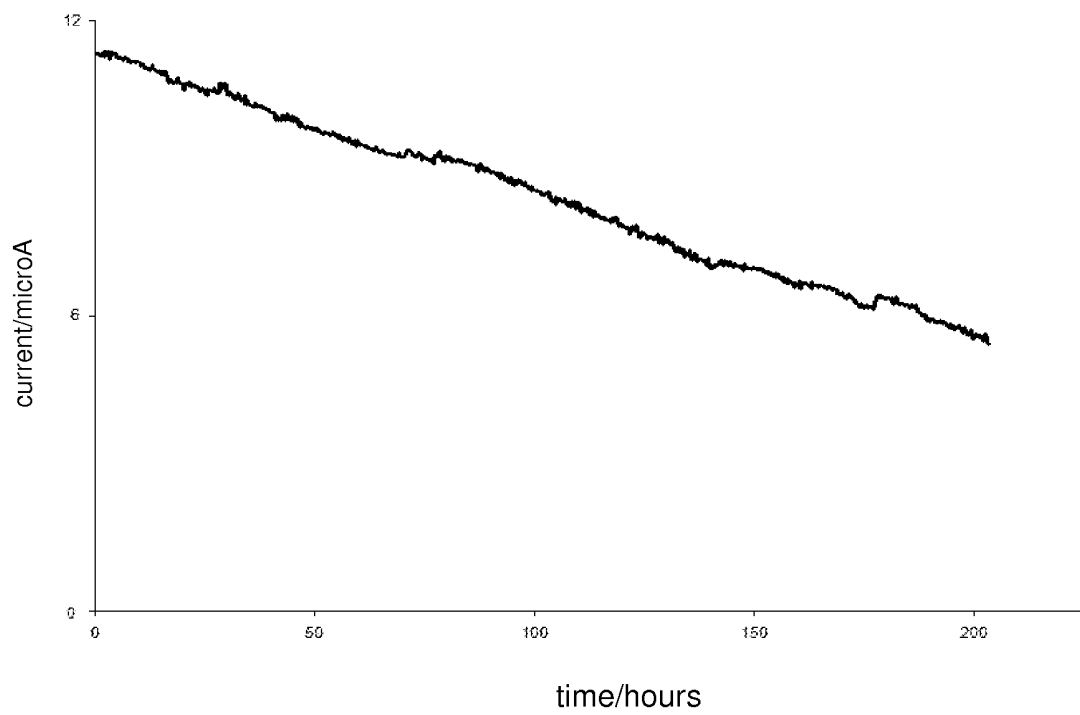


Figure 5b



REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 5876581 A [0005] [0006]

Non-patent literature cited in the description

- **RICCI et al.** *Biosensors and Bioelectronics*, 2003, vol. 18, 165-174 [0009] [0039] [0050]

专利名称(译)	制备修饰电极的方法，用所述方法制备的电极和包含所述电极的酶促生物传感器		
公开(公告)号	EP1935331B1	公开(公告)日	2015-09-09
申请号	EP2007122884	申请日	2007-12-11
申请(专利权)人(译)	A. MENARINI INDUSTRIE FARMACEUTICHE RIUNITE S.R.L.		
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IPC分类号	C23C18/16 C23C18/12 A61B5/00 C12Q1/00 C12Q1/54 G01N33/543		
CPC分类号	C12Q1/001 C12Q1/003 C23C18/1204 C23C18/1601 G01N33/5438 Y10T436/144444		
优先权	FI2006000322 2006-12-13 IT		
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外部链接	Espacenet		

摘要(译)

描述了一种制备用于测量生物流体中分析物的修饰电极的方法，包括在丝网印刷电极上沉积普鲁士蓝，以及通过所述方法制备的修饰电极；还描述了包含所述修饰电极的酶电极和生物传感器以及使用所述修饰电极测定生物流体中分析物的方法。

Figure 1

