Description

A biosensor comprising a biomaterial which is covalently fixed to a signal-active surface

---

This invention relates to biosensors, particularly to biosensors which are based on the field effect, comprising a signal-active surface which is optionally silanised and to which the sensory biomaterial is bonded by means of a crosslinking agent.

Biosensors which make use of enzymatic reactions for the selective detection of defined analytes have already been in a state of extensive development for a long time.

Sensors such as these fundamentally comprise a surface which contains biomaterial, such as an enzyme for example, and which is exposed to the medium to be tested - particularly a liquid. The information supplied by the biomaterial under the action of the analyte is converted by a transducer element and is ultimately obtained in recordable form by means of electronic signal processing, which is optionally integrated signal processing. Potentiometric and amperometric electrodes, such as field effect structures etc., are being discussed in particular as transducers.

One particular problem of sensors containing biomaterial such as these is the satisfactory fixation, i.e. particularly the permanent fixation or immobilisation, of the biomaterial on the sensor surface with high specific activity per unit area and the avoidance, as far as possible, of a decrease in activation due to the type of immobilisation.

Different forms of sensory biomaterial have been considered, such as enzymes, microorganisms, cells, antibodies, antigens, organelles or tissue sections for example. The relevant literature predominantly comprises works on enzyme-containing biosensors,

however, so that enzyme-containing biosensors are considered below for the sake of simplicity.

Known immobilisation techniques comprise purely adsorptive deposition, binding into polymer layers, crosslinking of the enzyme molecules to each other and covalent bonding of the enzyme to the sensor. In order to effect covalent bonding of the enzyme to the predominantly oxidic sensor surface, the latter is usually silanised (mostly with an aminoalkylalkoxysilane) and coupling of the enzyme to the silanised surface is subsequently ensured by means of glutaraldehyde (see Anal. Chim. Acta 245 (1991) pages 89 - 99). However, the silanisation of Si_3N_4 surfaces with subsequent covalent bonding by means of different crosslinking agents has also already been described (see R.A. Williams et al., Biosensors & Bioelectronics 9 (1994) 159-67).

In addition, the use has also been recommended of thiol-terminated silanes, with subsequent protein immobilisation by means of a heterobifunctional crosslinking agent, e.g. with N-hydroxy-succinimide esters such as N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDS), N-succinimidyl-p-iodoacetylamido-benzoate (SIAB), N-succinimidyl-4-(p-maleimido-phenyl) butyrate (SMBP) or N-succinimidyl-m-maleimido-benzoate derivatives (MBS derivatives) (see S.K. Bathia et al., Anal. Biochem. 178 (1989) 408-13).

The direct bonding of enzymes and microorganisms to an Si_3N_4 surface which is produced by plasma polymerisation is described in JP 61 087 699 of 1986, wherein glutaraldehyde or diisocyanate are used for this purpose.

The reactivity of the functional groups, both of the homo- or bifunctional crosslinking agent and of the bonding agent concerned, enables oligomer formation and diversification of the couplings to take place, so that it appears that the covalent bonding of a biomolecule to the signal-active surface specifically via a molecule of a crosslinking agent cannot be ensured.

A new type of biomaterial immobilisation on sensor surfaces has therefore been developed which results in definite products having a high sensitivity and a long life.

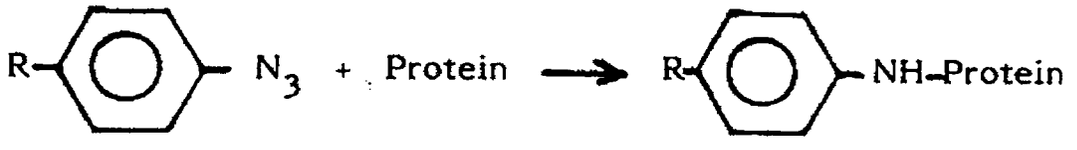
The biosensors according to the invention of the type cited at the outset are essentially characterised in that the crosslinking agent is formed by a heterobifunctional crosslinking agent comprising firstly a group (A) which is reactive specifically towards the signal-active surface and secondly a photo-activatable group (B) which is reactive towards biomaterial.

With a heterobifunctional crosslinking agent such as this, comprising a group which is reactive towards biomaterial but which is does not react until photo-activation is effected, a definite coupling of the biomaterial to the surface can be achieved by first reacting the crosslinking agent with the surface, via a group which is correspondingly reactive, with the avoidance of photo-activation. The removal of excess (non-bonded) residual crosslinking agent is then followed by the reaction with biomaterial under corresponding illumination.

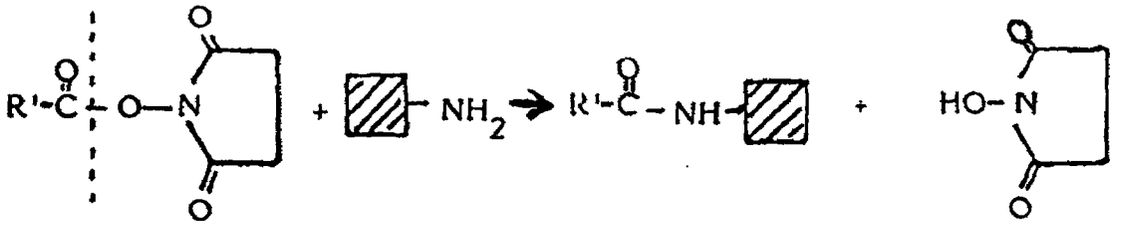
Substances which are particularly suitable as surfaces include semiconductor materials such as germanium or silicon, as well as oxide materials such as silica, alumina, tantalum oxide or the like, in reactive form or silanised, wherein silanisation layers are deposited, e.g. on silica, which comprise mercapto or amino groups in particular. An Si_3N_4 surface layer comprising reactive groups, particularly NH_x- groups which are available for coupling with aldehyde groups, ester groups or imidoester groups in particular, is also advantageous.

Alicyclically or aromatically bonded nitro or azido groups which react with protein material under illumination are particularly suitable as photo-activatable groups which can react with biomaterial. Substances which are known in this respect include crosslinking agents which contain azidophenyl radicals or azidosalicylic radicals as group B and which contain a N-hydroxy-succinimidoester group as group A. Crosslinking agents such as these are commercially available (see the "Immunotechnology Catalog and Handbook" of the Pierce company of 1992/3; E34-46, for example).

The photo-activated reaction of an azidophenyl compound with protein under irradiation by incident light of wavelength 265-275 nm proceeds, for example, according to the equation:



Radicals which are particularly suitable as R, due to their availability, are radicals which contain an N-hydroxy-succinimidoester group as the terminal group A, which can react with NH_x groups in the following manner



Disulphide groups are known as functions A of the crosslinking agent which react with SiH groups.

The formation of silicon nitride surfaces can be effected in particular by deposition by the CVD technique from an SiH_4/NH_3 mixture (see A. Garde et al. in ESSDERC 1994, 11 - 15 September 1994 Ed. C. Hill & P. Ashburn). They absorb oxygen from the air and exhibit a tendency to hydrolyse under the action of moisture, with the formation of Si-OH, Si-NH and Si-NH_2 groups. These groups, which react with group A of the crosslinking agent, can be utilised for the coupling of biomaterial to the nitride surface.

A surface which can be used particularly advantageously for the production of the sensors according to the invention is an Si_3N_4 surface which has been freed from oxides by treatment with dilute hydrofluoric acid, and the NH_2 groups, or the NH groups also, of which react with a crosslinking agent, the photo-activatable function B of which is subsequently reacted with the protein.

Apart from those groups already mentioned, suitable groups A, which are reactive towards NH_2, of the crosslinking agent also comprise halide, epoxide, imide or isocyanate functions. In addition, a multiplicity of possible reactions with amino groups is to be found in US-PS 5 234 820, for example.

The amino-specific reactions which are cited in detail below proceed at room temperature at a neutral to slightly alkaline pH. Raising either the temperature or the pH increases the rate of reaction, but also increases the rate of hydrolysis of the crosslinking agent. The buffer which is used should contain neither amines nor other compounds with which the functional groups of the crosslinking agent could react.

In particular, N-hydroxysuccinimide ester reacts specifically with primary amines. An amide bond is formed between the primary amine and the residual group of the ester used, with the separation of N-hydroxysuccinimide. Unless a water-soluble analogue is used, a crosslinking agent which contains this functional group first has to be dissolved in a small amount of an organic solvent (e.g. DMSO) and not until then is it diluted to the final concentration in an aqueous buffer. The ionic strength of the buffer should not be too high, in order to prevent salting-out effects. A slightly alkaline pH (7-9) ensures that the primary amines are in their unprotonated state.

Aldehydes contain a strongly reducing carbonyl group. This reacts with primary amines with the separation of water.

The reaction of primary amines with imidoesters proceeds in the pH range between 8 and 9. In the course of this reaction, the ester is cleaved and the primary amine forms a guanidino compound with the imido group.

If the crosslinking agent comprises a thiol-specific group such as maleimide, activated halide or pyridyl disulphide as its functional end A, the surface to be coupled must comprise a free sulfhydryl group (usually a mercaptoalkyl group of an alkoxysilane). In order to prevent the oxidation of this group, the buffer which is used has to be de-gassed.

Maleimide reacts in slightly acidic to neutral media (pH 6.5 - 7.5), whereas pH values greater than or equal to 7 are recommended for halides and pyridyl sulphide.

The invention can be used for the bonding of biomaterial, particularly protein biomaterial, especially very different enzymes such as hydrolases, oxidoreductases, transferases, lyases,

isomerases and ligases, or synthases, particularly penicillinase, urease, glucose oxidase, urokinase, acetylcholinesterase, butyryl cholinesterase or lactatoxidase. It has been tested for penicillin sensors in particular, for example, which is why the following description relates to the latter. Reference is made in this respect to the accompanying drawings, which comprise the following schematic illustrations:

Figure 1 illustrates the principle of a sensor (measuring arrangement);

Figure 2 shows a typical measurement curve for a concentration range from 10^{-4} to 10^{-1} mole/litre penicillin; and

Figure 3 shows the calibration curve of a sensor according to the invention.

Example:

An electrically non-conductive layer of silica with a thickness of 5 - 100 nm was first produced on p- or p^+-doped silicon wafers (1 mOhm.cm - 30 mOhm.cm), by thermal oxidation under dry conditions between 700 and 1200°C (at 1000°C here) in a diffusion furnace. Non-conducting silicon nitride with a thickness of 10 - 100 nm was likewise deposited thereon by chemical deposition in the gas phase (PECVD). The SiH_4/NH_3 ratio in the reaction gas was 2/1, the substrate temperature was 200 - 500°C (300°C here), and the pressure during the deposition operation was 1 - 3 torr (1.5 torr here). This was followed by an annealing step under an N_2 atmosphere (5-60 minutes at 700-1000°C). Finally, the unpolished side of the substrate was provided with an ohmic contact (e.g. 10 - 1000 nm Al, Au). The material used was deposited by thermal evaporation in a vacuum at a base pressure $<10^{-3}$ mbar. The deposition rate was between 0.1 and 10 nm/sec. The wafer was subsequently annealed at 150 - 500°C (400°C here) in an N_2 atmosphere in an RTA furnace.

Immediately before the commencement of the enzyme immobilisation process, the wafers were cleaned in acetone, 2-propanol and distilled water in an ultrasonic bath and were etched for 10 - 60 seconds (30 seconds here) in dilute hydrofluoric acid (1-10 % HF).

When the heterobifunctional crosslinking agent ANB-NOS (N-5-azido-2-nitrobenzoyl-oxysuccinimide) was used, this was first dissolved in a small amount of DMSO and was then diluted to a final concentration of 0.5-10 mM with 0.2 M triethanolamine buffer (pH 5-9). This solution was applied to the Si_3N_4 surface and was incubated at room temperature for between 5 and 40 minutes. Longer incubation times have to be used at lower temperatures. Molecules which were not bonded to the silicon nitride surface were removed by rinsing them off with triethanolamine buffer (TEA). Thereafter, the enzyme (penicillinase Type 1 from *Bacillus cereus*, sigma P 0389) was dissolved (1000-5000 units/ml) in a buffer which contained no amino groups (e.g. TEA, but especially not TRIS or glycine buffer) and was applied to the silicon nitride surface which had been pretreated with the crosslinking agent. After an incubation period of 1 - 240 minutes (15 minutes here) at temperatures between 4 and 60°C, particularly at room temperature, bonding of the enzyme molecule to the functional group, which was still free, of the crosslinking agent was induced by light in the wavelength range from 320-350 nm. After the completion of the immobilisation process, the penicillin sensors, which were fit for use, were rinsed with 0.1 M TRIS buffer (pH 7-8) and distilled water and were dried for at least 10 minutes in air under N_2 or under an inert gas atmosphere.

Measurements were made with the field effect sensors produced in this manner, in order to determine the penicillin concentration in aqueous solutions.

Figure 1 is a schematic illustration of the measuring arrangement. The field effect sensor produced according to the invention, consisting of a silicon substrate 1, the insulating layer 2 (silicon dioxide and silicon nitride), the crosslinking agent layer 3 and the enzyme layer (penicillin layer) 4 were integrated in a measuring cell. The measuring cell was filled with an aqueous test solution which contained penicillin G in a concentration between 10^{-5} and 1 mole/litre. A reference electrode (e.g. Ag/AgCl) 7 was immersed in the test solution 6. The potentials were measured via the reference electrode 7 and a contact electrode 8 on the silicon substrate.

Figure 2 illustrates a typical measurement curve, which was recorded in CONCAP (COnstant CAPacitance) mode in the concentration range between 10^{-4} and 10^{-1} mole/litre penicillin. For

this purpose, the sodium salt of penicillin G sigma P 3032) was dissolved in 10 mM TRIS buffer, pH 7. As the penicillin concentration increased, the concentration of the penicillinic acid formed increased, and there was thus an increase in the concentration of the hydrogen ions in the immediate vicinity of the silicon nitride area which acted as a pH transducer. This resulted in a shift of the potential at the silicon nitride/electrolyte interface towards more positive or more negative values of the voltage. The recording as a function of time enabled the concentration-dependent course of the potential of the enzyme reaction to be observed. The test solution was changed at the times marked.

Figure 3 shows the chemical transfer characteristic determined from **Figure 2**. This constitutes the calibration curve of the field effect sensor produced according to the invention. Data on the sensitivity of a potentiometric chemo- or biosensor are given as regards the underlying Nernst relationship in the linear region of this curve, i.e. in the region in which a logarithmic relationship exists between the penicillin concentration and the associated potential. In the sensor produced according to the invention, this range falls between a p[penicillin] G of 2.3 and 3.3, which corresponds to 0.5 and 5 mM. The sensitivity is 50 mV per decade.

Claims

1. A biosensor, particularly a biosensor based on the field effect, comprising a signal-active surface which is optionally silanised and to which the sensory biomaterial is bonded by means of a crosslinking agent,
characterised in that
the crosslinking agent is formed by a heterobifunctional crosslinking agent comprising firstly a group (A) which is reactive specifically towards the signal-active surface and secondly a photo-activatable group (B) which is reactive towards biomaterial.

2. A biosensor according to claim 1,
characterised in that
the crosslinking agent is bonded to the signal-active surface via a coupling reaction of its group (A) with -OH, -SH or -NH_x groups of the signal-active surface.

3. A biosensor according to claim 2,
characterised in that
the crosslinking agent is bonded, by means of a coupling reaction of its group (A), to a silanised surface or to an Si_3N_4 surface comprising reactive NH_x groups.

4. A biosensor according to any one of claims 1 to 3,
characterised in that
the crosslinking agent is bonded to the protein of the biomaterial via a photo-activated coupling reaction of an azido or nitro group (B) of the crosslinking agent.

5. A biosensor according to claim 4,
characterised in that
the photo-activated coupling reaction is effected with an aromatically bonded nitro or azido group (B).

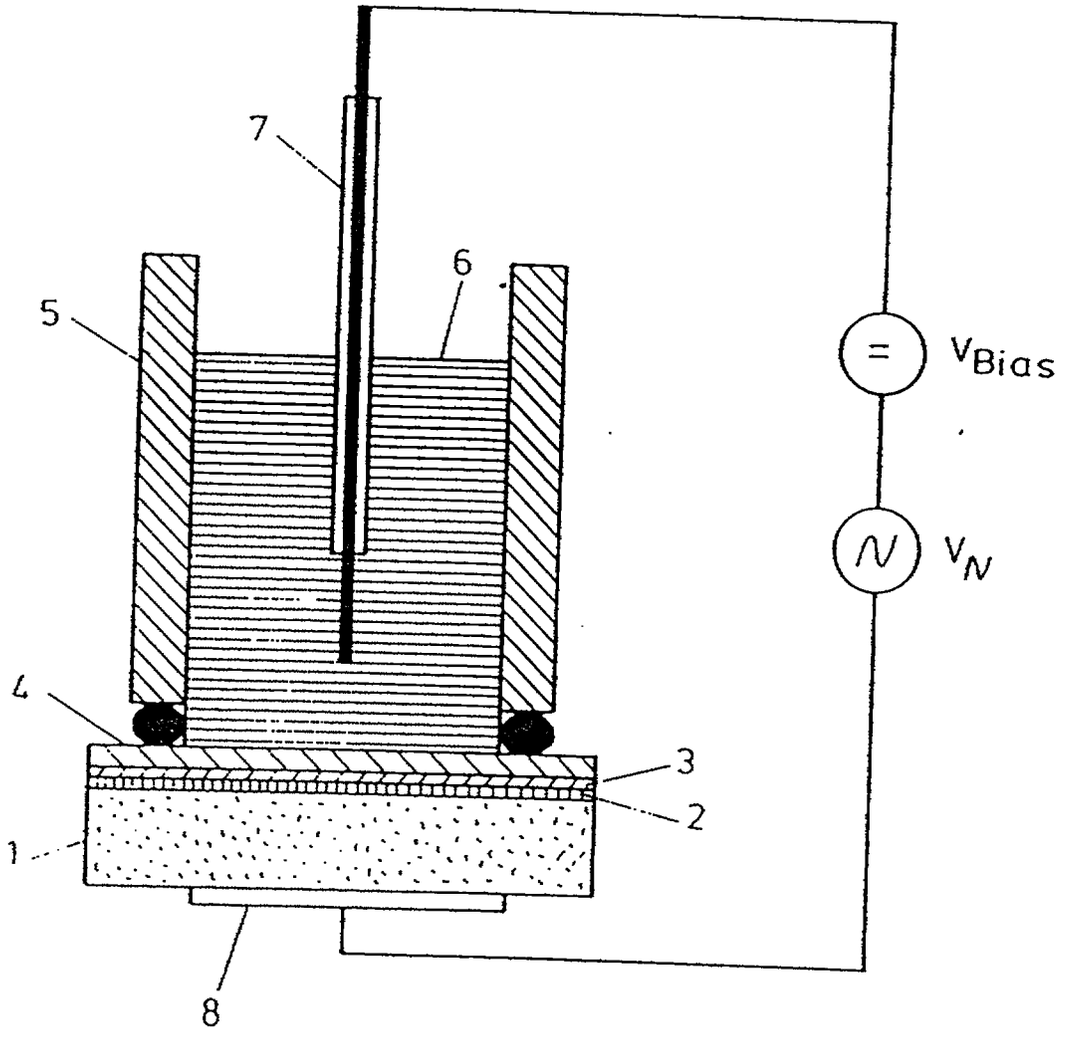
6. A biosensor according to claim 5,
characterised in that
coupling is effected by means of a crosslinking agent comprising an azidophenyl group.

7. A biosensor according to claim 2,
characterised in that
the crosslinking agent is bonded to the surface by means of a coupling reaction of an aldehyde, ester or imidoester group (A).

8. A biosensor according to claim 7,
characterised in that
the crosslinking agent is bonded to the surface by means of a coupling reaction of an N-hydroxy-succinimide ester group (A).

9. A biosensor according to any one of the preceding claims,
characterised by
a signal-active surface having an Si_3N_4 surface layer which is 10 - 1000 nm thick and which is deposited on an SiO_2 surface by CVD from an SiH_4 /NH_3 mixture.

Fig. 1



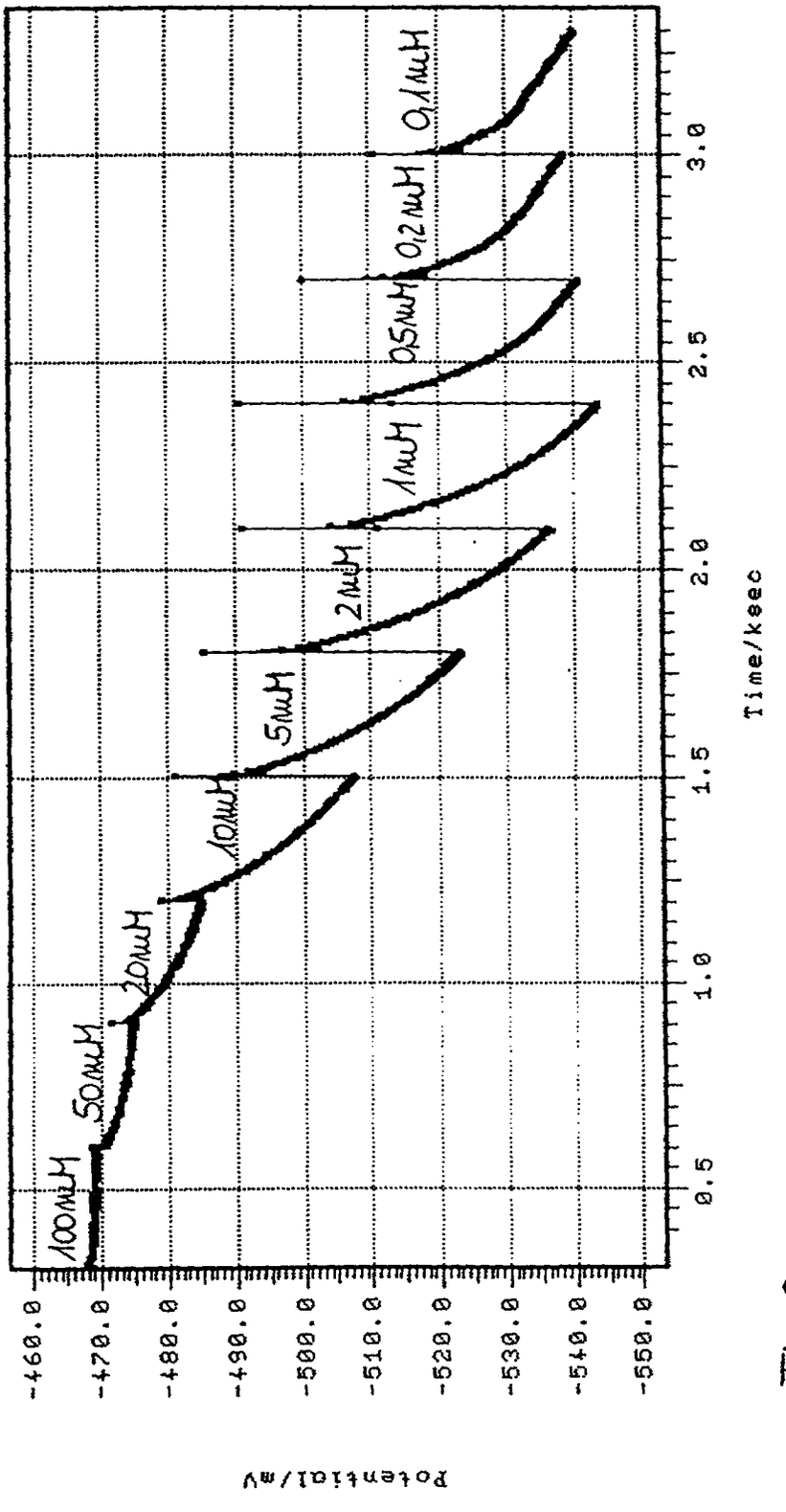

100mN
50mN
20mN
10mN
5mN
2mN
1mN
0,5mN
0,2mN
0,1mN
-460.0
-470.0
-480.0
-490.0
-500.0
-510.0
-520.0
-530.0
-540.0
-550.0
0.5
1.0
1.5
2.0
2.5
3.0
Time/ksec
Potential/mV

Fig. 2

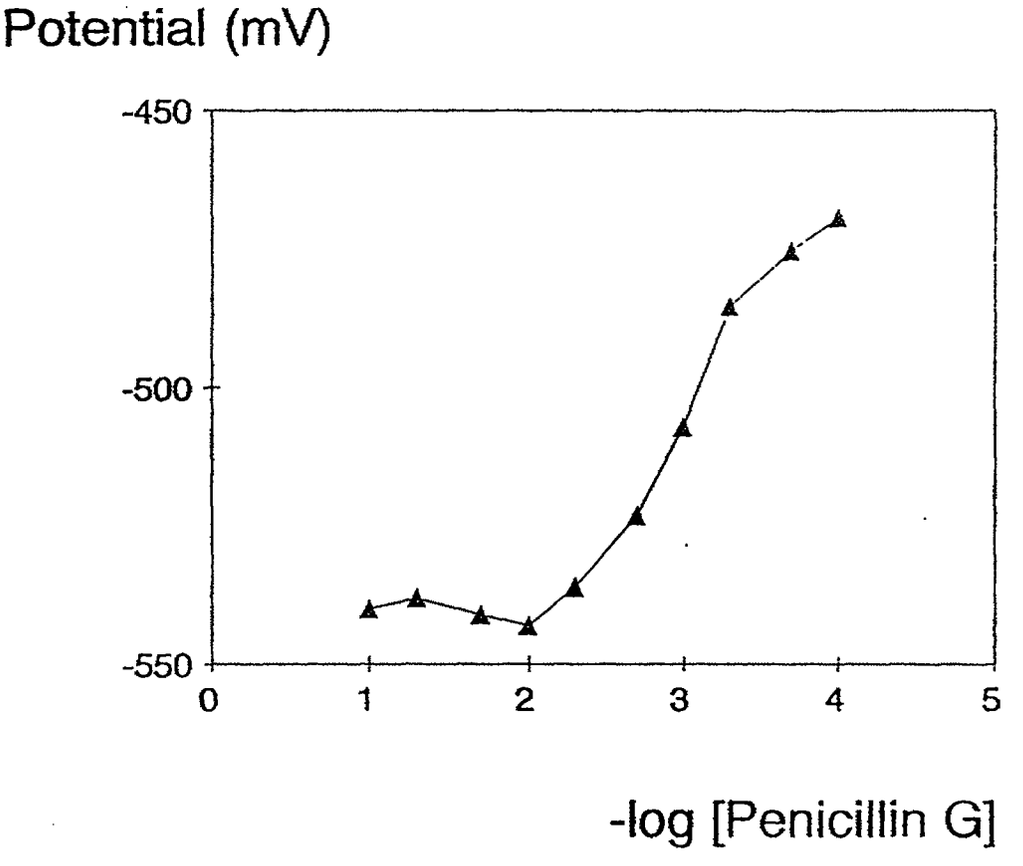


Fig. 3