Description

A method of immobilising biomaterial on a substrate

This invention relates to a method of immobilising biomaterial on a substrate comprising an Si_3N_4 surface to which the biomaterial is covalently bonded via bonding agents.

The immobilisation of biomaterial is of considerable importance for the utilisation of bioactivity, particularly for liquid contact. It plays a large role both in process technology and in analysis during separation operations or for the repeated utilisation of the bio-function. Other fields of interest are to be found in the pharmaceutical and medical sphere, and in environmental technology.

Numerous investigations are known which are aimed at the immobilisation of biomaterial on various surfaces, including those of a mineral type such as glass, which is generally silanised first.

The immobilisation of urease on a quartz crystal which is provided with a thin silver layer, on to the surface of which a silicon nitride layer is sputtered, is described by E. Tamiya et al. in J. Mol. Catal. 43 (1988) 293-301. The crystal which is treated in this manner was stored in air for 24 hours, washed and dried in an air bath. This was followed by the vapour deposition of γ-amino-propyl-triethoxysilane, followed by the vapour deposition of glutaraldehyde. The thin organic surface film which was produced in this manner, which was about 100 Å thick, was described as being only slightly porous. Linking of silane and aldehyde could not really be observed.

The enzyme which was deposited on the film from aqueous solution had an activity of only 2.25 % in relation to that of free urease. This immobilisation concept does not appear to be completely satisfactory.

The object of the present invention is a new type of fixation of biomaterial which involves a small number of treatment steps and results, in a reproducible manner, in relatively stable products.

The method according to the invention of immobilising biomaterial on a substrate of the type cited at the outset is essentially characterised in that an Si_3N_4 surface is provided on the immobilisation substrate comprising NH_x groups which are active as regards bonding and which are reacted with a heterobifunctional crosslinking agent, which firstly contains an aldehyde, ester, halide, epoxide, imine or isocyanate group which is reactive towards NH_2 and secondly contains a group which is reactive towards biomaterial, and to which the biomaterial is then coupled.

Further particular features of the invention follow from the claims.

The invention is useful for the immobilisation of enzymes, microorganisms, cells, antibodies, antigens, organelles, tissue sections, etc., on substrates such as semiconductor substrates, foils, wall areas or on granules or structural members of mineral type in particular, and can be used in fields of application such as those cited at the outset.

Amongst other methods, silicon nitride surfaces can be deposited by the CVD technique from an SiH_4/NH_3 mixture (see A. Garde et al. 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 can be utilised as reactive functions for the coupling of biomaterial to the nitride surface via crosslinking agents.

In a particularly advantageous embodiment of the method according to the invention, an Si_3N_4 surface is used which has been freed from oxides by treatment with dilute

hydrofluoric acid, and covalent enzyme coupling is ensured in two steps, by selecting a crosslinking agent, one function of which reacts firstly with the NH_2 groups, or the NH groups also, of the nitride surface, and the second function of which is subsequently reacted with the protein. Alternatively, the crosslinking agent may also firstly be reacted with the protein and the product is then reacted with the Si_3N_4 surface.

Suitable groups of the crosslinking agent which are reactive towards NH_2 comprise aldehyde, halide, epoxide, imide or isocyanate functions in particular. Moreover, a multiplicity of possible reactions with amino groups is to be found in US-PS 5 234 820, for example. In practice, various compounds are already being offered for sale by the Pierce company in the "Immuno technology Catalog & Handbook: of 1992/3.

Crosslinking agent functions which are capable of covalent bonding via functional groups of the enzyme, and which in particular contain terminal carboxy groups or side chain groups such as -SH, -COOH or -OH groups or aromatic rings, are utilised for the reaction with the biomaterial. According to the invention, the aim is therefore to effect bonding of the biomaterial to the nitride surface by utilising a heterobifunctional crosslinking agent. The term "heterobifunctional crosslinking agent"is to be understood here to mean a crosslinking agent which comprises two functions which are basically of the same type chemically but which exhibit different reactivities towards the different co-reactants.

The heterobifunctional crosslinking agents are reacted in steps; with the Si_3N_4 surface and then with the protein. 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.

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.

Bonding of the protein is then effected by means of the second functional group, which is still free, of the crosslinking agent. This group can react specifically or non-specifically with thiol, carboxyl or carbohydrate groups of the protein.

If the crosslinking agent comprises, as its second functional end, a thiol-specific group such as maleimide, an activated halide or pyridyl disulphide, the protein to be bonded must have a free sulfhydryl group (usually from a cysteine residue). If the latter is not present, it can be generated by the reduction of protein disulphides. Alternatively, primary amines of the protein can be modified so that sulfhydryl groups are available (Traut's reagent). In order to prevent the oxidation of these groups, the buffer which is used must be de-gassed. Addition of the complexing agent EDTA prevents oxidation by any metals which are present in the solution.

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

Glycolysed proteins can be crosslinked via the hydroxy group on the side chains of the sugar. If a carbohydrate-active crosslinking agent is used which comprises a hydrazide,

for example, as a functional group, the carbohydrate group of the protein first has to be oxidised to an aldehyde (e.g. with $NaIO_4$). The resulting carbonyl group then reacts with the hydrazide to form semicarbazone.

One option for a direct linkage between carboxy and amino groups results from the reaction with carbodiimides. In an acidic pH range (4-5), carbodiimides convert the carboxy group into an activated ester bond. This reacts with primary amines with the formation of an amide bond and the separation of urea.

If a crosslinking agent is used, the non-amino-specific functional end of which comprises a photo-activatable group (e.g. azidophenyl), the entire immobilisation operation has to be conducted in a dark room under red light. The azido group of azidophenyl is activated by irradiation with incident light of wavelength 265-275 nm.

The invention can be used for bonding very different biomaterials to substrates or supports of a very general type. It has been tested in particular for penicillin sensors 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 $<10^{-5}$ 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 power of ten.

The exact position of the linear measuring range and the absolute sensitivity essentially depend on the choice of buffer composition and on the concentration thereof, i.e. on the buffering capacity and pH. The measuring range which is required for a measurement can be adjusted in a targeted manner by suitably selecting these parameters. For example, when IMIDAZOL buffer (pH 7) is used, the linear measuring range is between 2 and 20 mM penicillin, and for HEPES buffer it is approximately between 1 and 10 mM. Increasing the pH shifts the position of the linear ranges of the calibration curve towards higher penicillin concentrations; lowering the pH correspondingly shifts it to lower concentrations.

The sensors produced according to the invention exhibit a prolonged long-term stability of more than 140 days. The sensitivity is 50 mV per penicillin decade.

It is possible to produce a field effect transistor which comprises, in its gate region, a similar structure to that of the capacitive layer structure described in the invention.

Claims

1. A method of immobilising biomaterial on a substrate comprising an Si_3N_4 surface to which the biomaterial is covalently bonded via bonding agents, characterised in that an Si_3N_4 surface is provided on the immobilisation substrate comprising NH_x groups which are active as regards bonding and which are reacted with a heterobifunctional crosslinking agent, which firstly contains an aldehyde, ester, halide, epoxide, imine or isocyanate group which is reactive towards NH_2 and secondly contains a group which is reactive towards biomaterial, and to which the biomaterial is then coupled.

2. A method according to claim 1, characterised in that the group or function of the crosslinking agent which is reactive towards biomaterial is a group which reacts with terminal or side chain functions of proteins.

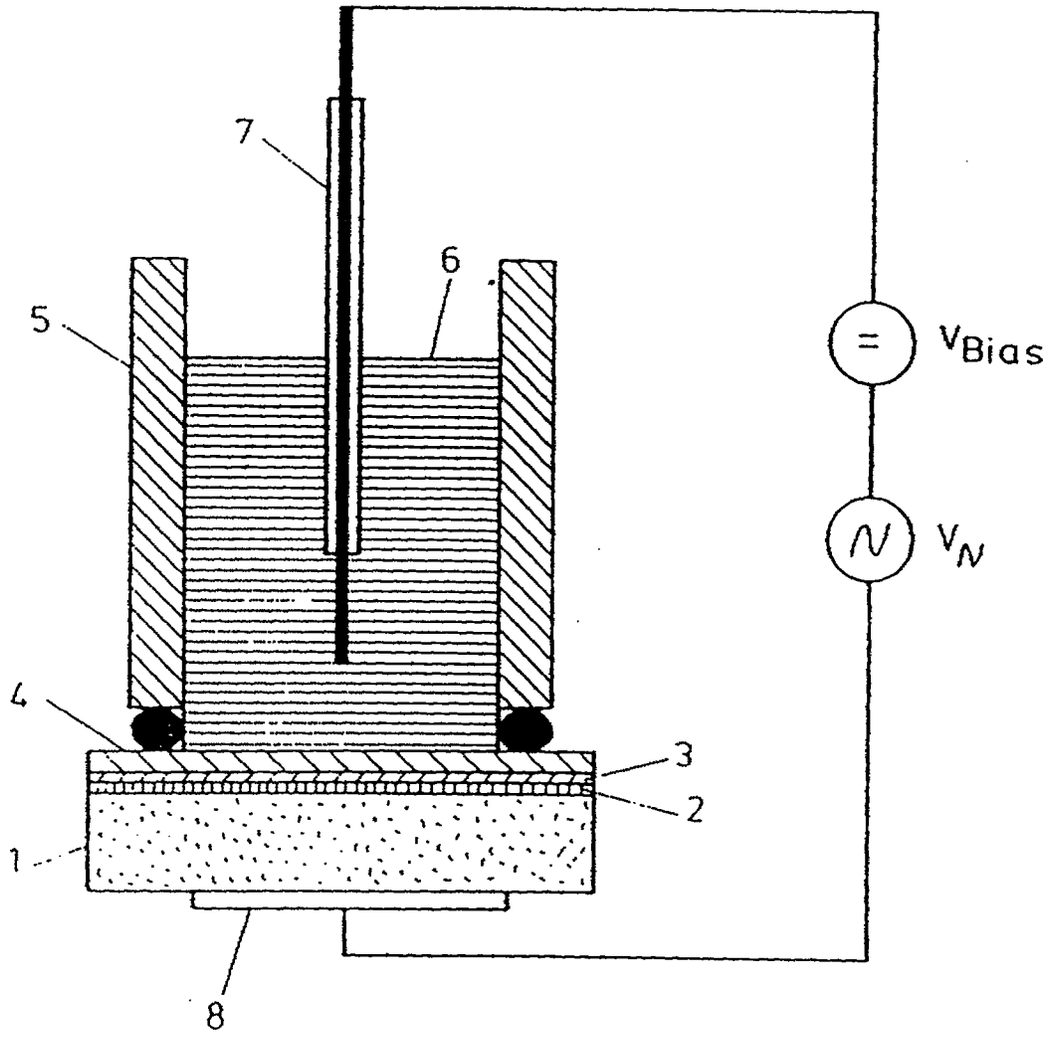
3. A method according to claim 1 or 2, characterised in that a crosslinking agent is selected which contains a biomaterial-active group which reacts with carboxyl, sulfhydryl or hydroxy groups or which couples to aromatic rings.

4. A method according to any one of the preceding claims, characterised in that an Si_3N_4 layer 10 - 1000 nm thick is deposited on the immobilisation substrate by CVD from an SiH_4/NH_3 mixture and is provided with bonding-active NH_x groups by a hydrolysing surface cleaning operation.

5. A method according to any one of the preceding claims, characterised in that enzymes, microorganisms, cells, antibodies, antigens, organelles or tissue sections are fixed as the biomaterial on semiconductor substrates, foils, wall areas or on granules of mineral type in particular.

6. A method as a modification of claim 1, characterised in that for the bonding by means of the crosslinking agent the latter is first reacted with the biomaterial and its linking to the Si_3N_4 surface is thereafter ensured.

Fig. 1



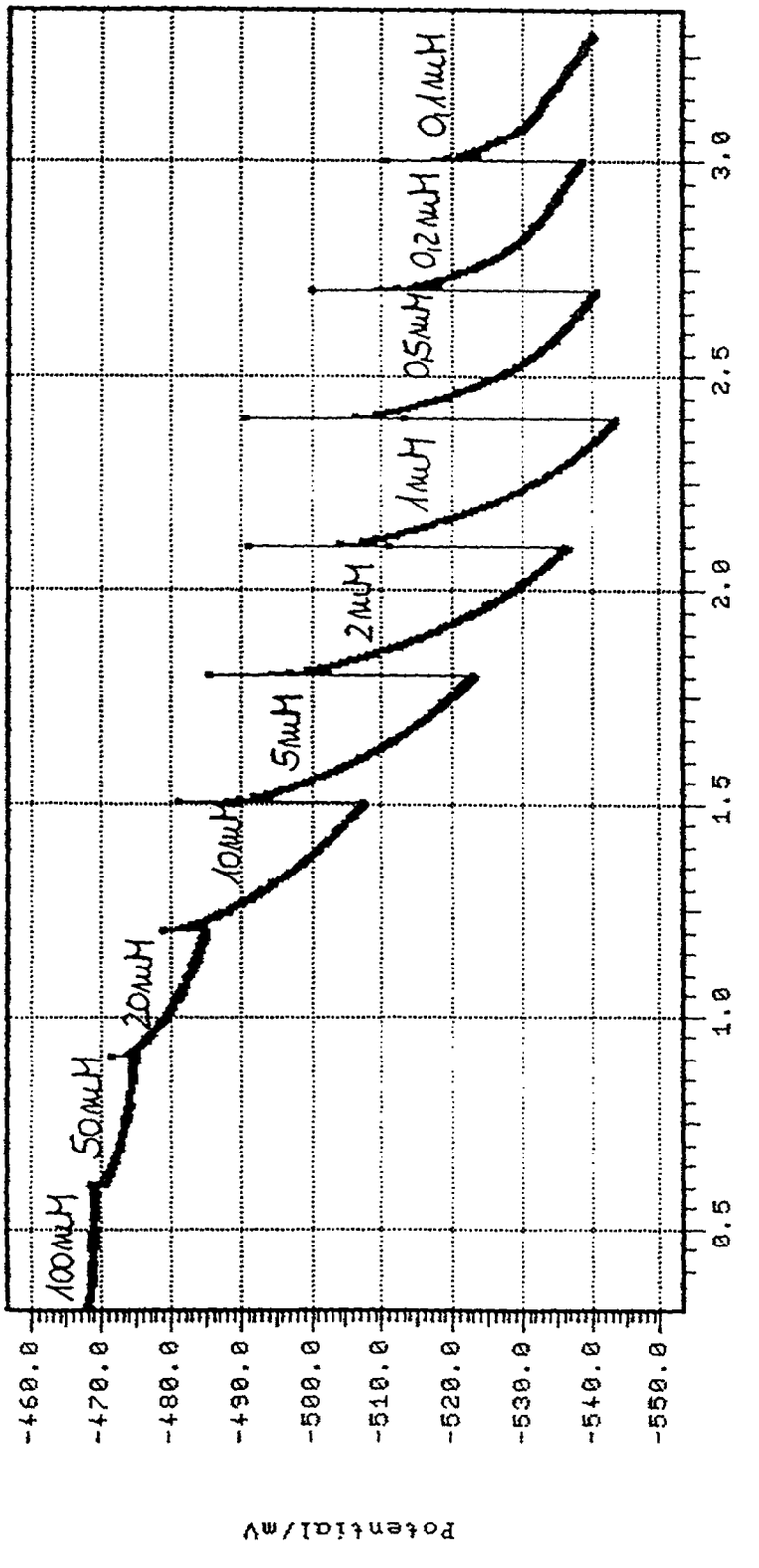

100mN
50mN
20mN
10mN
5mN
2mN
1mN
0.5mN
0.2mN
0.1mN
-460.0
-470.0
-480.0
-490.0
-500.0
-510.0
-520.0
-530.0
-540.0
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0.5
1.0
1.5
2.0
2.5
3.0
Time/ksec
Potential/mV

Fig.2

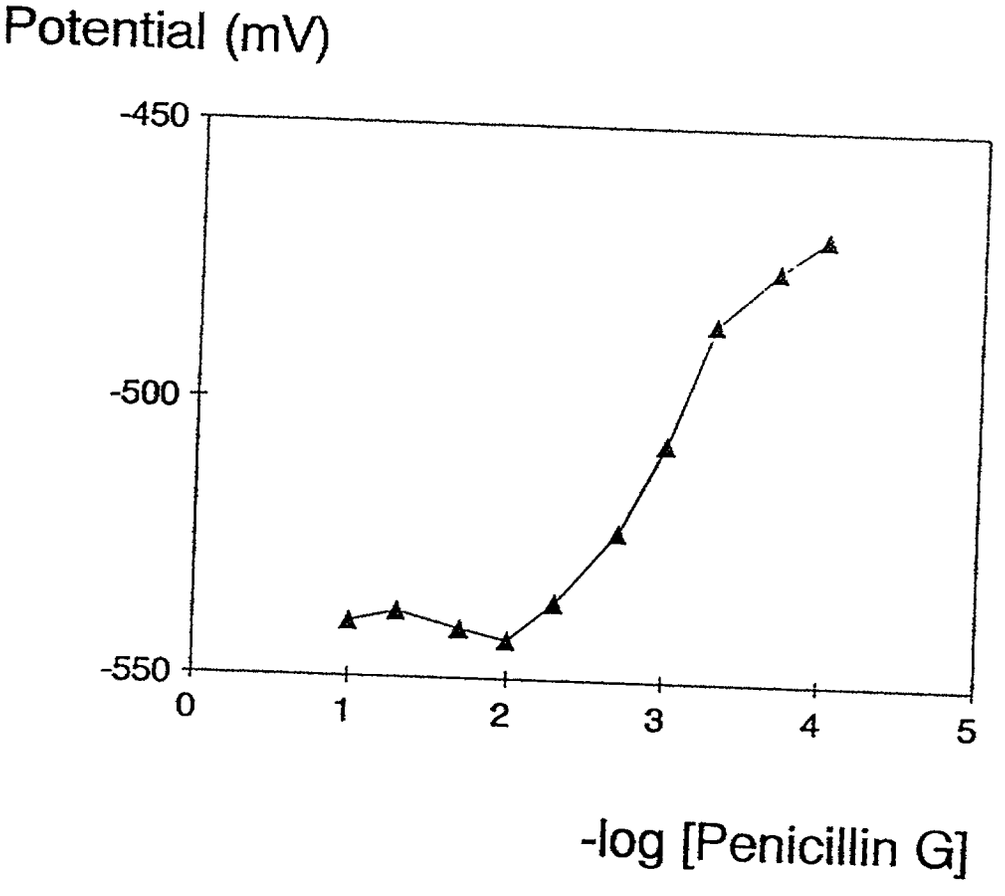


Fig. 3