Title: SILANIZATION OF SURFACES

Abstract

Method at the detection of organic molecules and biomolecules by means of surface-sensitive detection methods, the molecules becoming covalently bonded to the solid, plane test surface that has been given by means of silanization groups being functional for the covalent bonding. The invention relates to a silanization technique, which combines a special cleaning technique for the surfaces to be silanized with a silanization process at a low pressure and with the silane in gas phase and at an enhanced temperature of the surfaces to be silanized. The method gives reproducible surfaces provided with stable, homogeneous and functional silane layers of monolayer character. Said surfaces are then used for covalent coupling of highly specific organic molecules and biomolecules to the surfaces. The coupling may be carried out directly by means of a reactive group on the silane or by means of a bifunctional coupling reagent such as for instance N-succinimidyl-3-(2-pyridylidithio)propionate, carbodiimide or glutardialdehyde.
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SILANIZATION OF SURFACES

The present invention relates to a method at the detection of organic molecules or biomolecules, e.g. proteins.

Biomolecules may be detected by utilizing the biospecificity between two or more different molecules. The reaction product thus obtained may then be determined and measured in a number of different ways. The most used detection methods today are based on some kind of labelling, for instance with an enzyme or radioactive isotopes, of one or more of the measuring system components. Such a method is time-consuming, requires a costly and relatively complicated equipment, is not suitable for automatization and, when radioactive isotopes are used, it is not to be preferred from working environment reasons.

In surface-sensitive detection methods the labelling of the components is eliminated, and the reaction product obtained is measured directly. Examples of detection methods of this kind are ellipsometry and simplified variants thereof (Ellipsometry and Polarized Light, Azzam, R.M.A. and Bashara, N.M., North Holland Publ., Amsterdam, 1977), chemically sensitive field-effect transistor, so-called CHEM-FET (Theory, Design and Biomedical Applications of Solid State Chemical Sensors, Cheung, P.W, Flemming, D.G., Neuman, M.R. and Ko, W.H. (Eds.), CRC Press Inc., 1977) and other electrochemical detection systems such as measurement of differential capacitance and potential changes.

The advantages of the surface-sensitive detection methods are their simplicity, rapidity and low cost. Characteristics in common in these surface-sensitive detection methods, that distinguish them from conventional detection methods, are the fact that they measure the average change at the surface of some physical-chemical parameter, e.g. the quantity per surface unit, the change of the charge per surface unit, and that the change can be measured
continuously. These characteristics are essential conditions for miniaturization, rapidity and accuracy as kinetic parameters can be measured. Moreover, the test surface can be made very small, which considerably cuts down the consumption of the bio-
molecules present in the measurement system.

The test surface is a solid, plane surface of a magnitude of some \( \text{mm}^2 \) down to \( 10^{-3} \text{ mm}^2 \) with reproducible physical-chemical surface qualities, e.g. surface energy, optical refractive index, electric potential. The qualities of the bulk material are dependent on the measurement principle (e.g. ellipsometry-light reflecting, CHEM-FET semiconductors, electrochemical measurement principles-electrically conducting or semiconductors).

In the measuring of a biospecific reaction, e.g. an immunochemical reaction, one component is in the simpliest case adsorbed to the surface. However, the adsorption of biomolecules causes problems as to the sensitivity and specificity of said methods. Adsorbed molecule films are never completely stable in a liquid phase but are to a certain extent in a dynamic equilibrium with other molecules present in the liquid. Besides, an adsorption process is very difficult to control, and reproducible molecule films, as far as biological activity and physical-chemical qualities are concerned, are difficult to obtain. Biomolecules, especially proteins, are generally adsorbed irreversibly to surfaces, which means expendable systems and makes automation more difficult.

The present invention relates to a method for the treatment of the test surface in order that the biospecific molecules will become covalently bonded to the surface in a reproducible and well-controlled manner, which means that the surface-sensitive detection methods can be utilized. The characteristics of the invention are evident from the patent claims.

Thus, the test surface is a reproducible, solid, plane surface which is chemically modified by means of the silanization process of the invention in that it is given reactive groups suitable for
the covalent bonding. Examples of such surfaces are oxides or oxide-coated metals, semiconductors and insulators, e.g. Si/SiO$_x$, Si/Si$_{3.4}$/Si$_{3.4}$, Ti/TiO$_x$, Pt/PtO$_x$, Pd/PdO$_x$, Al/AlO$_x$, Ni/NiO$_x$. 

5 needs to be well-defined.

It has previously been possible to produce oxide-coated surfaces having reproducible physical-chemical qualities that have hydrated hydroxyl groups on the surface after a suitable cleaning procedure, but said groups per se are not suitable for the direct covalent bonding of other molecules. It is known that the surface can be chemically modified by the use of a reaction with an organosilane or an alcohol (P. F. Cox, US Patent 3,831,432 (1974). Methods have been given to modify the hydroxyl groups into chloride groups (W. Hertl, US Patent 3,924,032 (1975), and possibilities are mentioned for the reaction with thionyl chloride or the direct condensation of carboxyl or hydroxyl groups in the biomolecules with the surface (T. Chiyou, Japanese Appl. No. 53-83604 (1978). Stability in water phase for surfaces that have been modified in said manners is only expected for the reactions with the organosilanes. This is the case because the bond Si-C has higher hydrolytical stability that the bond O-C which is the result of the other modification techniques.

25 Organosilanes are of three main kinds: chlorosilanes, alkoxysilanes and silasanes. They can be obtained as mono-, di- or trifunctional. Reactions between said silanes and oxide-coated surfaces have been studied in detail, and a number of reaction conditions are known. The majority of said studies are concerned with porous or solid pellets or spheres of small size. Here it is known that the pretreatment of the spheres or the pellets and the temperature and the time for the reaction are critical parameters (see for instance W. Hertl, J. Phys. Chem., 72 (1968), 1248 and F. Buzek and J. Rathousky, J. Colloid Interface Sci., 79 (1981), 47. On the other hand, the silanization of solid, plane surfaces has not attracted attention to the same extent.
On plane surfaces the reproducibility, homogeneity and stability of the reaction can be studied in simple ways. The most usual method to silanize surfaces is to let the reaction between the surfaces and organosilanes occur in liquid phase (Mosbach K. (ed.), Methods in Enzymology, Vol. 19, pages 139-140, Academic Press, New York, 1976). It is known that the presence of water in the systems will cause polymerization of the silane with heterogeneous and un-reproducible silane layers as the result (see for instance W.D. Bascom, Macromolecules, 5 (1972), 792). Strictly waterfree reaction conditions are complicated to achieve and technically difficult to control. Besides, the presence of previously more or less polymerized silanes cannot be completely eliminated at a reaction in liquid phase. It is also known that the reaction between chlorine and alkoxy-silanes and the oxide surfaces will occur at an appreciable speed only at higher temperatures (150-600°C) (F. Buzek and J. Rathousky, J. Colloid Interface Sci., 72 (1981), 47).

Said conditions are difficult to obtain at a reaction in liquid phase. Said problem can be eliminated by vacuum distillation of the silane and bringing the surfaces in contact with the silane in the gas phase (e.g. K.L. Mittal and D.F. O’Kane, J. Adhesion, 8 (1976), 93). However, from a reaction at room temperature no water-stable layers are obtained (I. Haller, J. Am. Chem. Soc., 100 (1978), 8050). A method to silanize fibre glass in the gas phase at an enhanced temperature has been described (J. Eakins, U.S. Patent 3,276,853 (1966)). However, said method is specialized for fibre glass and in the described embodiment it is not suitable for the silanization of the surfaces here described. Besides, the process is carried out at atmospheric pressure and in contact with the ambient air.

However, if the surface modification is carried out according to the method of the present invention those reproducible and stable silane layers are obtained that are required for an instrumental detection of organic molecules, such as biomolecules, using surface-sensitive detection methods. The present invention combines special cleaning procedures of the solid, plane surfaces with a low pressure distillation of the silane over to a vessel comprising
the oxide-coated surfaces that are kept at an enhanced temperature
suitable for the reaction between the organosilane and the surface,
for instance 75-250°C, preferably 100-200°C.

5 The cleaning procedures will free the surfaces from organic and
inorganic contaminations and will give the surfaces a hydrophilic
character. The procedure described in example 1a can be used
preferably for test surfaces that are part of a bulk material, e.g.
the surface of single crystal silicon or foils of titanium.

10 Test surfaces consisting of thin films coated on a bulk material
of for instance glass or plastic require a milder cleaning program
(example 1c), and those programs must be adapted to the special
surface.

15 The distillation of the silane will make sure that no polymerized
silanes will leave the silane solution and come into contact with
the surfaces. As the process is carried out at a low pressure
and at an enhanced temperature, the amount of free water in the
system is minimized, which reduces the risk for polymerization of
the silanes when they are in the gas phase. Moreover, a mild treat-
ment is made possible of thermally unstable silanes because of the
boiling point depression. As the surfaces are kept at a temperature
required for the reaction, there is ensured that the silanes react
covalently with the surfaces and form a water-stable modified surface.

20 **The temperature of the surfaces are, however, not enhanced so much
that the water that is bonded to the surfaces completely disappears.
The bonded water together with the water that is left in the system
make hydrolysis possible of the silane before the reaction with the
surface hydroxyl groups can occur.** The temperature is so chosen
that an optimal result is obtained as regards to reproducibility,
stability and functionality of the modified surfaces. The time
during which the silanization occurs, is also a variable parameter.

The surfaces that are silanized with said technique are reproducible
35 and give polymer-free silane layers of monolayer- or submonolayer
character (verified with contact angle measurements, ellipsometry,
scanning transmission electron microscopy and electron spectroscopy.
for chemical analysis). The surfaces are functional and stable in an environment comprising water of different pH, which can be verified by coupling chemically reactive gel beads to the surfaces (example 4). Because a suitable method of examining the presence of reactive, functional groups on a silanized surface is to modify gel beads with organic molecules and bring them together with the surface. If functional groups are present on the surface, the gel beads are bonded covalently and specifically to the surface.

Then there can be bonded to the reactive groups either a spacer, i.e. a molecule that brings the biomolecule out a certain distance from the surface and thus prevents the molecule from being affected from the surface in a manner detrimental to the detection system, or a combination of spacer and inert molecules, i.e. molecules that do not take part in the biospecific reaction but for instance prevent an unspecific adsorption to the surface giving an environment that is physically-chemically favourable to the bonded molecule, e.g. by being hydrophilic. The biospecific molecules may also be bonded directly to the reactive groups on the surface.

The covalent bonding of organic molecules or biomolecules to reactive groups on the test surface, e.g. amino, thiol, epoxy, vinyl, pyridyldisulfide, succinimide, carboxyl, methoxy, ethoxy or methacryl, may be carried out with bifunctional coupling reagents, e.g. with glutardialdehyde, carbodiimide or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, Pharmacia Fine Chemicals, Uppsala, Sweden). This may be done by a direct bond between the reactive group and the desired molecule or via a link in between, a so-called spacer. This link or the direct bond may be cleavable at conditions that are not disturbing the bonding of the reactive group to the test surface, e.g. by reduction of the disulfide structure or thioldisulfide interchanges, oxidation of vicinal diol structures with periodate or enzymatically cleavable groups such as esters or glycoside bonds.

By the coupling with SPDP intramolecular couplings are eliminated,
which results in monolayers of the molecules bonded to the surfaces.

In order to eliminate modifications of organic molecules or biomolecules in solution, the bond to the test surface is achieved by inserting directly reactive groups on the test surface, e.g. N-hydroxysuccinimide ester or epoxides. This is done by modifying said reactive groups as is evident from example 3 or by direct synthesis of a silane so that it is given directly reactive groups.

The invention may be exemplified as follows:

a. Spontaneously oxidized silicon is cleaned according to Fig. 1a. The surfaces that are blown dry in nitrogen gas are then kept at 150-180°C and are silanized at a low pressure in gas phase with a silane (e.g. A-189 from Union Carbide) which modifies the surface so that thiol groups are introduced. These thiol groups on the silicon surface are modified with SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate) in an acetate buffer at pH 4.5 because of the thioldisulfide interchange reactions that occur. N-succinimidyl which is in this way introduced on the silicon surface, can react and bond amino-comprising compounds, e.g. protein at higher pH.

b. The silicon surfaces are silanized as under a. Amino-comprising organic molecules or biomolecules are modified with SPDP and can then be bonded to the thiol silanized surface via thioldisulfide interchange reactions.

c. The silicon surfaces are silanized with an amino group introducing silane (e.g. A 1120 from Union Carbide) as under a. The amino groups introduced on the silicon surface are modified with SPDP the same way as the amino-comprising molecule. One part is reduced to thiol and the covalent bonding occurs via thioldisulfide interchange reactions.

d. The silicon surfaces are silanized as under c. Carboxyl-comprising molecules are coupled to the surface by means of carbodiimide
reagents, e.g. 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-p-toluenesulfonate or N,N'-dicyclohexylcarbodiimide.

e. The silicon surfaces are silanized as under c. Amino-compromising molecules are coupled to the surface by means of glutaraldehyde.

f. The silicon surfaces are silanized with an epoxyintroducing silane (e.g. A 187 from Union Carbide) as under a. The epoxymodified silicon surface can then covalently bond amino-compromising, thiol-compromising or hydroxyl-compromising molecules.

g. The silicon surfaces are silanized with mixtures of silanes so that multifunktional surfaces are formed on the silicon. These different functional groups can be used to bond different molecules described under a.-f.

h. Silicon is silanized with a N-succinimidyl-introducing silane as under a. The N-succinimidyl-modified silicon surface can then covalently bond amino-compromising organic molecules or biomolecules.

i. Silicon is silanized with a pyridyldisulfide-introducing silane as under a. The pyridyldisulfide-modified silicon surface is then reduced in water phase with e.g. dithiothreitol, in which way free SH-groups are introduced on the surface. These can be covalently bonded to SPDP-modified organic molecules or biomolecules.

J. Silicon coated with silicon nitride is cleaned according to a. and silanized according to a.-i.

k. Titanium is evaporated or sputtered onto glass surfaces to a thickness of 500-10 000 Å, is cleaned according to Fig. 1b and is silanized according to a.-i.

l. Machined titanium is cleaned and silanized accroding to a.-i.

The characteristic of the surface-sensitive detection methods to
register the average changes on the test surface has as a result that at content determinations the magnitude of the signal is a measure of the surface concentration of the substance that is bonded to the surface, while conventional solid phase methods such as RIA and ELISA measure the amount of the substance that is bonded to the surface independent of its size. The consequence of this difference is that the test surface can be miniaturized and thus the sample volume and the amount of the substance coupled to the test surface.

Another effect of said difference between measuring for instance a radioactive disintegration and determining the qualities of surfaces is that one should of necessity try to utilize every part of the surface for the coupling of specifically active molecules. Every molecule coupled to the surface should have the desired specificity and be orientated in such a way that it is not hindered from sterical reasons to take part in its specific interaction. This means that every substance that is coupled to the surface should have as high purity as possible.

For biospecific systems based on immuno reactions it is essential to change over from serum antibodies, being a mixture of inactive and active antibodies, to purified antibodies or hybridom antibodies. Serum antibodies can be purified by affinity chromatography, and the antibodies obtained in such a way are all directed towards the same antigen but will react with different parts of the antigen and have different equilibrium constants.

Monclonal antibodies or hybridom antibodies have the characteristic of being antibodies with only one specificity and one equilibrium constant. If those antibodies are coupled to the test surface, you will get a more uniform surface with affinity for the antigen where all antibodies on the surface can bind its antigen. This is an essential contribution in order to increase the resolution and the sensitivity of the measurement methods, which is not apparent from earlier patents and publications referring to surface-sensitive detectors.
Monoclonal antibodies can be obtained towards a very large number of different antigens in low- and high-molecular organic compounds. This means that using assay methods based on surface-sensitive detection technique with monoclonal antibodies bonded to the surface, you can measure low-molecular substances, such as hormones and substances that are toxic and dangerous to the environment, and high-molecular substances, such as proteins, polysaccharides, nucleic acids, viruses or bacteria. Optical and electrical methods give different sensitivities dependent on the size and charge of the antigen and its ability to induce potential changes on the test surface.

Enzymes are proteins that catalyze a chemical reaction transferring a substrate into a product. Many substances that are toxic and dangerous to the environment exercise their action by inhibiting enzymes and specifically bind to the active surface. This gives a possibility to selectively measure enzyme inhibitions by covalently binding the enzyme to the test surface and then measure the change that will occur when the inhibitor is bound to the enzyme.

Content determinations of enzymes can be carried out either by means of enzyme activity measurements or by means of conventional immunological methods, which however will not give any information about the enzyme activity. By coupling a specific enzyme inhibitor covalently to a plane test surface, the enzyme content can be measured by means of surface-sensitive detection methods because of the bonding of the enzyme to the inhibitor on the solid phase.

Protein A from Staphylococcus aureus is a protein that can bind immunoglobulins from several different species in its Fc-part. Every Protein A molecule has several bonding spots like that, and the protein is often used to detect immunoglobulins in content measurement methods and microscopic section-cuttings. By covalently couple Protein A to plane test surfaces, the IgG-contents of samples can be measured as is evident from example 2b.

The example shows that it is essential that every molecule coupled
to the surface take an active part in the biospecific reaction
in order to obtain as high surface concentration as possible.
Protein A has also the ability of orientating the antibodies so
that they will expose their antigenbonding structures out into
the solution by binding to the Fc-part of the immunoglobulin.
Said ability can be utilized in order to increase the efficiency
in the antigen-antibody reaction.

The bond between Protein A and the Fc-part of the immunoglobulin
is cleavable at a low pH (example 2b). This can be used to regenerate
the test surface. The bond between the immunoglobulin and an antigen
is in general also cleavable at a low pH. In those cases where
antibodies have been orientated by means of Protein A on the test
surfaces, the cleavage between said molecules can be prevented by
means of mild cross-linking of Protein A and the antibodies (e.g.
with glutaraldehyde). When rinsing in a low pH (2-3) only the
bond between the antibody and the antigen is cleaved, and the test
surface can be used again.

Molecules that may be coupled to the test surface after the surface
modification according to the invention are for instance antibodies,
preferably monoclonal antibodies, antigens, Protein A from Staffylo-
coccus aureus, enzymes, nucleic acids, enzyme inhibitors and enzyme
substrates, and as examples of the detected substance there can
be mentioned antigens, antibodies, nucleic acids, enzymes, enzyme
inhibitors and enzyme substrates as well as larger complexes such
as viruses and microorganisms.

The invention will now be described in more detail in the following
examples with reference to the attached drawings, where

Fig 1a shows the cleaning procedure for e.g. the surface of a bulk
material,
Fig 1b shows a milder cleaning procedure at room temperature for
e.g. a surface that consists of a film disposed on a bulk
material,
Fig 2 shows a suitable embodiment of the silanization equipment.
Fig 3 is a diagram over the adsorption and coupling of immunoglobulin G from rabbit to different silicon surfaces, and the meaning of the symbols used is:

- cleaned surfaces
- surfaces that have been exposed to the silanization procedure but without the addition of silane
- aminosilanized surfaces
- aminosilanized surfaces that have been modified with SPDP
- aminosilanized surfaces that have modified with SPDP and reduced with dithiothreitol. To said surfaces a covalent bonding of the molecules are expected to occur.

The symbols give average values of two separate experiments.

Fig 4 is a diagram visualizing the interaction of immunoglobulin G from rabbit with Protein A (pA), covalently bonded to silicon surfaces, and the meaning of the symbols used is:

- interaction of immunoglobulin G with Protein A
- the same surfaces after being cleaned at a low pH

Example 1a.

Introduction of amino groups on hydrophilic single crystal silicon.

Single crystal n-doped silicon (Wacker Chemitronic, Western Germany) is cut into 5 x 5 mm pieces and cleaned according to Fig. 1a. The silicon is hydrophilic after this treatment with a critical surface tension of about 39 dynes/cm, with a polar contribution of about 50 dynes/cm and a dispersion contribution of about 19 dynes/cm, measured by means of contact angle measuring.

The silanization is carried out in the apparatus according to Fig. 2.

All parts are here made of glass with silicone grease in the joints. The silicon pieces, blown dry in nitrogen gas, are put into the right vessel, which is kept at a temperature of about 160°C. In the left vessel there are poured some mLs of N-(3-aminopropyl-aminopropyl-liquidysilane (Union Carbide, A-1120), once distilled before the use. The system is evacuated to about 0.1 torr and is kept in this condition for 3-4 h, the water of the system partly disappearing. Then the silane is vacuum distilled over into the right vessel by
Raising the temperature of the left vessel to about 85°C. The silicon pieces are kept the whole time at about 160°C, and the silanization is now carried out during 2-4 days. Then the vacuum distillation of the silane is stopped, and the system is kept for about another 12 h at an enhanced temperature and low pressure, whereafter the heating is stopped and the system is allowed to cool down while keeping the evacuation. Finally the system is filled with dried air and the pieces are taken out.

The aminosilanized pieces are reproducible after this process and have a silane coating of submonolayer character (verified by means of electron spectroscopy for chemical analysis and ellipsometry). The surfaces are homogeneous and comprise no silane polymers (scanning transmission electron microscopy). Furthermore, the surfaces have functional amino groups (contact angle measurement and gel bead test (see example 4), that are stable in water at pH 1 to pH 11 (gel bead test (see example 4)).

Example 1b.

Introduction of sulfhydryl groups on hydrophilic, single crystal silicon.

Silicon is cleaned according to example 1a and silanized with γ-mercaptopropyl-trimethoxysilane (Union Carbide, A-189). The silanization is carried out according to example 1a but with the difference that the silane temperature during the vacuum distillation is 25-35°C.

The sulfhydrylsilanized pieces are characterized according to example 1a with a similar result.

Example 1c.

Introduction of amino groups on Ti/TiO_x-films.

Electrodes can be made of thin metal or semiconductor films. These films are coated on some suitable substrate, e.g. glass or plastic. In the present example titanium was evaporated in a 3000 Å thick
film on glass surfaces. For these thin films a milder cleaning program had to be used than according to example 1a. The titanium-coated glass surfaces were cleaned according to Fig. 1b. The pieces have a hydrophilic surface structure after this treatment. The surfaces were then silanized according to example 1a.

Example 2a.
Covalent bonding of immunoglobulins to amino-modified silicon.

Silicon silanized according to example 1a was modified with SPDP solved in 99.5 % ethanol to a final concentration of 2mM during 0.5 h in 0.1 M NaCl, 0.1 M phosphate and 1 mM ethylenediamine tetraacetate, pH 7.5 (coupling buffer). After cleaning in coupling buffer the modified silicon surface was reduced with dithiothreitol, 50 mM, for 0.5 h. After renewed cleaning in coupling buffer the surfaces were reacted with rabbit immunoglobulin G (IgG) in the concentrations of 1, 3, 10, 30, 100 and 300 μg/mL, that had been modified with SPDP according to Carlsson, J. et al, Biochem. J., 173 (1978), 723. The modified IgG comprised an average of 3.5 SPDP/molecule after the modification. As reference surfaces were chosen cleaned silicon, silicon that had been exposed to the silanization procedure but without an addition of silane, amino-modified silicon and amino-modified silicon that had been modified with SPDP but not reduced as above. For all the last-mentioned surfaces no covalent coupling is expected to occur. Instead an unstable adsorption of IgG to the surfaces are here obtained. No adsorption of IgG to the cleaned surfaces does occur because of the hydrophilic character of the surfaces.

The result of the bonding was evaluated by means of ellipsometry without the pieces being air dried in any moment. Here the results are given as the change of the ellipsometric parameter Δ. This parameter is for silicon approximatively proportional to the amount of material per surface unit. Fig. 3 shows the change of Δ as a function of the IgG concentration. It was found that those surfaces, where the covalent coupling is expected to function, got the highest amount bonded IgG. The stability of the surfaces was then tested
at pH 2.5 for 30 min. Only those surfaces, where covalent coupling was expected to occur, showed unchanged values, while all the other surfaces lost material.

Example 2b.
Covalent bonding of Protein A and its biospecific interaction with IgG.

Pieces of aminosilanized surfaces were modified with carboxypyridyldisulfide (synthesized from dithiopyridine (Fluka) according to Carlsson, J. et al, Biochem, J., 173 (1978), 723) 10 mM in dichloromethane comprising 10 mM dicyclohexylcarbodiimide (Aldrich) during 2 h. A base catalyst, e.g. 2,6-Luhydrin, can be added which speeds up and improves the reaction. The pieces were then rinsed in dichloromethane, acetone, ethanol and water and stored in 50% ethanol.

The pieces were given pyridyldisulfide groups by this modification. They were reduced with dithiothreitol according to example 2a, and then coupling was carried out for 12 h with SPDP-modified Protein A (Pharmacia Fine Chemicals), in average 3 SPDP/molecule, 100 μg/ml. After rinsing and measuring in the ellipsometer the pieces were reacted with rabbit IgG in the concentrations of 1, 3, 10, 30, 100 and 300 ng/ml and 1, 3, 10 and 30 μg/ml. The result is shown in Fig. 4. The surfaces could be regenerated by cleaning them in a glycine buffer, pH 2.5, and the clean Protein A surface was obtained again. It could then become recoupled with IgG.

Example 3.
Silicon was silanized according to example 1b. The thiolated silicon surface was modified with SPDP 2 mM in 0.1 M NaCl, 0.1 M acetate, 1 mM EDTA pH 4.5 by means of thiol-disulfide interchanges for 1 h.

After cleaning in the coupling buffer there was added about 50 μg/ml rabbit IgG in 0.1 M NaCl, 0.1 M phosphate, 1 mM EDTA pH 7.5. After cleaning with phosphate-buffered sodium chloride (PBS) there was added pig anti rabbit Ig purified with regard to IgG 5 μg/ML.

The coupling of protein to the silicon surface by the reaction between introduced N-hydroxysuccinimideester and amino groups in IgG was evaluated by means of ellipsometry. Here there has been
estimated a refractive index of 1.42 for the films. The film thickness can then be calculated from ellipsometry data.

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<td>Rabbit IgG</td>
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Example 4a.

Test of functional groups.

Thiolcomprising silicon surfaces can be tested with regard to functional thiol groups by means of covalent bonding to the surfaces of 100 μl Thiopropyl Sepharose gel beads (Pharmacia Fine Chemicals, Sweden) in suspension in 0.1 M NaCl, 0.1 M phosphate, 1 mM EDTA, pH 7.5 during 1 h. By means of thioleisulfide interchange the gel beads are bonded and cannot be rinsed away. The results can be directly and visually evaluated without aids.

Table 1. The specificity of thiopropyl-modified gel beads for SH-modified surfaces in comparison with other gel bead types.

<table>
<thead>
<tr>
<th></th>
<th>Unmodified gel beads</th>
<th>Negatively charged gel beads</th>
<th>Positively charged gel beads</th>
<th>Thiopropyl-modified gel beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfaces only cleaned</td>
<td>0</td>
<td>0</td>
<td>Bonding</td>
<td>0</td>
</tr>
<tr>
<td>Surfaces exposed to the silanization procedure without silane</td>
<td>0</td>
<td>0</td>
<td>Bonding</td>
<td>0</td>
</tr>
<tr>
<td>Aminosilanized surfaces</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aminosilanized surfaces treated with SPDP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aminosilanized surfaces treated with SPDP and reduced</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Bonding</td>
</tr>
</tbody>
</table>
Table 1 shows the specificity of Thiopropyl Sepharose for reduced, aminosilanized and SPDP-modified silicon surfaces. Unmodified gel beads (Sepharose 6B) and positively and negatively charged gel beads (DEAE and CM Sepharose, all from Pharmacia Fine Chemicals, Sweden) were chosen as controls. In the table "0" means no bonding, while "Bonding" gives a lasting bonding of gel beads. From the table is evident that unmodified and negatively charged gel beads do not bond to any surface. Positively charged beads, however, bond to cleaned silicon and silicon that has been exposed to the silanization procedure without silane. This is not surprising as the oxide has on its surface negatively charged groups (Th. F. Tadros and I. Lyklema, J. Electroanal. Chem., 17 (1968), 267) at the pH that is used in the coupling process. When the ion concentration in the buffer was raised (3 M NaCl instead of 0.1 M), the bonding did not occur to said surfaces. The increase of the ion concentration did not affect the bonding of Thiopropyl Sepharose to aminosilanized surfaces that had been SPDP-modified and reduced.

Example 4b.

Stability in water solutions at different pH.

Table 2 shows the coupling of Thiopropyl Sepharose to aminosilanized surfaces after storage in a refrigerator for 2 days in different pH. In the table "Bonding" and "0" have the same meaning as in table 1. After the storage the pieces were thiol-modified (according to example 2a) and gel bead coupled. The controls were stored in a similar way but not thiol-modified, which shows that the aminosilane structure has remained intact.

For those pieces that were stored at pH 12, the coupling was only partial. This is probably due to the known instability of silicon dioxide at a pH above 11.

Table 2. Stability test of A-1120 silanized silicon after storage for 2 days in water solutions of different pH.
<table>
<thead>
<tr>
<th>pH</th>
<th>SH-modified surfaces</th>
<th>Cleaned surfaces</th>
<th>Silanized surfaces without SH-modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 3</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 5</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 6</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 7</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>pH 9 Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 10</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 11</td>
<td>Bonding</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
| pH 12| Bonding              | 0                | only partially                           

A similar test was also carried out with A-189 silanized surfaces (example 1b). Here coupling of gel beads did only occur for pieces stored at pH 7.
Patent claims:

1. Method at the silanization of a solid, plane surface for chemical modification thereof in order to make it appropriate for use as a test surface in instruments for surface-sensitive detection methods, the surface being made for instance of an oxide or an oxide-coated metal, semiconductor or insulator, whereby there is obtained on the surface a reproducible, stable silane layer with groups being functional for covalent bonding of organic molecules, biomolecules or particles, e.g. proteins, nucleic acids, microorganisms or viruses, characterized in that the surface before the silanization reaction is activated by being freed through cleaning from organic and inorganic contaminations and given a hydrophilic character, and then the surface is blown dry and silanized at a low pressure and an enhanced temperature with silane in gas phase, and then after the silanization a part of the surface may be tested for the presence of groups being functional for covalent bonding by bringing the surface together with gel beads modified with organic molecules, and to the silanized surface there may be added a bifunctional coupling reagent that covalently bonds the organic molecule, biomolecule or particle to a functional group on the silanized surface, possibly via a so-called spacer molecule or a spacer molecule and inert molecules.

2. Method according to claim 1, characterized in that the surface of a bulk material, for instance single crystal silicon, is made hydrophilic by cleaning it at an enhanced temperature in a basic solution comprising an oxidant, rinsing it repeatedly in water and cleaning it at an enhanced temperature in an acid solution comprising an oxidant, followed by repeated rinsing in water, whereafter the surface is blown dry and silanized at a low pressure and enhanced temperature with a silane distilled at a low pressure.

3. Method according to claim 1, characterized in that the surface of thin films, for instance of a metal or semiconductor, disposed on a suitable substrate, e.g. of glass or plastic, is made hydrophilic by cleaning it with organic solvents followed by a detergent in water solution and then the surface is rinsed in water.
and blown dry to be silanized.

4. Method according to any one of claims 1-3, characterized in that an organosilane is distilled at a low pressure, for instance at $10^{-6}$-1 torr, preferably $10^{-2}$-$10^{-1}$ torr, from a first vessel over into a second vessel comprising the surface that has been cleaned and blown dry, said second vessel being kept at a low pressure and a temperature suitable for the reaction between the organosilane and the surface, for instance 75-250°C, preferably 100-200°C.

5. Method according to claim 4, characterized in that the silanization process is carried out in gas phase and vacuum with N-β-(aminoethyl)-γ-aminopropytrimethoxysilane at a temperature on the surface of about 180°C, introducing amino groups on the surface, or with γ-mercaptopropytrimethoxysilane, introducing sulphydryl groups on the surface.

6. Method according to claim 1, characterized in that the surface, that has been given the functional group thiol by means of the silanization process, is brought together in a coupling buffer with thiopropyl-modified gel beads, the gel beads becoming bonded to the surface by means of a thiol-disulphide interchange reaction.

7. Method according to claim 1, characterized in that the surface is given the functional group thiol, succinimide, pyridyldisulfide, amino, carboxyl, epoxy, vinyl or methacryl by means of the silanization process, thereafter the covalent bonding of the organic molecules, biomolecules or particles is carried out by means of a thiol-disulfide interchange reaction, reaction with carbodiimide or with glutardialdehyde.

8. Method according to claim 7, characterized in that the bifunctional coupling reagent is N-succinimidyl-3-(2-pyridyldithio)propionate.
\[
\text{NH}_4\text{OH}, \text{H}_2\text{O}_2, \text{dist.} \text{H}_2\text{O}, \\
1:1:5, 85^\circ \text{C}, 5 \text{ minutes}
\]

Rinse 5 times in dist.\text{H}_2\text{O}

\[
\text{HCl}, \text{H}_2\text{O}_2, \text{dist.} \text{H}_2\text{O} \\
1:1:6, 85^\circ \text{C}, 5 \text{ minutes}
\]

Rinse 5 times in dist.\text{H}_2\text{O}

25\% ethanol

Blow dry in nitrogen gas and silanize

FIG. 1a
DICHLOROMETHANE, 2 min.

ACETONE, 2 minutes

ETHANOL, 2 minutes

RINSE IN DIST. H₂O 5 times

BASIC DETERGENT e.g. HELLMANEX (Herlma, GDR), 12 h.

RINSE IN DIST. H₂O 5 times

25% ETHANOL

BLOW DRY IN NITROGEN GAS

FIG. 1b
**INTERNATIONAL SEARCH REPORT**

**International Application No PCT/SE83/00027**

### I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC:

- G 01 N 33/50
- C 07 G 7/00
- C 03 C 17/30
- C 12 N 11/06
- C 12 Q 1/00

### II. FIELDS SEARCHED

Minimum Documentation Searched:

<table>
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<tr>
<th>Classification System</th>
<th>Classification Symbols</th>
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<tr>
<td>IPC 3</td>
<td>C 03 C 17/28,30, C 23 C 13/00,04, C 07 G 7/00,02</td>
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<tr>
<td></td>
<td>C 12 N 11/06, C 12 Q 1/00, G 01 N 33/50,52,54</td>
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<tr>
<td>US Cl 435;7</td>
<td>260:112,112;5 195:63,68</td>
</tr>
</tbody>
</table>

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched:

- SE, NO, DK, FI classes as above

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Doc.</th>
<th>Citation of Document, with Indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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<tr>
<td>P</td>
<td>CA, A, 130 228 (BORDEN CO Ltd) 24 August 1982</td>
<td>1-3</td>
</tr>
<tr>
<td>A</td>
<td>GB, A, 1 486 826 (GENERAL ELECTRIC CO) 28 September 1977</td>
<td>1</td>
</tr>
<tr>
<td>Y</td>
<td>GB, A, 1 512 052 (PHARMACIA DIAGNOSTICS AB) 24 May 1978 &amp; SE, 387 746</td>
<td>1-8</td>
</tr>
<tr>
<td>Y</td>
<td>Handbook of Thin Film Technology (ED. MAISSEL L I, GLANG R) published 1970 by McGraw-Hill Inc, see pp 6-37 to 6-42</td>
<td>1-5</td>
</tr>
</tbody>
</table>

* Special categories of cited documents:
  - "T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
  - "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
  - "A" document member of the same patent family

### IV. CERTIFICATION

- Date of the Actual Completion of the International Search: 1983-09-12
- Date of Mailing of this International Search Report: 1983-10-18
- International Searching Authority: Swedish Patent Office
- Signature of Authorized Officer: Carl-Olof Gustafsson
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<tbody>
<tr>
<td>A</td>
<td>DE, 834 002 (DOW CORNING CORP.) 14 February 1952</td>
<td>1</td>
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</table>

Form PCT ISA 210 (extra sheet) (October 1981)