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PII: S0956-5663(97)00053-5

Biosensors & Bioelectronics Vol. 12. No. 9–10, pp. 977–989, 1997

0956–5663/97/$17.00

Capacitive monitoring of protein immobilization and antigen–antibody reactions on monomolecular alkylthiol films on gold electrodes

Vladimir M. Mirsky*, Michael Riepl & Otto S. Wolfbeis

University of Regensburg, Institute of Analytical Chemistry, Chemo- and Biosensors, 93040 Regensburg, Germany

(Received 11 December 1996; revised version received 26 March 1997; accepted 17 April 1997)

Abstract: Self-assembled monolayers of ω-mercaptohexadecanoic acid and ω-mercaptohexadecylamine on gold electrodes are stable at neutral pH and display pure capacitive behavior at frequencies around 20 Hz. Different methods of covalent immobilization of proteins on these monolayers are compared. Various reagents including succinimides, thionylchloride, *p*-nitrophenol and carbodiimides were used to activate the carboxy groups of the adsorbed monolayer of ω-mercaptohexadecanoic acid. Glutaraldehyde, cyanuric chloride and phenylene diisocyanate were used to activate the amino groups of the monolayer of ω-mercaptohexadecylamine. The immobilization of albumin on the activated surface was studied by capacitive measurements. The *N*-hydroxysuccinimide and carbodiimide methods were identified as most suitable for protein immobilization in that they did not compromise the insulating properties of the alkylthiol layer and led to maximal increase of its dielectric thickness. These approaches were used for a layer-by-layer preparation of a capacitive immunosensor. Specifically, antibodies to human serum albumin were immobilized on the alkylthiol monolayer. Binding of the antigen led to a decrease of the electrode capacitance. The detection limit of the immunosensor is as low as 15 nM (1 mg/l). ©1997 Elsevier Science Limited

Keywords: protein immobilization, self-assembly, immunosensor, impedance, capacitive sensors

INTRODUCTION

Immunosensors consist of a receptor (antibody or antigen) and a transducer which is able to detect the binding event between antigen and antibody (Janata, 1989; Scheller & Schubert, 1989; Hall, 1990; Turner *et al.*, 1987). Numerous transducers are known now, most being based on either optical (Place *et al.*, 1985; Wolfbeis, 1991; Wolfbeis *et al.*, 1991; Brecht *et al.*, 1995) or mechano-acoustic phenomena (Lu & Czanderna, 1984; Nieuwenhuizen & Venema, 1991; Suleiman & Guilbault, 1996). Some devices, for example the widely used BIAcore system from Pharmacia Biosensor, are commercially available. An alternative method for detecting antigen–antibody

*To whom correspondence should be addressed. Email: vladimir.mirsky@chemie.uni-regensburg.de

binding can be the measurement of electrical capacitance of the receptor layer. It is a traditional method of classical electrochemistry and has been widely used to study the structure of electrical double layers and the adsorption of different species on plain metal electrodes. The use of the capacitive method for chemical sensing and immunosensing represents an interesting extension.

The first step in this method involves immobilization of a receptor (for example antibody or antigen) at the interface. Early attempts to make such a sensor by means of the Langmuir–Blodgett technique or by direct adsorption of receptor proteins on the electrodes were less successful (Bergveld, 1991), probably because specific capacity changes after antigen–antibody binding (about 60 nF/cm^2 in our work) are too small compared to the large capacity of the electrical double layer (about 40 μF/cm^2). Relative changes in the capacity of this sensor whose surface is covered by 50% cannot exceed 0·15%. Besides, an application of this sensor for real samples containing only minute quantities of redox-active impurities can lead to a large error because of pseudo-capacitance effects caused by Faradaic processes. These problems can be overcome by deposition of an ultra-thin insulator layer on the surface of the electrode. This is accomplished by formation of an oxide layer on the surface of a doped semiconductor electrode (Bataillard *et al.*, 1988; Billard *et al.*, 1991; Barroud *et al.*, 1993) or by exploiting the tendency of alkylthiols to form self-assembled and stable monomolecular insulating layers on a gold surface (Finklea *et al.*, 1987; Bain *et al.*, 1989; Dubois & Nuzzo, 1992; Bard *et al.*, 1993; Ulman, 1996). It is noted here that we do not consider the metal-supported self-assembled lipid membranes (Stelzle & Sackmann, 1989; Tien & Salamon, 1989; Hianik *et al.*, 1993; Puu *et al.*, 1995; and others) which are not chemically bound to the electrode surface. The gold–alkylthiol system was recently applied for detection of peptide-antibody binding (Rickert *et al.*, 1996), surfactant adsorption (Krause *et al.*, 1996; Mirsky *et al.*, 1996b) and phospholipase activity (Mirsky *et al.*, (1996a, b)); here, the hydrophobic surface formed by methyl groups was used as a receptor for surfactants, whilst a phospholipid layer served as a receptor for phospholipases.

Capacitive immunosensors based on the alkylthiol-coated gold electrodes have a sandwich structure of the type Au-S-$(CH_2)_n$-coupler-receptor. This structure can be prepared in two ways. The first is based on the binding of the alkylthiols to the bioreceptor and subsequent adsorption of this complex onto the gold electrode. Because of steric constraints, this leads to the formation of a functionally active receptor monolayer with a considerable number of defects in the insulating alkylthiol layer (Knichel *et al.*, 1995). These defects can be filled by subsequent or simultaneous adsorption of alkylthiol (Rickert *et al.*, 1996). Such a sensor (with a peptide as the receptor) was recently used for detection of antibodies, although its electrical capacity was about two times higher (and therefore its sensitivity was considerably lower) than the theoretical value. This was probably due to certain defects in the structure of the alkylthiol layer. The second way is based on subsequent adsorption of an ω-functionalized alkylthiol layer on the gold electrode, activation of this layer by a coupling reagent and subsequent covalent immobilization of the receptor molecules. This approach allows an insulating layer of alkylthiol which is essentially free of defects to be obtained. This method also allows the optimal solvent to be used for every stage of sensor preparation and to perform an immobilization of receptor molecules in aqueous electrolyte under physiological conditions without addition of detergents or use of organic solvents.

Though methods of immobilization of proteins on different traditional supports are already well established (Lyn, 1978; Kennedy & Cabral, 1987; Staros *et al.*, 1986; Taylor, 1996), new materials such as the gold–thiol self-assembled structures demand a new evaluation of these methods, since an optimal method of immobilization should not only result in the formation of a densely packed monolayer of functionally active protein, but also prevent any damage of the gold–thiol binding during this procedure. This aspect is especially important for the preparation of capacitive immunosensors, because even minute damages of the alkylthiol layer lead to large changes in capacitance. The identification of the best method was a major goal of this work.

EXPERIMENTAL

The basic method applied in the present work is measurement of capacity, which served two purposes (Fig. 1). The first was to test the insulating

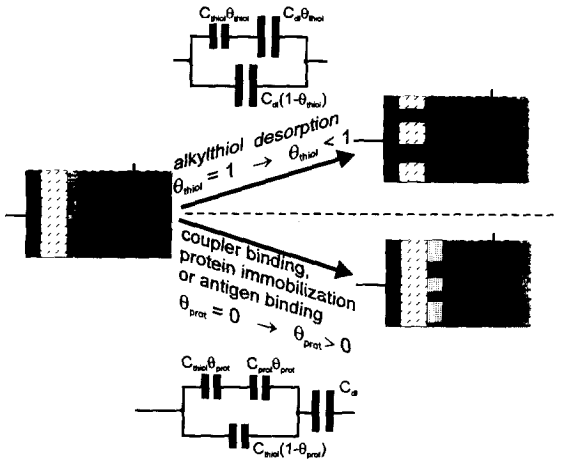

Fig. 1. Application of capacitance measurements for monitoring the desorption of alkylthiols and the deposition of an additional molecular layer (activation reagent, immobilized protein or bound antigen) on the electrode. C_{dl} is specific electrical capacitance of double ion layer (typically 30–80 μF/cm²), C_{thiol} and θ_{thiol} are specific electrical capacitance of the monolayer of ω-functionalized alkylthiol and its fractional coverage. C_{prot} and θ_{prot} are specific electrical capacitance and fractional coverage of the monolayer of coupler groups, protein or antigen for investigation of the alkylthiol activation, protein immobilization and antigen binding correspondingly.

monolayers made from ω-functionalized alkylthiols. The specific electrical capacity of these layers is about 100 times less than that of an electrical double layer (i.e. capacitance of the bare gold electrode). Therefore, even a minor desorption of the organic monolayer would result in an essential *increase* in capacity. For example, the desorption of only 0·5% of the monolayer gives a 50% increase in the capacity. The second application was to measure changes of the effective dielectric thickness of the insulating layer during chemical modification, protein immobilization, or antigen binding. In these cases a *decrease* of the electrical capacity can be expected.

All measurements were performed with a two-electrode system. The reference electrode was a Ag/AgCl electrode with a macroscopic surface of about 1 cm². Gold electrodes for measurements in the small cell (450 μl) were prepared by sputtering a gold layer on the silicon support and were generously provided by Prof. V. Tvarozek from the Technical University of Bratislava (Slovakia). Measurements in the large cell (20 ml) were performed with gold wire electrodes (diameter 0·05 mm, purity 99·995) received from Alfa (Karlsruhe, Germany). Monolayers of ω-functionalized alkylthiols were adsorbed on the gold electrodes, following the usual procedure. The electrode was cleaned with a hot mixture of piranha solution (a 1:3 mixture of 30% H_2O_2/conc. H_2SO_4), rinsed with water and dried. Then, the electrode was immersed into a 5 mM solution of 16-mercaptohexadecanoic acid or 16-mercaptohexadecylamine in chloroform for at least 12 h and washed briefly with chloroform. The macroscopic surface of the sensitive electrode was about 2·4 mm² for the electrodes on the silicon support, and about 4 mm² for the gold wire electrodes.

The electrode capacity was measured by registration of the 90° component of the capacity current by means of a lock-in amplifier (PAR, Model 121) at 20 Hz. The amplitude of the sine voltage on the electrode was 10 mV. A home-made current amplifier was used, the typical amplification being 10^4 V/A. The sensitivity of the capacitance measurements was limited by the capacitance drift and typically better than 0·1%. All measurements were performed at an electrode potential of + 300 mV (ref. Ag/AgCl, 100 mM KCl) at room temperature (about 22°C). To prevent formation of air bubbles, the electrolyte was degassed under vacuum before the experiment.

6-Mercaptohexanoic and 11-mercaptoundecanoic acids were synthesized according to Ferri (1978) *via* a Bunte salt. The purity of the 6-mercaptohexanoic acid was controlled by thin-layer chromatography (TLC). 11-Mercaptoundecanoic acid was characterized by TLC, IR and NMR spectroscopy.

16-Mercaptohexadecanoic acid was synthesized with xanthogenic acid ester as starting material according to Ferri (1978) and Morri & Nakamura (1969) with some modifications. 16-Hydroxyhexadecanoic acid (4·8 mmol) was refluxed for 3 h with 16 mmol of 47% HBr in glacial acetic acid, dried and purified. The 3·0 mmol of 16-bromohexadecanoic acid obtained were dissolved in 35 ml acetone and refluxed for 24 h with 4·5 mmol of potassium ethyl xanthogenate. The product (1·9 mmol of 16-ethyl xanthogenate-hexadecanoic acid) was stirred with 20 ml ethylenediamine under nitrogen for 24 h at 30°C. The mixture was added dropwise to 40 ml of ice-cooled 1:1 solution of H_2SO_4/H_2O to give a white precipitate. The resulting 0·8 mmol of 16-mercaptohexadecanoic acid was extracted with toluene from the precipitate and used without further

purification. No impurities were detected by TLC, IR or NMR spectroscopy.

16-Mercaptohexadecylamine was synthesized as described in the literature (Wieland & Hornig, 1956; Taylor, 1958; Morri & Nakamura, 1969) and adapted to longer alkyl chains. Concentrated sulfuric acid (13 ml) was added dropwise during 1 h to the suspension of 12·2 mmol of 1,16-hexadecanediol and 43·1 mmol of sodium bromide in 15 ml water, then refluxed for 20 h and slowly cooled to give a light brown precipitate. The precipitate was filtered, washed with water and purified by column chromatography with chloroform as the eluate. The 10·8 mmol of 1,16-dibromo-hexadecane obtained was refluxed for 5 days with 9·8 mmol of potassium phthalimide in 25 ml dry ethanol. The reaction product (5·3 mmol of 16-bromo-hexadecyl-1-phthalimide) was purified by column chromotography with chloroform as eluate and refluxed for 70 h with 15·2 mmol potassium ethyl xanthogenate in 10 ml acetone to give 0·4 mmol of dithiocarbonic acid-*S*-(16-phthalimido-hexadecyl) ester *O*-ethyl ester which was then stirred in 4 ml ethylenediamine under nitrogen for 24 h at 30°C. The 0·1 mmol of 16-mercaptohexadecylamine was extracted with toluene from the resulting precipitate, washed with water and purified by column chromatography using a methanol/chloroform (1/30, v/v) mixture as eluate. No impurities were detected by IR or NMR spectroscopy.

Chemical activation of amino or carboxy groups of the adsorbed ω-functinalized alkylthiols was performed according to Lyn (1978). Deionized water was additionally purified by passing it through a Millipore-Milli-Q system, the final resistivity being at least 18 MΩ cm. Monoclonal anti-HSA (from mouse, IgG2a, purity 26% from total protein), polyclonal anti-HSA (from the rabbit IgG fraction of antiserum, purity 38% from total protein) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were from Sigma. Sulfo-*N*-hydroxy-succinimide (S-NHS) was from Pierce, *p*-nitrophenol from Janssen Chimica, 16-hydroxyhexadecanoic acid (98%) and 1,16-hexadecanediol (98%) were from Aldrich, potassium ethyl xanthogenate and ethylenediamine from Fluka, and *N*-hydroxy-succinimide (NHS) and other compounds and solvents from Merck.

RESULTS AND DISCUSSION

Electrical properties and stability of insulating sublayers

A necessary component of any capacitive sensor is an insulating layer coating the conductive electrode and thus blocking any Faradaic processes. In this work we used insulating layers from mercaptohexadecanoic acid and mercaptohexadecylamine. These materials were chosen because of the formation of self-assembled monolayers on the gold electrodes. The impedance spectrum of the gold electrodes covered by an mercaptohexadecanoic acid is presented in Fig. 2. At low frequencies (10–30 Hz) electrical properties of this electrode are mainly capacitive, and the capacitive current (the imaginary component) is about 100 times higher than the conductivity current (the real component). For the gold electrodes covered by mercaptohexadecylamine, the real component was somewhat higher but also at least 10 times lower than the imaginary component.

Both long chain monolayers were very stable at neutral pH: no capacity changes were observed during 4 days' incubation in the aqueous electro-

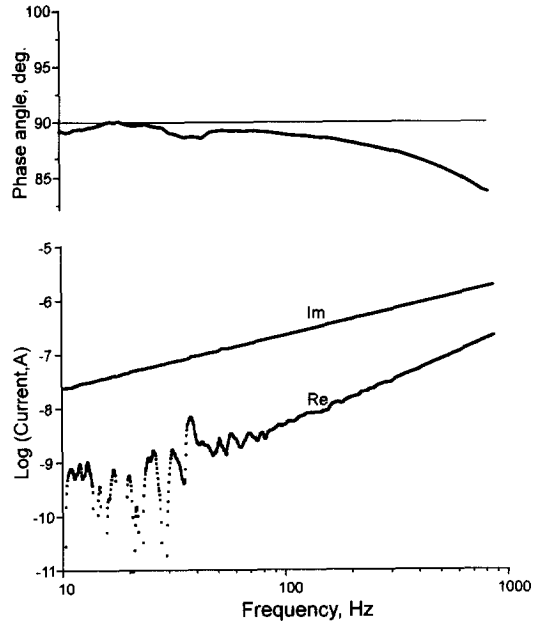

Fig. 2. EImpedance spectrum of a gold electrode covered by a monolayer of 16-mercaptohexadecanoic acid. Electrolyte: 100 mM KCl, 5 mM phosphate buffer, pH 7·2.

lyte (Fig. 3) except for a 0·5–2% change during the first several minutes after immersion of the dry electrodes in the aqueous solution. This effect is probably caused by electrode hydration. For comparison, the stability of the capacitance of gold electrodes covered by mercaptoundecanoic, mercaptohexanoic and mercaptopropionic acids was studied. A fast increase of the electrode capacitance was observed for all the short chain compounds.

This effect can be explained only by a desorption of these compounds from the electrode surface. Desorption was faster for the compounds with shorter alkyl chains, and both reversible and pH-dependent. For example, in the experiments with mercaptoundecanoic acid, the addition of this acid to the aqueous phase led to a decrease in the electrical capacity, i.e. to the adsorption of the mercaptoundecanoic acid on the electrode. At acidic pH only a very slow desorption of mercaptoundecanoic acid was observed. The increase of the rate of desorption, which was observed with decrease of the chain length, the reversibility and the pH-dependence of the adsorption/desorption suggest that these processes are governed by the solubility of these compounds in water.

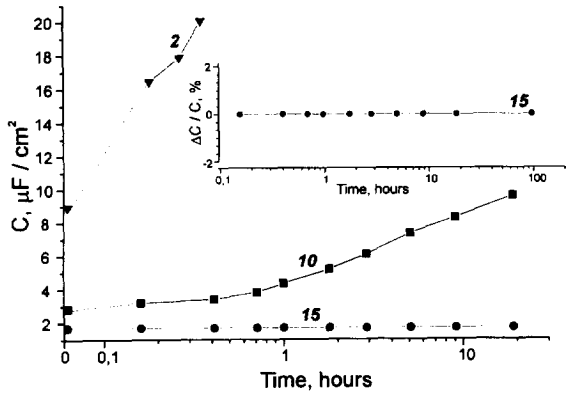

Fig. 3. Test of stability of adsorbed monolayer from different mercaptoalkyl acids expressed as capacity changes due to long time incubation in electrolyte. The values indicate the number of methylene groups of the alkyl chain. Electrolyte: 100 mM KCl, 5 mM phosphate buffer, pH 7·2. The capacitance values at zero time for mercaptopropionic acid and mercaptoundecanoic acid were calculated from the value for mercaptohexadecanoic acid assuming linear dependence between reversed molecule length and electrical capacity of the monolayer consisting of these molecules. The capacitance scale in the inset figure is magnified 200 times in comparison to the main figure.

It is important to note that at neutral pH the increase of the electrode capacity due to desorption of mercaptoundecanoic acids in the first 5 min is already higher than a typical signal of the capacitive sensor. These changes are even more dramatic for shorter chain compounds (Fig. 3). Therefore, one cannot expect low signal drift and high sensitivity with capacitive sensors based on the short chain ω-functionalized alkylthiols. Also, the design of alkylthiol-covered electrodes which are stable in a wide pH range requires the use of long alkyl chains.

Chemical activation of carboxy and amino groups on the surface

The methods used for activating carboxy and amino groups of the monolayers of mercaptohexadecanoic acid and mercaptohexadecylamine are shown in Fig. 4. The reactions proceeding in aqueous solutions (II, V and VI in Fig. 4) were continuously monitored by means of capacitive measurements. For the reactions in organic solvents, capacitance was measured before and after reaction. Chemical activation in organic solution was destructive for the monolayer; an especially high increase of the electrode capacitance was observed for activation of mercaptohexadecylamine (Fig. 5). The alkylthiol desorption from monolayers on metal surfaces exposed to organic solvents was also observed by Schlenoff *et al.* (1995). In contrast, the activation in aqueous solutions usually did not lead to such an enormous increase in the electrical capacity and therefore did not damage monomolecular layers (Fig. 5).

Both carbodiimides (EDC and CMC) showed a saturation of the capacitance effect at about 3 mM (Fig. 6(A)). The reaction kinetics at saturated concentrations of the carbodiimides (10 mM) was well represented by a bi-exponential function $A_1(1 - \exp(-t/t_1)) + A_2(1 - \exp(t/t_2))$ with parameters: A_1 = 2·1%, t_1 = 0·74 min, A_2 = 2·2%, t_2 = 7·0 min for EDC and A_1 = 0·67%, t_1 = 2·14 min, A_2 = 4·6%; t_2 = 17·3 min for CMC (Fig. 6(B)).

Albumin immobilization

Firstly, non-specific adsorption of albumin on the non-activated monolayers of mercaptohexadecanoic acid and mercaptohexadecylamine was tested by capacitive detection. When exposed to

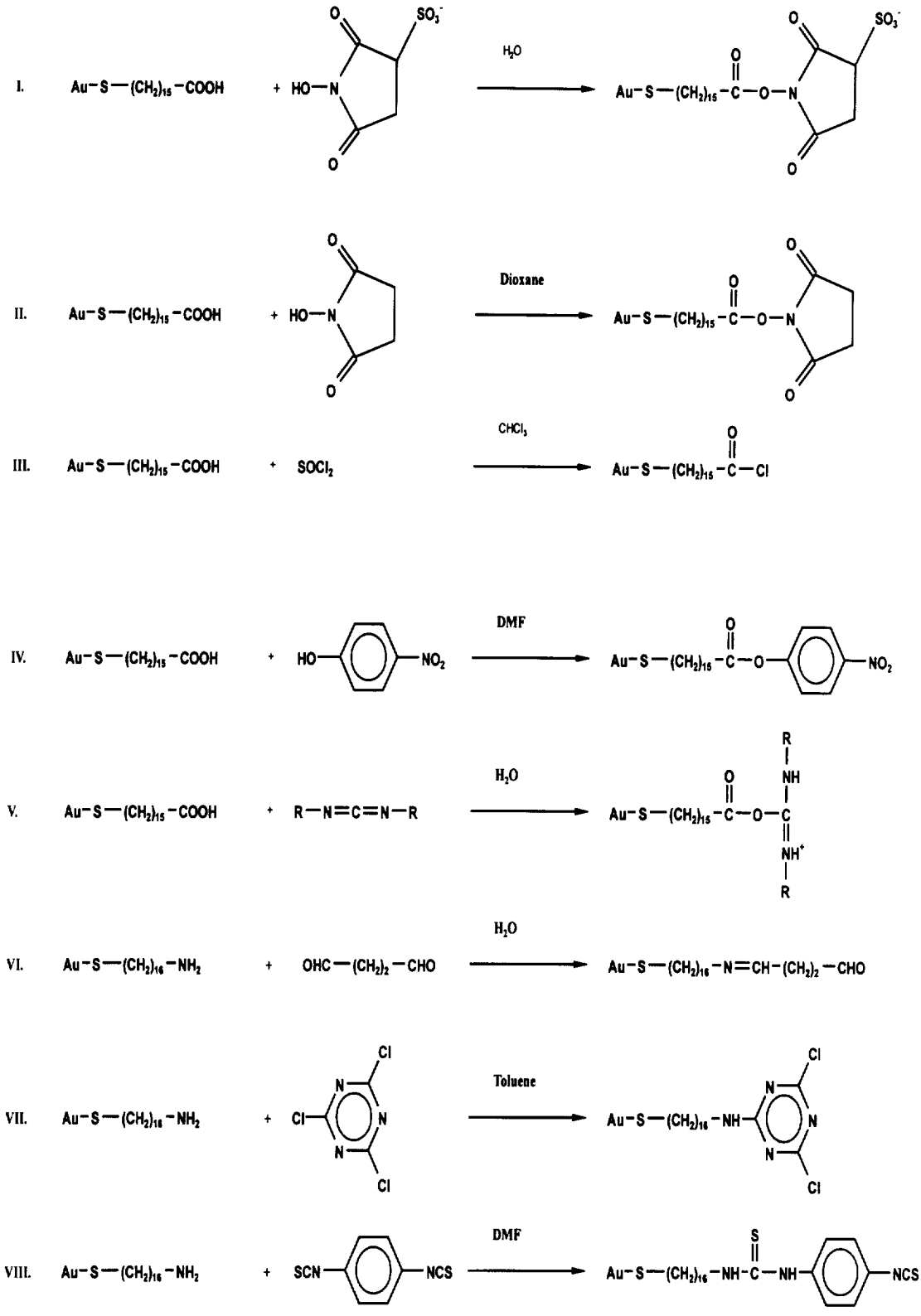

Fig. 4. Overview of methods of surface activation used in the present work for immobilization of proteins on the monolayers of 16-mercaptohexadecanoic acid and 16-mercaptohexadecylamine on the gold electrodes.

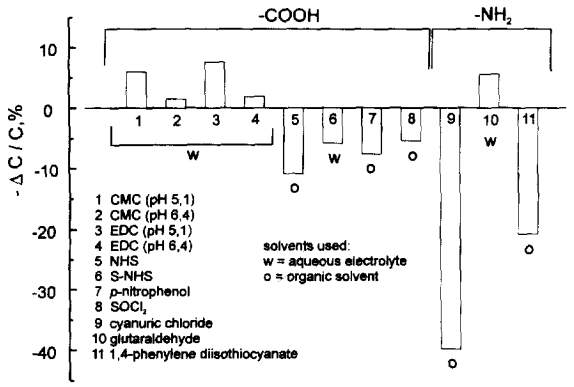

Fig. 5. Capacitance changes of the gold electrodes covered by monolayers of 16-mercaptohexadecanoic acid (COOH) or 16-mercaptohexadecylamine (NH_2) due to surface activation as summarized in Fig. 4.

40 μg/ml solution of albumin at pH 5·1, no changes of the capacitance were observed for the acidic monolayer and only a slight effect of about 1% was observed for the mercaptohexadecylamine monolayer. These capacitance changes could easily be reversed by rinsing the electrode with water.

The various reactions leading to protein immobilization on the surface activated according to Fig. 4 are shown in Fig. 7. Bovine serum albumin (BSA) was used as an inexpensive model protein to optimize immobilization conditions. Changes in the capacitance after protein immobilization by different methods are presented in Fig. 8. The results show that methods based on the use of either NHS, EDC, CMC or glutaraldehyde provide a higher increase in the dielectric thickness. The maximal capacitive effect was observed when the concentration of the protein exceeded 2–3 mg/ml. Both the reaction kinetics and the capacitance effect also depend on pH (Figs 8 and 9(A, B)). The optimal pH values for immobilization are 5·0–5·5 for NHS and both carbodiimides, and 6·0–6·5 for glutaraldehyde. The typical reaction time for protein immobilization with NHS at optimal pH was about 40 min (Fig. 9(A, C)).

To test for non-specific protein adsorption, which can occur during immobilization and can be as high as 75% of the total loading (Williams & Blanch, 1994), we have studied the long time stability of the electrical capacitance of the electrodes with immobilized proteins in the aqueous solutions (100 mM KCl, 5 mM phosphate buffer) of pH 6·4 and pH 7·2. The incubation

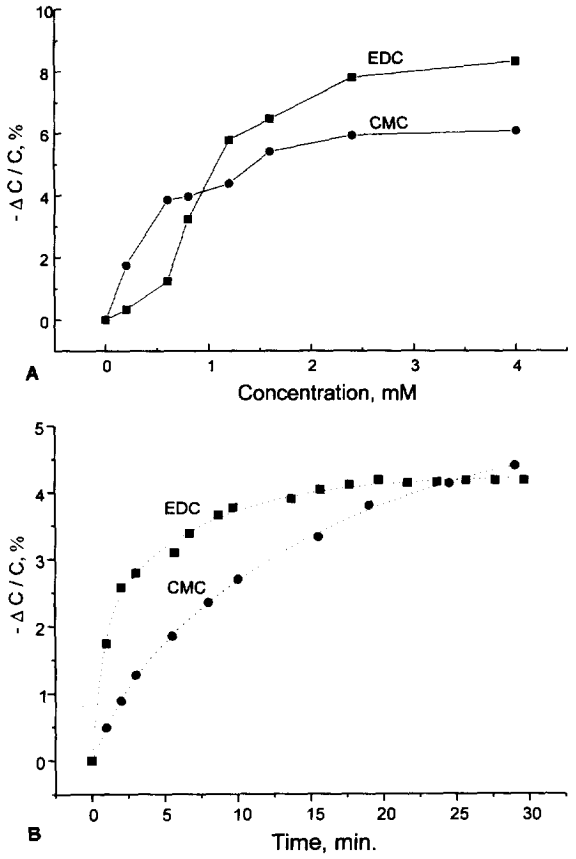

Fig. 6. Concentration dependence (A) and kinetics (B) of the capacitance changes due to activation of carboxy groups of mercaptohexadecanoic acid by carbodiimides (EDC: 1-ethyl-3-(dimethylaminopropyl)carbodiimide; CMC: 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate). The addition of 10 mM of the carbodiimides was used to study the reaction kinetics. The experimental points were fitted by a function $A_1(1 - exp(-t/t_1)) + A_2(1 - exp(t/t_2))$ *with parameters:* $A_1 = 2{\cdot}1\%$; $t_1 = 0{\cdot}74$ *min;* $A_2 = 2{\cdot}2\%$; $t_2 = 7{\cdot}0$ *min for EDC and* $A_1 = 0{\cdot}67\%$; $t_1 = 2{\cdot}14$ *min;* $A_2 = 4{\cdot}6\%$; $t_2 = 17{\cdot}3$ *min for CMC.*

time was about 12 h for electrodes prepared by means of NHS or EDC methods and 2–3 h for the other electrodes. No changes in capacitance (i.e. no desorption of any non-immobilized proteins) were observed. It is not quite clear why we did not observe any effects related to non-specific protein adsorption. Probably, this non-specific adsorption occurs mainly at uncovered gold surfaces and is negligible in our case because these electrodes are practically free of defects. In addition, non-specifically adsorbed proteins probably do not form a densely packed layer on a hydrophilic electrode surface and there-

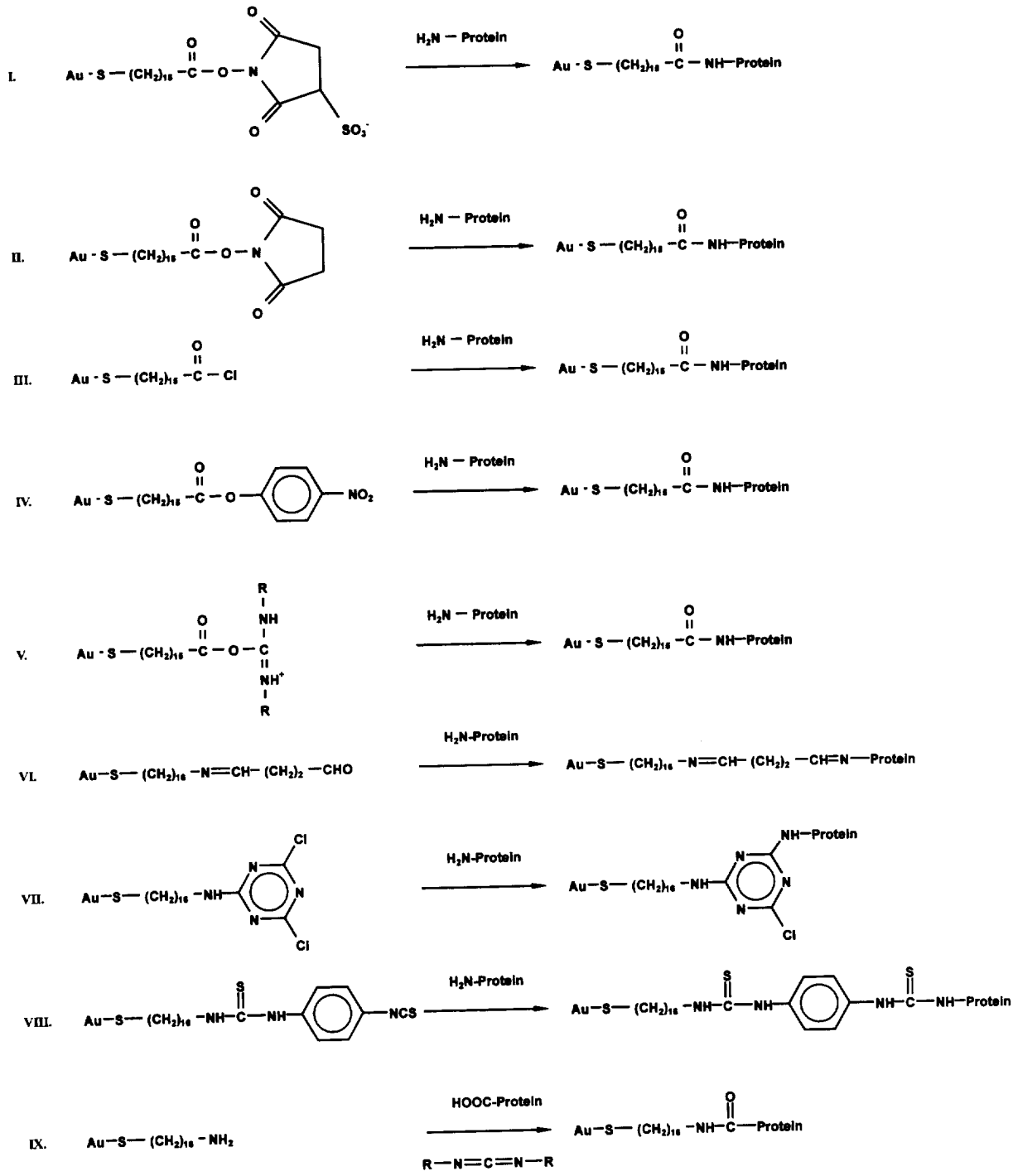

Fig. 7. Overview of methods for covalent immobilization of proteins on a surface activated as shown in Fig. 4.

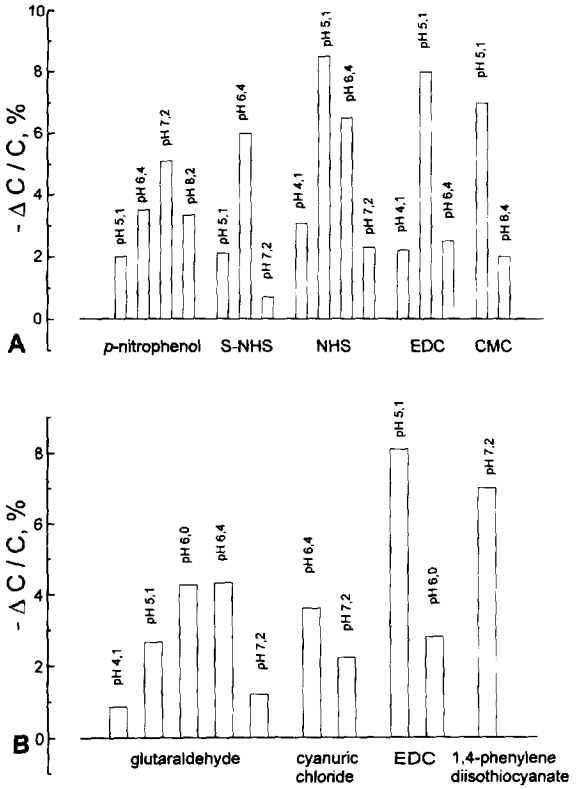

Fig. 8. Capacitance changes of gold electrodes covered by activated monolayers of 16-mercaptohexadecanoic acid (A) or 16-mercaptohexadecylamine (B) due to bovine serum albumin immobilization according to Fig. 7.

fore make only a minor contribution to the electrode capacitance.

Immunosensor

Once the method had been optimized using BSA, it was applied for immobilization of antibodies to human serum albumin (HSA) in order to obtain a capacitive immunosensor. The immunosensor has a sandwich-type molecularly organized structure, Au-S-$(CH_2)_{15}$-coupler-antibody, which is especially stabilized because of its covalent links on either side of the alkyl chain. The decrease in capacitance due to immobilization of anti-HSA by means of the NHS and EDC methods has nearly the same pH-dependence as for albumin immobilization (Figs 8(A) and 9(D)); the maximal effect was 1·2–1·8% (24–36 nF/cm^2) for polyclonal antibodies and 6–9% (120–180 nF/cm^2) for monoclonal antibodies (Fig. 9(C)). The last effect is comparable with the capacitance decrease due to immobilization of albumin (Figs 8 and 9(A, B)). The lower effect for polyclonal antibodies implies probably that they do not form a densely packed layer with low electrical conductivity. This may be the reason for the finding that the capacitance effect due to antigen immobilization on the semiconductor as observed by Billard *et al.* (1991) was only about 1·5 nF/cm^2.

Binding of HSA to the antibody layer leads to an increase of the effective dielectric thickness of the layer and therefore to a decrease of the electrical capacity (Fig. 10). The concentration dependence of the signal is presented in Fig. 11. For an immunosensor prepared with polyclonal antibodies, the decrease in capacitance due to antigen binding was about 3% at pH 7·2; at more acidic pH the effect was lower (Fig. 11(A)). Such a decrease of the efficiency of antigen binding at light acidic pH can be explained by electrostatic interactions between antigen and antibody. Isoelectrical points of HSA and BSA are, respectively, 5·85 and 5·1 (Patrickios & Yamasaki, 1995), isoelectrical points of antibodies are more basic. Therefore, one can expect maximal response at pH between the isoelectrical points of antigen and antibody, where both species have opposite charges.

When monoclonal antibodies were used for the immunosensor preparation, the resulting capacitive effects were significantly higher. The deviations of magnitude of the capacitance effects between different electrodes were about 10% at low antigen concentrations, but 30% at high antigen concentrations. For the immunosensor based on monoclonal antibodies, these deviations can be practically excluded by normalization of the sensor response to the capacitance changes during the immobilization of antibodies (Fig. 11(B)).

We have previously suggested proportionality between capacitance effect and the number of adsorbed molecules (Krause *et al.*, 1996). This allows one to use an adsorption isotherm to fit the dependence of the capacitance effect on the antigen concentration. By using the Langmuir adsorption isotherm $Kc/(c_{1/2} + c)$, a good fit was obtained with K = 0·84 and $c_{1/2}$ = 19 mg/l = 0·27 μM for the immunosensor based on monoclonal antibodies (Fig. 11(B)). It corresponds to an antigen–antibody binding constant of 3·58 × 10^6 M^{-1}, a value that is close to the data in the literature, e.g. 2·5 × 10^6 M^{-1} for the high affinity pool of mouse anti-HSA (Steward & Petty, 1972).

The maximal changes in capacitance due to

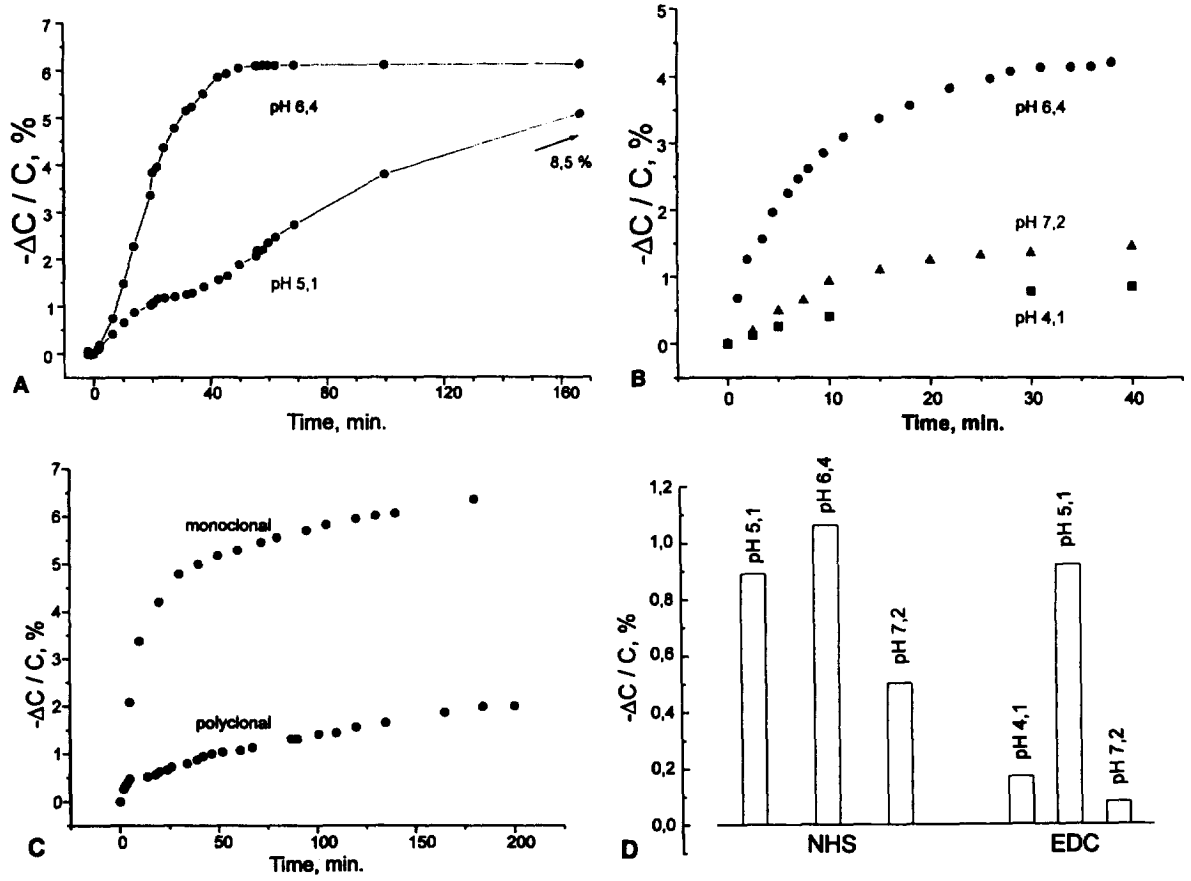

Fig. 9. Kinetics of protein immobilization, monitored as the electrode capacitance changes. (A) Addition of 600 μg/ml of BSA. The electrode was covered by 16-mercaptohexadecanoic acid and activated by NHS according to Fig. 4, II. (B) Addition of 80 μg/ml of HSA. The electrode was covered by 16-mercaptohexadecylamine and activated by glutaraldehyde according to Fig. 4, VI. (C) Addition of 57 μg/ml of monoclonal anti-HSA or 50 μg/ml of polyclonal anti-HSA antibodies; pH 6·4. The electrodes were covered by 16-mercaptohexadecanoic acid and activated by NHS according to Fig. 4, II. (D) Capacitance changes due to immobilisation of 50 μg/ml of polyclonal anti-HSA according to Fig. 7II and V.

antigen–antibody binding were found to be about 100 nF/cm^2. This effect is much higher than that observed by Billard *et al.* (1991) with a capacitive immunosensor based on a silanized silicon–silica heterostructure. Probably, our thiol–gold system provides a distinctly higher surface density of the receptor layer. One may expect a further increase in the sensor sensitivity by using F_{ab} fragments of antibodies.

The test on the cross-selectivity to BSA revealed that the response of the immunosensor with polyclonal antibodies was about a factor of two less than for HSA. For an immunosensor with monoclonal antibodies, this factor was about four to five. This rather limited selectivity can be explained by the very high homology of both albumins.

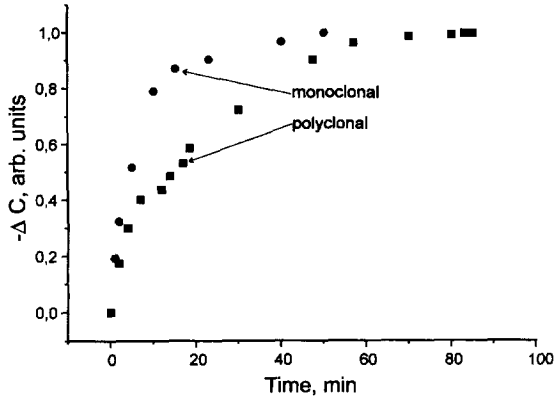

Fig. 10. Response of the capacitive immunosensor based on either polyclonal or monoclonal anti-HSA antibodies to addition of 16·5 μg/ml HSA. Both curves are normalized for maximal values.

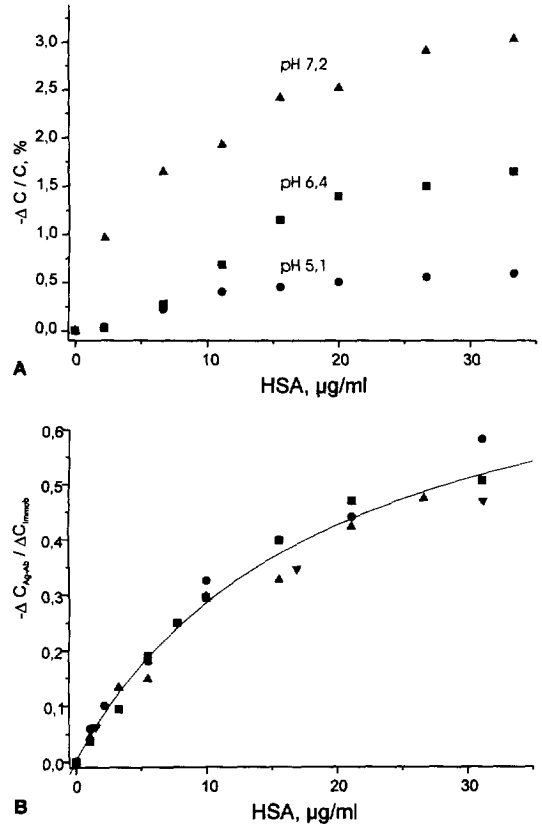

Fig. 11. Dependence of the response of the capacitive immunosensor on the antigen concentration: (A) pH effect (polyclonal antibodies); (B) data from four different electrodes (monoclonal antibodies) at pH 7·2. Changes of the electrode capacitance due to antigen binding (ΔC_{Ag-Ab}) were divided to the changes of the electrode capacitance due to immobilization of antibodies on this electrode (ΔC_{Immob}). Fitting curve: Langmuir adsorption isotherm $Kc/(c_{1/2} + c)$, where K = 0·84, $c_{1/2}$ = 19 mg/l.

A time interval between chemical activation and experiment with antibodies was at least 3 h. Both EDC and NHS intermediates are very unstable: a half-time of NHS intermediate hydrolysis at pH 7·5 is 14 min (Parker *et al.*, 1995), the stability of the EDC-activated groups is even less. Also in our work, no capacitance effect was observed when albumin was added to the EDC- or NHS-activated electrodes after 3 h incubation of these electrodes in aqueous solution. Therefore, in the experiments with immunosensors, one can exclude a binding of antigen with some remaining activated groups which did not react with antibodies.

CONCLUSION

We have demonstrated that the design of highly sensitive capacitive immunosensors is practically impossible unless complete coverage of the electrode surface by an ultra-thin insulator layer is achieved. The use of alkylthiols seems to be one of the most promising methods for obtaining defect-free layers. We have also demonstrated that, in spite of the generally accepted opinion on practically irreversible gold–thiol binding, short chain ω-substituted alkylthiols spontaneously desorb from the gold electrode at neutral pH. This effect was not observed, however, with long chain ω-substituted alkylthiols of 15–16 methylene groups. The results also show that carboxy-substituted alkylthiols are preferable over amino-substituted ones. However, activation of the alkylthiol monolayers in organic solvents usually leads to considerable desorption of these compounds from the gold electrode. The most promising methods for protein immobilization (without simultaneously compromising the insulating properties of the ω-substituted alkylthiol monolayers) are based on the chemical activation of carboxy groups by NHS or EDC. These methods were used for preparation of the capacitive immunosensor for HSA. The results demonstrate that this sensor provides selective measurements of antigen concentrations in the range from 1 to 20 μg/ml, which is the range for diagnosis of microalbuminuria. A more detailed investigation of this sensor as well as an amplification of the sensor response by subsequent adsorption of polyclonal antibodies will be reported later.

ACKNOWLEDGEMENTS

We are grateful to Prof. V. Tvarozek (Technical University of Bratislava) for providing us with gold electrodes, to Dipl. Chem. C. Krause for synthesis of 11-mercaptoundecanoic acid, to Dr. E. Terpetschnig for valuable advice on organic synthesis and to Dr. J. Ramsden (Biozentrum Basel) for reading the manuscript and useful discussion. The research was partially supported by European Project 'COPERNICUS CIPA CT94-0231'.

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