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# Sensitive Immunodetection Through Impedance Measurements onto Gold Functionalized Electrodes

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## Abstract

This article deals with a direct electrochemical method of detecting antigens using new methods of functionalization of gold electrodes. Based on the reacting ability of gold with sulfhydryl groups, three protocols for the fixation of antibodies have been explored. They are based on either the self-assembling properties of functional thiols bearing long alkyl chains or the possibility of a direct coupling of antibody moieties. Coverage rates as high as 97% can be reached. The analysis of the electrochemical impedance behavior of such layers can lead to a sensitive method for the direct detection of the antibody/antigen interaction. The addition of a redox couple in the tested solution, acting as an amplifier, allowed detection limits for the antigens as low as a few picograms/milliliter to be reached.

**Index Entries:** Biosensors; electrochemical impedance; modified gold electrodes; functional thiol.

## Introduction

Devices that would allow direct monitoring of the antibody-antigen interaction without any labeling, because they could compete with classic immunoassays, have attracted the attention of many researchers. An overview of some proposed approaches based on either optical or electrochemical techniques can be found in ref. *1*. For developing such a direct immunodetection concept, biosensors based on impedance measurements appear quite promising.

A first capacitive approach based on silicon-based heterostructures was investigated, assuming for the building up of the biolayers an

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electrically blocking behavior *(2)*. Detection limits of a few nanograms/milliliter can be reached but with severe constraints of frequency, polarization, and signal treatments, which render the detection not really in real time. The drawbacks of this capacitive concept for biodetection have been recently overcome, either by using the specific electrical properties of the detected specie itself *(3)* or by applying an appropriate electrical perturbation *(4)*. But such treatments cannot be easily generalized. Another way to exploit the electrical properties of the biolayer is by using conductive thin phtalocyanine films *(5)*.

In a previous publication, we presented the simple concept for an impedimetric immunosensor using metallic transducers functionalized with silanes and operating in a differential mode *(6)*. The performances of such platinum-based immunosensors functionalized with various polymeric layers were compared and interpreted in terms of permeability against the analyte. For electropolymerized polypyrrole-based biosensors, detection levels can be reached as low as 0.5 μg/mL *(7)*. But with these devices, because they need polarization, a rapid degradation associated with a loss of reproducibility was observed. These two effects can be interpreted in terms of degradation mechanisms of the polymeric functional layer owing to the electrical polarization. Probably such a polarization can also induce high electrical fields, near the metallic surface of the electrode, able to denature the immunospecies. To minimize the first drawback, one possible method could be the use of well-engineered layers that can also contribute to better orientation of the recognizing species *(8)*. Furthermore, this stabilization effect should be reinforced if such molecularly engineered layers are covalently bound or strongly stuck to the sensing electrode. Thus, it appears that thiol derivatives could be good candidates if the platinum electrode substrate is replaced by a gold-based material, the nature of the electrode material being itself of importance *(9)*. For instance, self-assembled monolayers of alkanethiols have been deposited on gold for molecular recognition purposes *(10)*.

In this study, we investigated the functionalization abilities of gold-based immunoimpedimetric-sensitive devices through the specific gold reactivity of thiol reagents or even of thiol residues obtained after cleavage of antibodies. Three methods, developed in order to obtain dense structured layers, were explored. Two are based on the ability of functional long alkyl chain thiols to form compact self-assembled monolayers on gold surfaces; the functional group, either an acidic or a hydroxyl group, can further be coupled to an antibody. The third method uses the reactivity of free sulfhydryl groups of antibody moieties.

## Materials and Methods

### *Reagents*

2-Mercaptoethylamine (2-MEA) (cysteamine), *N*-hydroxysuccinimide (NHS), ethyl-dimethyl-aminopropylcarbodiimide (EDC), γ-cyanopropyl-(dimethyl)chlorosilane, immunospecies (goat antirabbit IgG, specific rab-

bit IgG, nonspecific sheep IgG), polyethylene glycol (PEG) (average molecular weight 6000), and phosphate-buffered saline (PBS) tablets were from Aldrich-Sigma. Thiol alcohol (10-hydroxy decane thiol) and acidic thiol (undecanoic thiol acid) were synthesized at IBCP-CNRS Lyon. Polyoxyethylen sorbitan monolaurate (Tween-20) was purchased from Merck. All other chemicals were of analytical grade. Dissolving one tablet of PBS in 200 mL of distilled water gave a 10 m*M* phosphate buffer, 2.7 m*M* potassium chloride, and 137 m*M* sodium chloride (pH 7.4) at 25°C. In some cases, 0.2% (v/v) Tween-20, as surfactant, was added in buffer solutions in order to reduce the nonspecific interactions when washing electrodes functionalized with antibodies.

### *Electrode Design and Immobilization Protocols*

Gold disk electrodes (1.0 mm diameter), after sealing in a Mecaprex resin, were successively polished with emery cloth and diamond pastes of 6.3 and then 1 μm. The electrodes were cleaned in water and pure ethanol in an ultrasonic bath. Then they were dried in a stream of pure nitrogen after they had been thoroughly rinsed with deionized water.

The proper choice of an immobilization process on such electrodes, along with the knowledge of antibody structures, allows a good orientation of the recognizing molecule allowing an optimal binding capacity of antigens. For this aim, as previously stated, three immobilization protocols were explored.

The first protocol exploited the ability of long chain thiol alcohols to form self-assembled monolayers on gold surfaces. Once formed, such layers have free hydroxyl groups that can, through silane reagents acting also as spacers, react to give cyano derivatives as shown in Eq. 1:

$$\begin{aligned} &\text{Au-SH-}(CH_2)_{10}\text{-OH} + \text{CN–}\ (CH_2)_3\text{-Si}\ (CH_3)_2\text{-Cl} \rightarrow \\ &\text{Au-SH-}(CH_2)_{10}\text{-O-}\ (CH_3)_2\text{Si -}(CH_3)_2\ \text{-CN} + \text{HCl} \end{aligned} \quad (1)$$

As previously shown *(11)*, these free cyano groups can react with the antibodies' glycoresidues, thus preserving the accessibility and the recognizing properties of the antibodies.

Another, more classic coupling method developed for the Biacore instrument *(12)* consists of fixing a long alkyl chain acidic thiol on the gold surface. These acidic groups can react with NHS in the presence of EDC. Then the antibody is coupled through the active *o*-acylisourea. However, during this last reaction the antibody can present various orientations.

The third method is based on the ability of the antibodies to be cleaved. The disulfide bonds in the hinge region, which hold the two heavy chains together, can be selectively reduced with cysteamine (2-MEA) to give two half IgG molecules, each keeping through an antigen-binding site its recognizing properties *(13)*. Proper protocol allows a limited reduction in the disulfide bonds between the antibody heavy and light chains. The obtained molecules contain free sulfhydryls that can directly react on gold surfaces.

The gold surfaces will be directly covered by antibody moieties and eventually by some residual molecules of 2-MEA used for the cleaving process. Thus, after cleaning and before inserting in the flow cell, the electrodes were dipped for 12 h in a solution of reduced antirabbit antibodies, and then rinsed in a PBS Tween-20 solution.

### *Instrumentation*

Electrochemical impedance measurements were performed on a cell *(7)* allowing differential measurements with a working and a reference sensor and an Ag/AgCl reference electrode. This cell was made of two assembled blocks, separated by a flat silicone seal cut at its central part in order to form a well-defined volume. The electrodes (sensors and counterelectrode) were inserted in the blocks in such a way that the sensors were facing each other. The counterelectrode was a platinum disk (0.4-$cm^2$ area) and had the same geometry as the sensors. Before inserting in the flow injection cell, the electrodes were thoroughly rinsed in a PBS Tween-20 buffer. The flow injection system operated in "closed circuit" mode. The same container was used at the same time as input and output for the flow circuit. This system could be used for long incubation periods and had the advantage of consuming small amounts of reagents. The flow rate was fixed at 1 mL/min, corresponding to a linear speed of 20 cm/min at the electrode level. An impedance analyzer (Princeton Applied Research model 6310) was used, connected to a microcomputer for the signal treatment. Impedance measurements and cyclic voltammograms were obtained using, respectively, the M398 and M270 commercial softwares. The equivalent electrical circuit was obtained using the Equivcrt software of EG&G. Using such a model, the functionalized electrode can be modeled by a resistance in series with two elements consisting of a capacity and a resistance in parallel. The first element is formed by the double-layer capacitance in parallel with a charge transfer resistance. The second, representing the biolayer, is composed of a resistance in parallel with a fictive element (rather than a capacity) called constant phase element, which allows for membrane irregularities or porosities. This model allows for the amplifying effect of the $[Fe(CN)_6]^{3-}/'^{4-}$ redox couple.

## Results and Discussion

### *Electrochemical Characterization*

Prior to the analytical tests on the final built-up layers including the recognizing molecules, the blocking characteristics of thiol layers, including the antibody moieties, because they can directly react on gold through their sulfhydryl residues, were tested by cyclic voltammetry. Such layers would allow the formation of an insulating layer, thereby preventing interferences from faradic origin. To test such a blocking character, electroactive species were added to the solution. The $[Fe(CN)_6]^{3-}/'^{4-}$ redox couple was

Table 1
Values of Faradic Currents
at 300 mV/AgAgCl for Various Functionalized Gold Electrodes

| Electrode | Current at 300 mV/AgCl (μA) |
|---|---|
| Gold bare electrode | 25.0 |
| Electrode modified with SAMs layers of acidic thiols | 1.5 |
| Electrode modified with SAMs layers of alcohol thiols | 2.5 |
| Electrode modified with antibody moieties | 5.0 |
| Electrode modified with cysteamine (2-MEA) | 17.5 |

SAMs: Self-assembled monolayers.

chosen because it constitutes an electrochemically reversible, well-defined system involving a one-electron redox reaction with a moderate normal potential. The voltammograms of the functionalized gold electrodes, obtained at a scanning rate of 100 mV/s, were compared with the one of a bare gold electrode. After obtaining reproducible voltammograms, the open circuit potential was checked against the reference electrode, and for further impedance measurements, the working electrode was maintained at such a value. Except for the 2-MEA-coated electrode, in which a small current appears with large overpotentials owing to ferrocyanide oxidation, most of the current observed on such electrodes was capacitive. Some typical current values, recorded at a potential of 300 mV/AgAgCl, are given in Table 1. It appears that, except for the 2-MEA-coated electrode, the thiol layers present a sufficient dense and compact character to block the faradic currents, even for the reduced antibodies. Thus, such reagents appear to be a good basis for further analytical investigations based on impedimetric measurements.

### *Determination of Coverage Rate for Gold-Modified Electrodes*

The use of an $[Fe(CN)_6]^{3-}/'^{4-}$ redox couple introduces in the electrical equivalent circuit a charge transfer resistance. This couple acts as a probe and, as a result, amplifies the impedimetric response. Because impedance spectra of the gold and modified gold electrodes were recorded using the same experimental conditions, the $R_{ct}$ value of the charge transfer resistance can be assumed to be affected only by the modification of the electrode area directly in contact with the test solution. At high frequency it is possible to access the $R_{ct}$ value, and thus an estimation of the electrode coverage rate, as $\theta = R_{ct}(\text{Au})/R_{ct}(\text{Au modified})$ *(14,15)*. Curves representing the real part of the impedance vs the inverse of the root square of the pulsation present a linear part, which extrapolated at high frequency, gives access to $R_{ct}$ values. Table 2 presents the results obtained from such an analysis. Except for cysteamine layers, which seem to present many defects allowing faradic currents, all the other thiol-based layers have a marked

Table 2
Coverage Rates of Various Functionalized Gold Electrodes
Evaluated from Charge Transfer Resistance Values

| Electrode | Coverage density (%) |
|---|---|
| Electrode modified with SAMs layers of acidic thiols | 97 |
| Electrode modified with SAMs layers of alcohol thiols | 87 |
| Electrode modified with antibody moieties | 87–92 |
| Electrode modified with cysteamine (2-MEA) | 47 |

electrically blocking character, even for the reduced antibody layers in which the observed coverage rate for different samples was between 87 and 92%. This fact can appear surprising; owing to the large size of such molecules, steric effects could hinder the binding. In fact, the coverage densities appeared quite good and the light discrepancies observed between several samples were probably owing to different amounts of residual 2-MEA, which, as stated, lead to layers not so compact as the one obtained with other reagents. A more detailed analysis of impedance measurements on such layers has been given elsewhere *(16)* showing high impedance values at low frequency. The good controlled molecular engineering of these species is thus promising for further analytical applications.

### *Immunodetection*

These impedance measurements, performed after functionalization with antibodies and incubation with various concentrations of specific antigens, can serve as a basis for immunodetection. A specific case for electrodes functionalized with antibody moieties is given in Fig. 1 that represents the impedance variations in a Nyquist representation. Thus, it appears that for a fixed value of the in-phase impedance, the out-of-phase impedance values are dependent on the antigen concentrations. Impedance acquisitions, for various antigen concentrations, were done after 30 min, allowing the antigen/antibody interaction to reach equilibrium, in a 10 m*M* PBS buffer (0.05% Tween) in the presence of a 5 m*M* concentration of the $[Fe(CN)_6]^{3-}/'^{4-}$ redox couple. When the antigens are recognized and fixed on the antibodies, the accessibility of the electrode surface for the oxydo-redox couple becomes more and more difficult, and, consequently, the electrochemical conductivity across the interface electrolyte/modified electrode is decreased. Taking into account the noise level of the electrochemical signal, Fig. 1 shows clearly that antigen concentrations as low as a few picograms/milliliter are easily detectable, which is quite competitive with earlier proposed direct detection methods of the antibody/antigen interaction.

Table 3 summarizes the analytical characteristics of the various layers functionalized with antibodies and their potentialities for immuno-

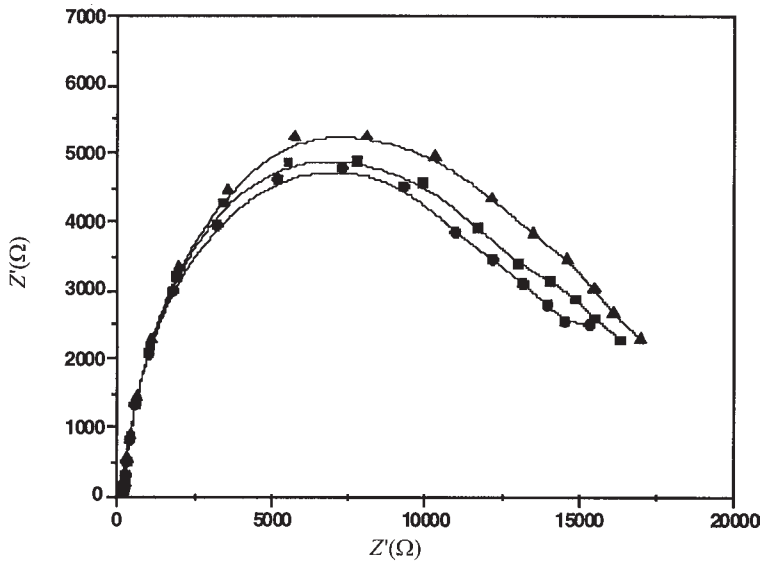


Fig. 1. Influence of specific antigen concentration on the out-of-phase impedance values vs the phase impedance values (Nyquist representation). Case of an immunosensor functionalized with antibody moieties. (▲), electrode functionalized with antibody moieties; (■), injection of specific antigens (5 pg/mL); one injection of specific antigens (50 pg/mL).

detection. These data were obtained from the analysis of the impedance variation, at a fixed frequency value, for each tested immunosystem vs variation in the specific antigen concentration. In this case, the data were compiled at a frequency value fixed at 3 Hz. From these results it appears that the three types of immunomembranes allowed the detection of antigens in the range of a few picograms/milliliter to about a few hundred milligrams/milliliter and thus could be of valuable interest for diagnosis purposes as well as the detection of toxic compounds. The best results are obtained for the acidic thiol-based layers in which no effect of nonspecific adsorption is observed. Thus, the injection of a nonspecific sheep IgG induced no significant variation in the impedance (<10 Ω/decade instead of 7300 Ω/decade for the specific rabbit IgG). These results, in which a quite low influence of nonspecific adsorption on acidic thiol-based layers was observed, are in accordance with the results of Tiefnauer et al. *(17)* showing that adsorption of nonspecific species could be strongly reduced by the use of negatively charged thiols.

In the case of electrodes based on alcohol thiol layers or directly coupled with antibody moieties, a strong interference of nonspecific species was observed, but it can be entirely removed by adding to the tested solution a low molecular weight PEG. In this case, a polymer of a molecular weight of 6000 was used and allowed complete exclusion of the nonspecific antigen (<10 Ω/decade for the nonspecific sheep IgG instead of 3400 Ω/decade and 1700 Ω/decade without PEG). This exclusion effect can be explained by

Table 3
Analytical Characteristics of Gold Electrodes Functionalized with Antibodies or Antibody Moieties[a]

| Type of electrode | Detection limit (pg/mL) | Saturation threshold (ng/mL) | Sensitivity in the linear range (10 pg/mL to 100 ng/mL) for specific antigen rabbit IgG (Ω/decade) | Sensitivity in the linear range (10 pg/mL to 100 ng/mL) for nonspecific antigen rabbit IgG (Ω/decade) |
|---|---|---|---|---|
| Electrode modified with SAMs layers of acidic thiols | 7 | 500 | 7300 | <10 |
| Electrode modified with SAMs layers of alcohol thiols | 10 | 70 | 8700 | 3400 |
| Electrode modified with antibody moieties | 5 | 500 | 3300 | 1700 |

[a]Results were obtained at a frequency of 3 Hz, in 0.05% PBS Tween-20 at 22°C.

a lowering of the dielectric constant of the solution, inducing a reduction in the protein solvatation by water, which can even lead to precipitation. These large-sized aggregates allow electronic transfers and do not contribute to an increase in impedance. Because the specific protein during incubation has a higher affinity for the specific antibody than the nonspecific one, it is preserved from this phenomenon and can be recognized.

## Conclusion

The direct binding of thiol derivatives onto gold surfaces allows a further building up of molecularly well-engineered layers, which combined with good recognizing properties and proper electrical characteristics, has led to immunosensors able to detect antigen concentrations at levels as low as a few picograms/milliliter. The presence of a redox couple allows measurement of the faradic impedance and thus following of the electronic transfer across the sensitive layers. Such a redox couple acts as a marker and an amplifier rendering the impedimetric detection well suited in the low concentration range.

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## References

1. Eggins, B. (1996), *Biosensors, An Introduction*, John Wiley & Sons, Chichester, UK.
2. Maupas, H., Saby, C., Martelet, C., Jaffrezic-Renault, N., Soldatkin, A. P., Charles, M. H., Delair, T., and Mandrand, B. (1996), *J. Electroanal. Chem.* **406,** 53–58.
3. Liu, M., Rechnitz, G. A., Li, K., and Li, Q. X. (1998), *Analyt. Lett.* **31,** 2025–2038.
4. Berggren, C., Bjarnason, B., and Johansson, G. (1998), *Biosens. Bioelectron.* **13,** 1061–1068.
5. Sergeyeva, T. A., Lavrik, N. V., Rachkov, A. E., Kazantseva, Z. I., and Elskaya, A. V. (1998), *Biosens. Bioelectron.* **13,** 359–369.
6. Maupas, H., Soldatkin, A. P., Martelet, C., Jaffrezic-Renault, N., and Mandrand, B. (1997), *J. Electroanal. Chem.* **421,** 165–171
7. Ameur, S., Maupas, H., Martelet, C., Jaffrezic-Renault, N., Ben Ouada, H., Cosnier, S., and Labbé, P. (1997), *Mater. Sci. Eng. C* **5,** 111–119.
8. Vikholm, I. and Albers, W. M. (1998), *Langmuir* **14,** 3865–3872.
9. Darbon, P., Michel, V., Math, F., Giorgi, H., and Machizaud, F. (1998), *Analyt. Chem.* **70,** 5072–5078.
10. Motesharei, K. and Myles, D. C. (1998), *J. Am. Chem. Soc.* **120(29),** 7328–7336.
11. Billard, V., Martelet, C., Binder, P., and Therasse, J. (1991), *Analytica Chimica Acta* **249,** 367–372.
12. Johnson, B., Löfas, S., and Lindquist, G. (1991), *Analyt. Biochem.* **198,** 268–277.
13. Hermanson, G. T., Mallia, A. K., and Smith, P. K. (1992), *Immobilization Affinity Ligand Techniques*, Academic, New York.
14. Blank, M. and Voddyanoy, I. (1994), *Biomembr. Electrochem.* **235,** 491–510.
15. Sabatini, E., Cohen-Boulakia, J., Bruening, M., and Rubinstein, I. (1993), *Langmuir* **9,** 2974–2981.

16. Ameur, S., Martelet, C., Chovelon, J. M., Ben Ouada, H., Jaffrezic-Renault, N., and Barbier, D. (1998), in *Eurosensors XII, Proceedings of the 12th European Conference on Solid State Transducers and the 9th UK Conference on Sensors and Their Applications, Southampton, UK, 13–16 September 1998*, vol. 2, White, N. M., ed., IOP Publishing, Philadelphia, pp. 797–800.
17. Tiefenauer, L. X., Kossek, S., Padeste, C., and Thiebaud, P. (1997), *Biosens. Bioelectron.* **12,** 213–223.