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High-sensitivity biosensor based on LB technology and on nanogravimetry

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Abstract

Quartz-resonator nanobalances are utilized here as effective transducers capable of determining with high sensitivity the mass changes due to specific protein–protein, antigen–antibody and ligand–receptor binding or self-assembly of functional complexes. The resulting highly sensitive biosensor is based on two quartz resonators (one active and the second used as a reference) and on Langmuir–Blodgett (LB) protein monolayers. The electronics are composed of two separate blocks, one designed to acquire by a personal computer the data coming from the other card, a 24-bit digital counter directly connected to the two oscillators. In the case of immunosensor application, the active and reference oscillators are covered respectively by antibodies specific to a given antigen and by antibodies non-specific to the antigen, in order to discriminate the physical adsorption effects. Deposition of antibody monolayers is performed by the LB technique in a surface pressure range of 20–35 mN m^{-1} onto gluteraldehyde pre-treated quartz resonators. A thermal treatment of the antibody layer up to 150 °C results in the reorganization of the film, and significantly improves the sensitivity and the properties of the immunosensor.

Keywords: Biosensors; Langmuir–Blodgett technique; Nanogravimetry

1. Introduction

Among different types of transducers used for immunosensors, the gravimetric type seems to be rather promising because it allows the mass of the antigens specifically bound to the antibody-sensitive layer to be measured directly [1]. Low price and high sensitivity [2] permit it to be considered as a promising tool for commercial immunosensors.

Several techniques are used for the formation of sensitive antibody layers. Most of them deal with chemical immobilization of antibody molecules on the surface of the transducer. Recently, the Langmuir–Blodgett (LB) technique began to be applied for the deposition of sensitive layers [3]. The technique allows dense monomolecular layers to be formed at the air/water interface and transferred onto solid substrates, providing a high density of molecules in the layer and covering the surface homogeneously. The technique does not demand a large amount of antibodies and provides good reproducibility of the properties of the deposited layers. Moreover, it was shown that dense organization of proteins (including antibodies) in the layer by the LB technique causes a marked improvement of the thermal stability of proteins [4]. This last finding is also very important for immunosensors, as it will allow them to be stored without particular precautions, for example.

The aim of the work is to develop and to characterize the properties of a gravimetric biosensor with a sensitive layer formed by the LB technique. In order to take into account non-specific adsorption, which would cause a decrease in the sensitivity, a differential scheme was implemented. The amount of bound antigen was estimated from the difference in frequency shifts according to the Sauerbrey equation [5]:

$$\frac{\Delta f}{f_0} = -\frac{\Delta m}{A\rho l} \tag{1}$$

where Δf is the frequency shift, f_0 is the initial quartz resonance frequency, m is the mass shift, A is the covered area and ρ and l are the quartz density and thickness respectively.

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2. Materials and methods

2.1. Dual quartz resonators

A differential scheme has been implemented in order to avoid the measuring problems related to physical adsorption onto the quartz resonators; this undesired contribution to the total signal can affect the measurements rather strongly, and should be carefully eliminated.

In order to overcome this problem, a method of parallel detection with two separate quartz oscillators was used, namely, one oscillator was used as the sensing element and the other as a reference. This set-up is commonly utilized and guarantees a better response with respect to a single-crystal system [6–9]. In addition to this commonly utilized differential scheme, both resonators (target and reference) were covered with monolayers of similar molecules, namely IgG 1 molecules (target having specificity to the antigen, reference having no specificity). This method additionally increases the sensitivity because physical adsorption effects can be considered equal for the two resonators, while specific binding takes place only on the surface of the target one. In this way the input in the mass change due to undesired phenomena such as physical adsorption is eliminated in the measurements.

In order to have the possibility of performing different kinds of experiments, a computer-based programmable system was developed; this system also allows sequentially acquired signals to be averaged.

The proposed hardware can be divided into three sections: a plug-in card provides the digital I/O interface to a precision counter, and two selectable driving circuits are connected to the measuring oscillators.

The circuits are designed according to TTL standards.

To count pulses from the oscillators, a fast eight-bit counter (74F579) is used as the first of three cascade counters to form a 24-bit device; 24 bits allow 10 MHz pulses to be counted for a time interval (gate) longer than 1 s, which ensures a proper resolution in the measurements.

In order to minimize the total number of components and to ensure absolutely equal counting for both oscillators, a single cascade counter has been designed; simple logic enables one oscillator at a time. The scheme is presented in Fig. 1. Here signals QB and QC allow the selection of the desired oscillator, while signal CKSR is necessary to reset the counter between two consecutive acquisitions. The parallel digital output is latched into three eight-bit registers, connected to the computer BUS through a bus buffer. The system is logically and practically divided into two devices, and therefore can be independently addressed and driven. Fig. 2 shows the circuit organization represented as a block diagram. The interfacing circuitry provides a suitable connection to a normal personal computer through a standard ISA bus. After a counting session data are stored in three eight-bit registers, and then transferred into the computer; each register is therefore addressed independently in order to enable its output to the internal bus. Additional logical devices are the resetting circuitry of the counters and the oscillators enables. The differential algorithm is performed by the software program after data acquisition; different averaging and acquisition procedures can be easily implemented because the overall system is very flexible.

Quartz resonators with a resonance frequency of 10 MHz were used.

The measuring procedure was as follows. Resonators with deposited layers were placed in the antigen solution for an increasing time. After each exposure the resonators were washed in order to remove non-specifically adsorbed antigens and measurements of the frequency shift were carried out after drying them with nitrogen.

2.2. LB protein monolayers

Anti-insulin antibodies from the Immunological Group of Bakh Institute, Russian Academy of Sciences, were used in the study. Rabbit anti-mouse antibodies were used for estimation of non-specific binding. Insulin was purchased from Sigma and working solutions were prepared at a concentration of 0.5 mg ml^{-1}.

Monolayers were prepared in an LB trough (MDT, Russia) according to the method described in Ref. [3]. Films were transferred onto substrates by the horizontal lift technique at 25 mN m^{-1} surface pressure.

Heating of the samples was carried out in a usual chemical oven at 150 °C for 30 min. X-ray measurements were done by a small-angle X-ray diffractometer with a linear position-sensitive detector, providing an angular resolution of 0.02° [10]. Surface-potential measurements were carried out by a home-made device using a vibrating electrode (Kelvin probe) [11].

In another series of experiments the resonator is first covered by a cytochrome P450 monolayer and then exposed to a solution of the corresponding coenzymes (adrenodoxin, adrenodoxin reductase) in 0.1 M Tris–HCl pH 8.0, NaCl 100 mM. Self-assembly has been conducted at 37 °C with careful shaking in the incubator. After exposure, the resonators were washed with distilled water and measurements of the frequency shift were taken.

3. Results and discussion

The accuracy of our differential apparatus is shown in Fig. 3, where a comparison between a commercially available high-precision frequency meter (Fig. 3(a)) and our system (Fig. 3(b)) is shown; in both cases, the

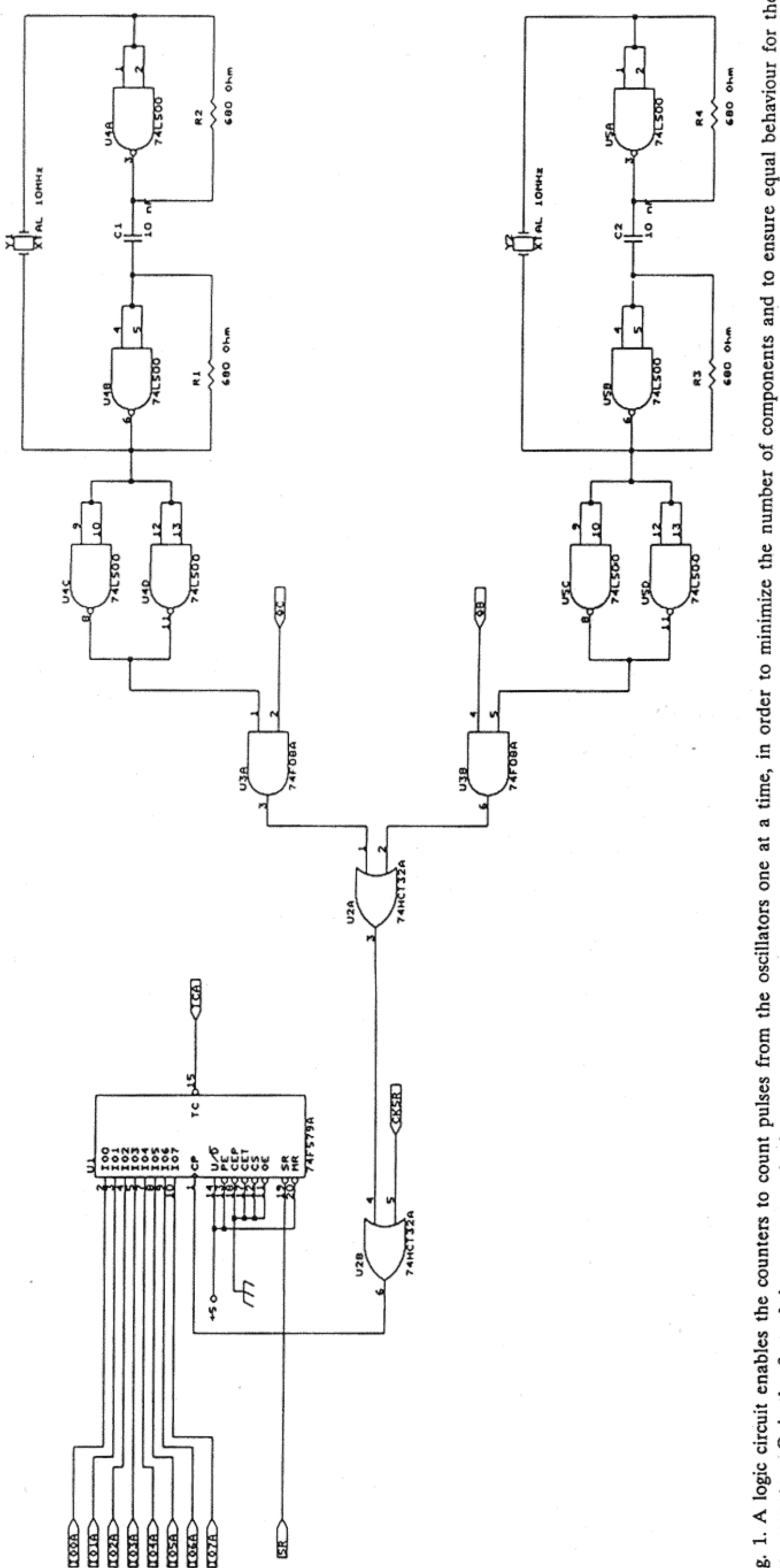

Fig. 1. A logic circuit enables the counters to count pulses from the oscillators one at a time, in order to minimize the number of components and to ensure equal behaviour for the two resonators. Only the first of three counters is shown.

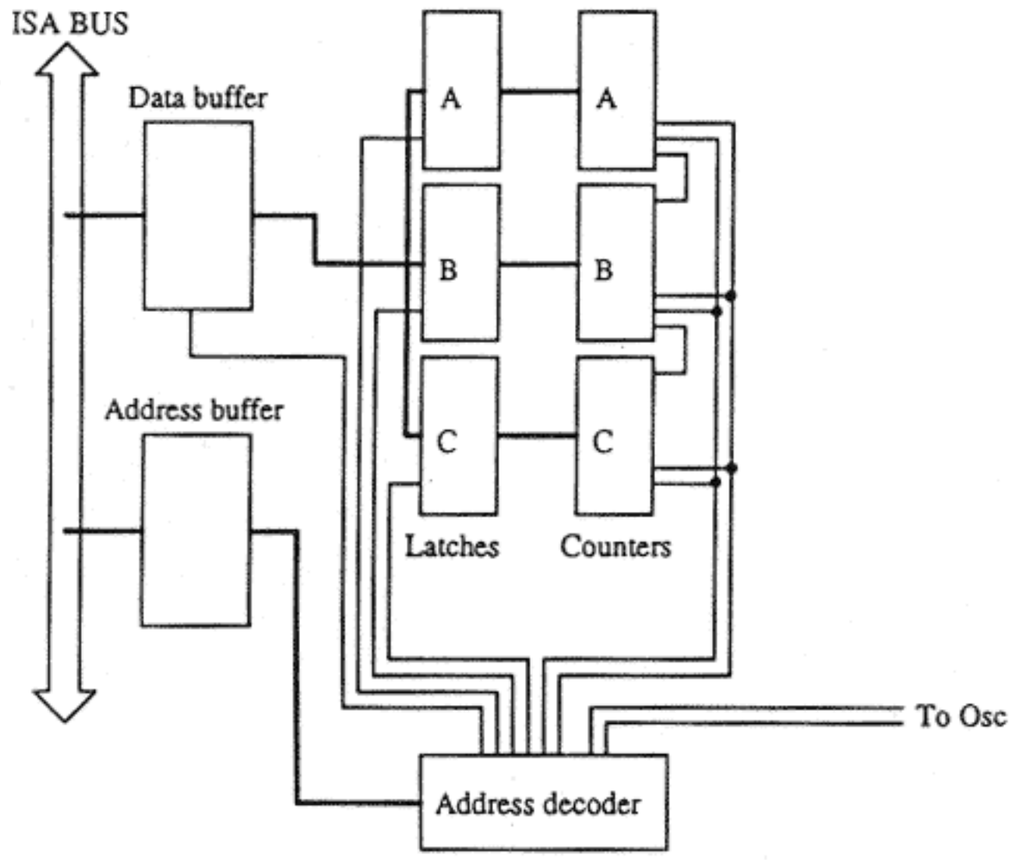

Fig. 2. Block diagram of the proposed hardware. A plug-in card allows direct computer interfacing of a 24-bit counter connected to the oscillators.

stability of the two resonators, with and without the metal cover, has been measured.

The usefulness of utilizing a differential approach as described above was shown with two experiments. A first experiment involved a working resonator and a reference one treated in the following way: both quartz resonators were incubated in gluteraldehyde (1% solution in water); one monolayer of rabbit antimouse (RAM) antibodies was deposited onto the working quartz by the LB technique; on the other hand, one monolayer of rabbit antiinsulin (RAI) antibodies was deposited onto the reference resonator.

The two antibodies show different specificity, so that dipping into a solution containing antigens specific for the working quartz resonator should not induce a mass variation in the reference. Moreover, the use of both resonators allows physical absorption effects on the measured signals to be neglected.

Both resonators have been dipped in a solution containing the antigen (mouse monoclonal antibodies) at a concentration of 10^{-9} M. The measurements have been performed after 5, 10, 20 and 30 min of dipping.

Fig. 4(a) shows the result of the experiment; the two curves are shifted along the *y*-axis due to the native difference in the oscillation of the two quartz resonators; however, the frequency decrease for the working quartz is evident, while the reference one shows a much slower trend. The immunocomplex was formed after 10 min of dipping into the antigen-containing solution.

The second experiment is presented in Fig. 4(b), which shows the baseline signals relative to the same quartz resonators, without any treatment after the removal of the metal cover; they have simply been dipped into the same buffer solution (TBS solution pH 7.3).

The experiment shows the usefulness of the differential approach; in fact the working resonator gives rise to a frequency decrease, as visible in Fig. 4(a), which is the immunoreaction effect when compared to the reference oscillator; the latter indeed presents a slow decrease due to the physical adsorption.

Taking the difference between the working and reference signals, it is possible to highlight the net frequency shift in the presence of the immunoreaction and in the reference situation, as seen in Fig. 5.

In conclusion, the experiments presented here show that a differential approach is mandatory in order to avoid artefacts, and allows a precise understanding of the immunoreaction taking place; in particular, it is possible to discriminate between the signal variation due to binding versus an eventual frequency decrease due to physical adsorption.

Quite interesting and unexpected results were obtained after thermal treatment of antibody LB films. Fig. 6 reports binding curves for two different LB samples, namely unheated and heated antibody. The striking sensitivity increase (of about one order of magnitude) is connected with the behaviour of the heated sample. First, antibody activity appears to be preserved in the LB films [3]. Secondly, the antibody–antigen reaction kinetics show that, after thermal treatment up to 423 K, the saturation level (reached when all the available binding sites are filled with antigens) was increased by 25% and a six-fold increase in the reaction rate occurred. As these phenomena are very unlikely to be due to modifications of the single antibody molecule activity, as induced by temperature, they could only be due to a modification in the film organization.

To explain the increased plateau level, it was shown [11,12] that the heating process provides enough energy to allow film recrystallization after returning to room temperature. Lipid [12] and protein [11] LB films showed a better order after such a heating procedure. In our case, due to the weak amphiphilic properties of antibodies, there is a certain statistical distribution in the orientation of IgG molecules at the air/water interface and, therefore, after deposition, not all the binding sites are exposed to the antigen solution. Moreover, there is the possibility of flip–flop transitions of molecules in deposited LB films [13]. Heat-induced recrystallization could provide a more regular distribution of binding sites, i.e., more Fab fragments could be oriented towards the antigen solution.

A larger amount of molecules oriented in the same direction on the solid substrate means that the monolayer becomes more anisotropic. In this case, charges and dipole distribution inside each antibody provide an effective electric field in the direction normal to the surface [11]. Comparison between the surface po-

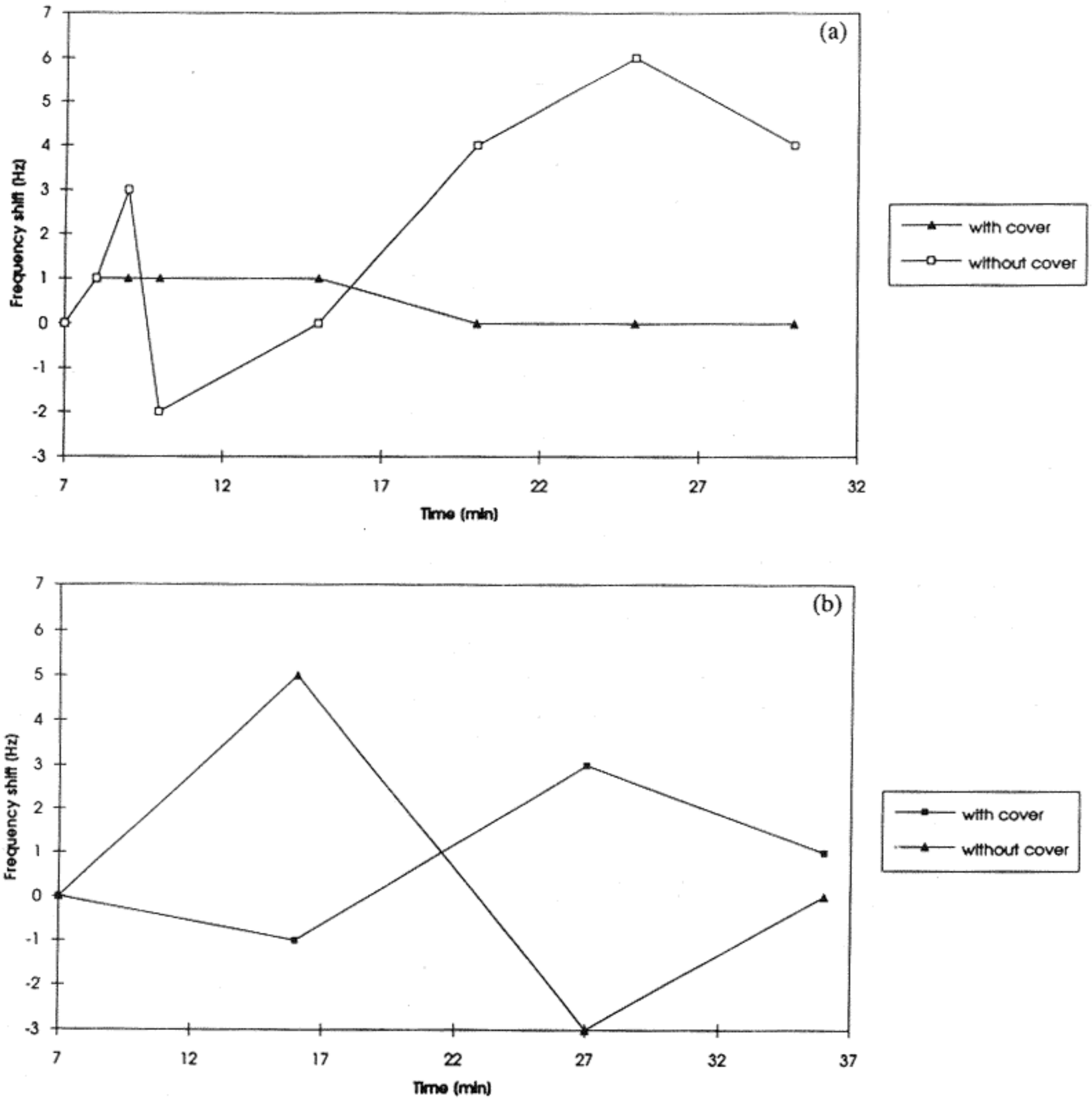

Fig. 3. (a) Test of quartz-oscillator stability with a commercial set-up utilizing two frequency meters. The gate interval is 1 s. (b) Test of the same oscillators with the differential scheme. The gate interval is greater than 1 s in order to ensure a proper resolution in the measurements.

tential of an unheated film and that of a heated sample has shown a marked increase in the surface potential, giving an insight into the phenomenon of the six-fold increase in reaction rate. In fact, the appearance of an electric field can easily account for the kinetic variation through electrostatic interactions between antibodies and antigens, as already shown for the interaction of the same IgG with other organic molecules [14].

To prove directly that the recrystallization process also takes place in antibody LB films, X-ray diffraction measurements yielded a periodicity of 115°, clearly pointing out a strong improvement of the order of the film [15]. The value of the spacing, in fact, corresponds to the length of an IgG molecule.

In another series of experiments the results of the complex P450 self-assembly are shown in Fig. 7.

The resonator covered by a cytochrome P450 monolayer was successively exposed to a 1 mg ml^{-1} solution of adrenodoxin, adrenodoxin reductase and adrenodoxin–adrenodoxin reductase mixture (1 mg ml^{-1} each) in 0.1 M Tris–HCl pH 8.0, NaCl 100 mM. Measurements were carried out as indicated previously.

The results obtained demonstrate the possibility of studying the self-assembing process by the nanogravimetric technique. Curves of the self-assembly of cytochrome P450 with adrenodoxin and with a mixture of both coenzymes are in good agreement with typical saturation curves. On the other hand, even the values of the saturation levels for adrenodoxin and adrenodoxin–adrenodoxin reductase mixture are in good agreement with theoretical suppositions.

Instead, absolutely different results were obtained with only adrenodoxin reductase. The shape of the curve suggests that mainly physical sorption takes place in this case. The results are in a good agreement with the hypothesis that the first step of the total complex formation is a specific attachment of adrenodoxin to P450 (1:1 molar ratio). Adrenodoxin reductase attaches only to the previously formed complex.

Thus, we have shown the kinetics of the formation of two-component (cytochrome P450–adrenodoxin)

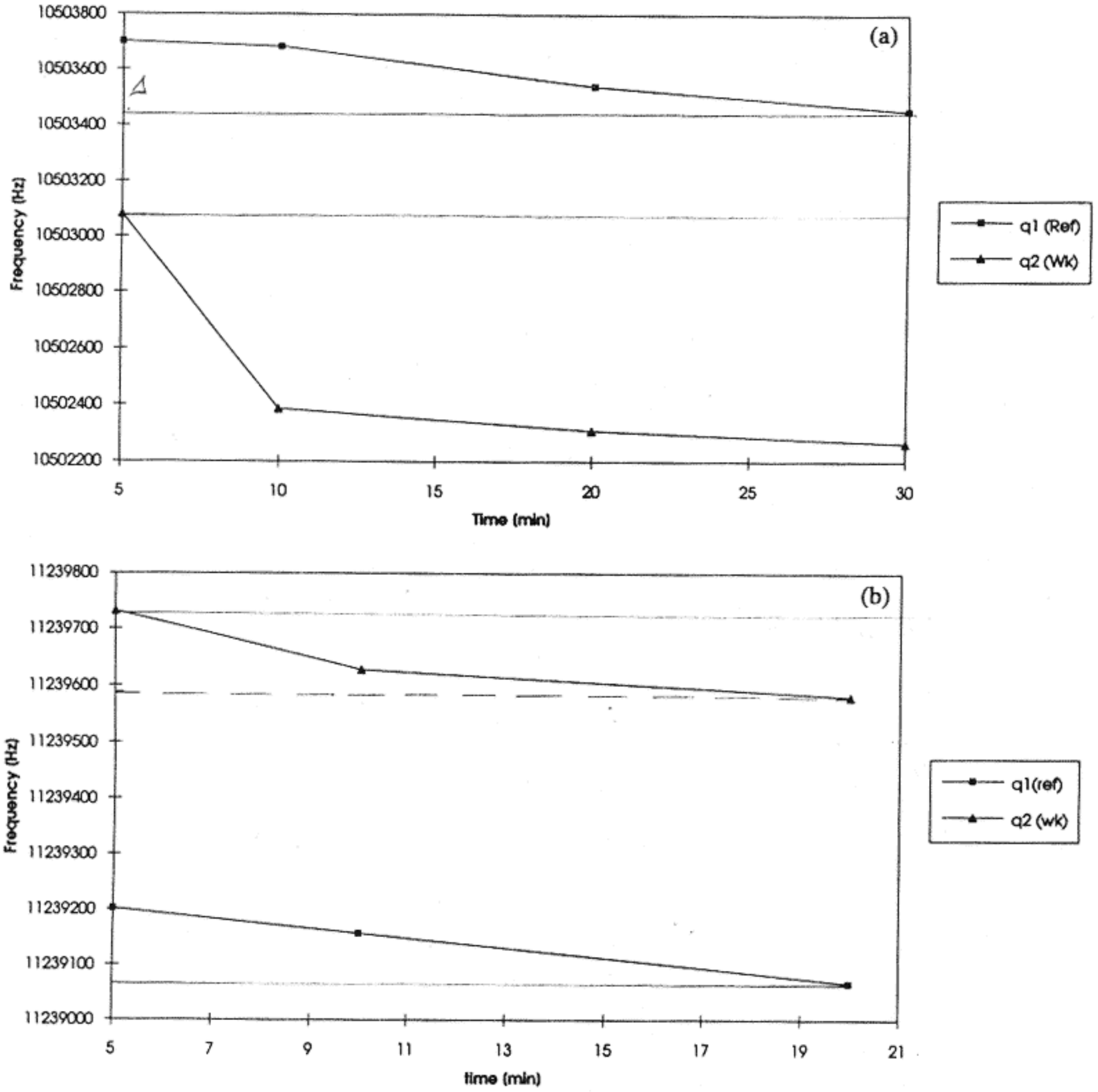

Fig. 4. (a) Result of an experiment with the differential scheme. A RAM monolayer was deposited on the working quartz resonator and an RAI monolayer on the reference quartz; both of them were exposed to a solution containing antigens specific for the working quartz resonator. (b) The same approach as above was used for testing the behaviour of the quartz resonators, without cover and without any layer, to the buffer solution (TBS buffer, pH 7.3).

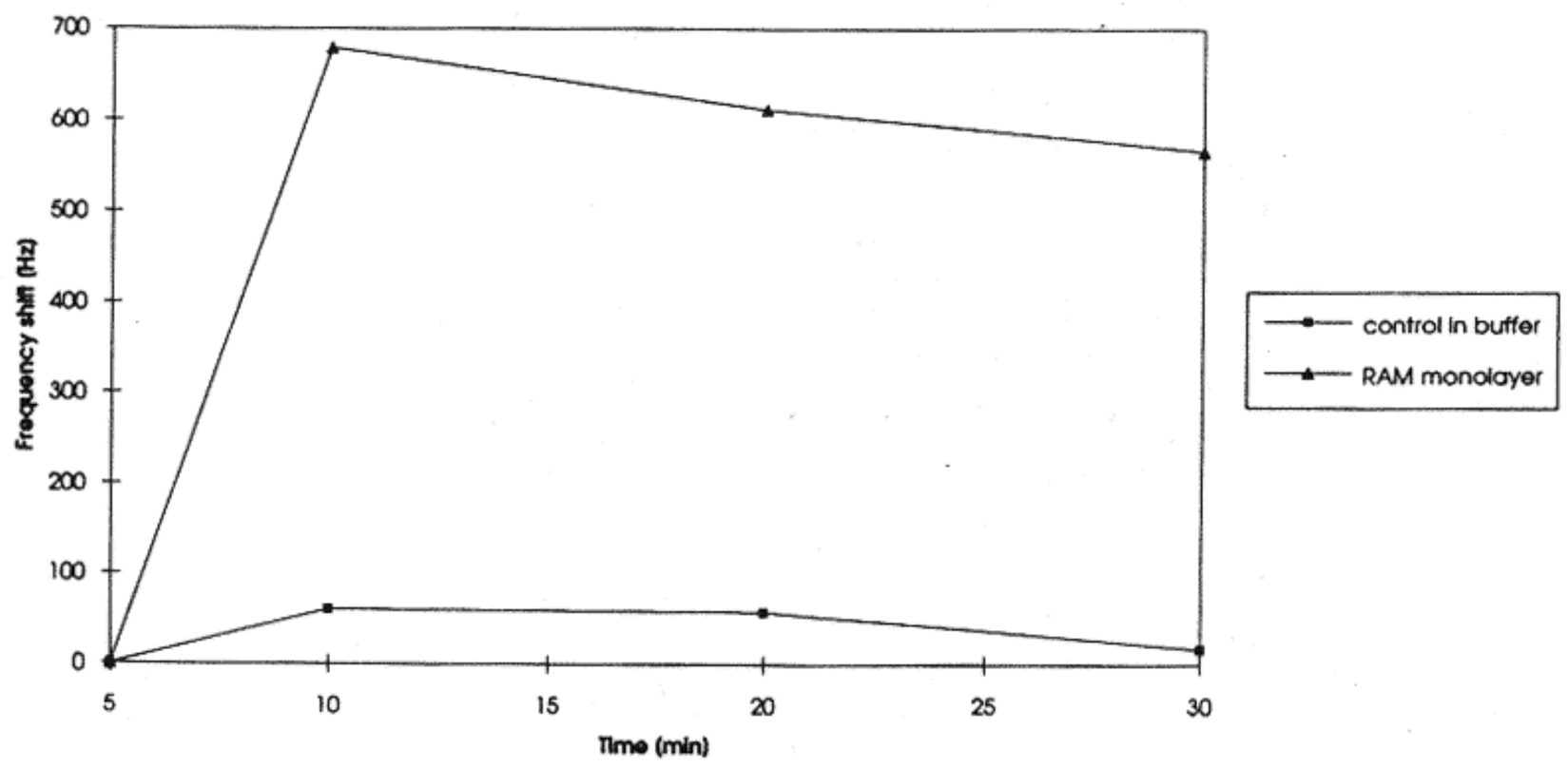

Fig. 5. The frequency shift between the two resonators, obtained from the experiments depicted in Fig. 4, vs. time.

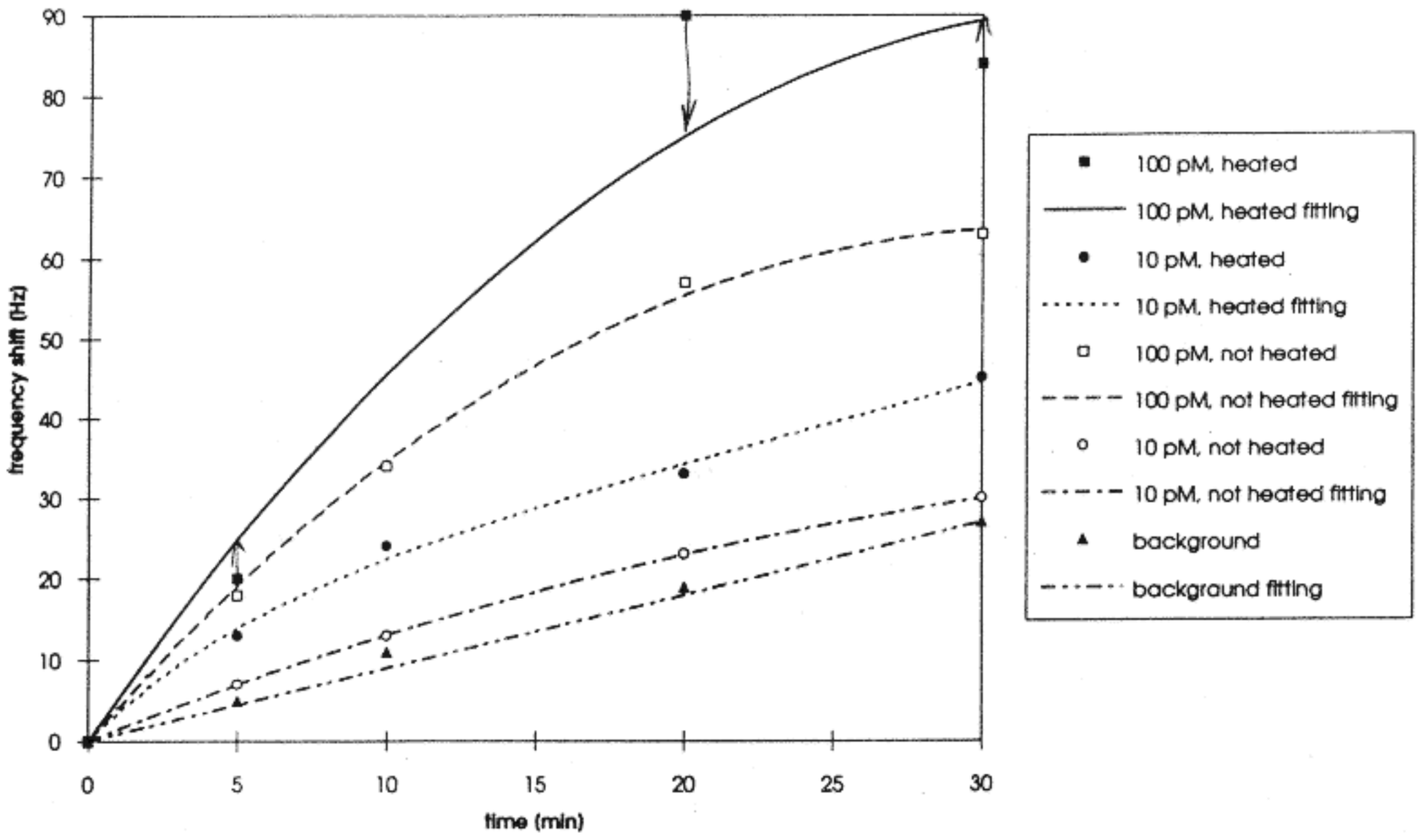

Fig. 6. Antibody–antigen reaction kinetics for heated and unheated antibody layers, with the experimental data fitting.

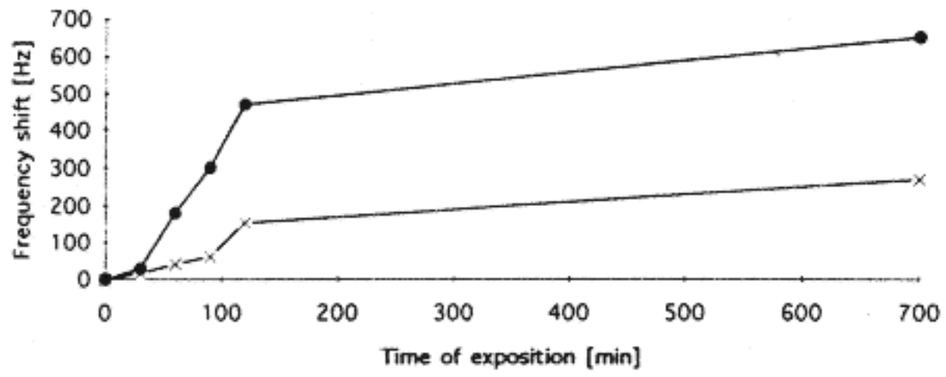

Fig. 7. Frequency shift vs. time for P450 monolayer exposed to a solution of adrenodoxin–adrenodoxin reductase (●) and of adrenodoxin only (×). See text.

and three-component (cytochrome P450–adrenodoxin–adrenodoxin–reductase) complexes, indicating the time for complex formation to be about 2 h (which corresponds well to the data obtained in solution). It was also shown experimentally that it is impossible to form a self-assembled complex between cytochrome P450 and adrenodoxin reductase if the complex between cytochrome P450 and adrenodoxin was not previously formed.

4. Conclusions

Summarizing the results, a new highly sensitive nanogravimetric biosensor was developed, based on a differential approach and LB technology. In the case of a layer of antibodies as the sensitive element, the best sensitivity was achieved when the antibody layer was deposited and thermally treated up to 150 °C. The performance of the device was improved by an order of magnitude, probably due to the reorganization of the layers toward more regular packing of molecules.

Acknowledgements

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References

[1] C. Barnes, C. D'Silva, J.P. Jones and T.J. Lewis, The theory of operation of piezoelectric quartz crystal sensors for biochemical application, *Sensors and Actuators A, 31* (1992) 159–163.
[2] P. Facci, V. Erokhin and C. Nicolini, Nanogravimetric gauge for surface density measurements and deposition analysis of Langmuir–Blodgett films, *Thin Solid Films, 230* (1993) 86–89.
[3] V. Erokhin, R. Kayushina, Yu. Lvov and L. Feigin, Langmuir–Blodgett films of immunoglobulin G as sensitive elements, *Il Nuovo Cimento, 12D,* (1990) 1253–1258.
[4] C. Nicolini, V. Erokhin, F. Antolini, P. Catasti and P. Facci, Thermal stability of protein secondary structure in Langmuir–Blodgett films, *Biochim. Biophys. Acta, 1158* (1993) 273–278.
[5] G.Z. Sauerbrey, *Z. Phys., 178* (1964) 457–462.
[6] A. Suleiman and G. Guilbault, Piezoelectric PZ immunosensors and their applications, *Anal. Lett., 24* (1991) 45.
[7] J. Ngeh-Ngwainbi, A. Suleiman and G. Guilbault, Piezoelectric crystal biosensors, *Biosensors Bioelectron., 5* (1990) 13–26.
[8] G. Guilbault and A. Suleiman, *Am. Biotechnol. Lab., 8* (1990) 28–32.
[9] M. Minunni, P. Skladal and M. Mascini, A piezoelectric quartz crystal biosensor as a direct affinity sensor, *Anal. Lett., 27* (8) (1994).
[10] L.Yu. Mogilevski, A.T. Dembo, D.I. Svergun and L.A. Feigin, Automatic small angle X-ray diffractometer with linear position sensitive detector, *Crystallography, 29* (1984) 587–591.

[11] P. Facci, V. Erokhin and C. Nicolini, Scanning tunnelling microscopy of a monolayer of reaction centres, *Thin Solid Films, 243* (1994) 403–406.
[12] L. Bourdieu, O. Ronsin and D. Chatenay, Molecular positional order in Langmuir–Blodgett films by atomic force microscopy, *Science, 259* (1993) 798–801.
[13] T. Kato, *Jpn. J. Appl. Phys., 27* (1988) 1358–1360.
[14] P. Facci, V. Erokhin, F. Antolini and C. Nicolini, Chemically induced anisotropy in antibody Langmuir–Blodgett films, *Thin Solid Films, 237* (1994) 19–21.
[15] V. Erokhin, P. Facci and C. Nicolini, Two-dimensional order and protein thermal stability: high temperature preservation of structure and function, *Biosensors Bioelectron.,* (1994) in press.