Quantum Electronics **30** (12) 1099 – 1104 (2000)

PACS numbers: 42.62.Be; 42.30.Rx
DOI: 10.1070/QE2000v030n12ABEH001876

Spectral-phase interference method for detecting biochemical reactions on a surface

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Abstract. A simple optical method is proposed for the direct detection of biochemical reactions on a surface, which is insensitive to variations in the radiation intensity and refractive index of a solution. The method is based on the detection of the spectrum of reflected or transmitted radiation modulated by the interference in a sensitive layer of a large thickness (several tens and hundreds of microns), which can be a microscope cover glass with a deposited receptor layer. A change in the phase of the interference pattern in this spectrum is used as an information signal about a change in the thickness of the sensitive layer caused by a biochemical reaction. The method was tested in studies of the reactions of binding and detachment of proteins in real time. The root-mean-square noise of the method expressed in the layer thickness is 3 pm.

1. Introduction

Direct optical methods for detecting biological and chemical interactions have gained wide acceptance in the last years. They have already become competitive in the sensitivity with traditional methods that use radioactive, enzyme, and fluorescence labels for detecting molecules involved in the reaction. In addition, optical methods have a number of advantages such as the possibility to monitor reactions in real time and a good reliability of the results that are obtained using fewer operations. These advantages stem from the fact that optical methods directly detect an increase in the thickness of a biomolecular layer on the surface of a sensitive element caused by the reaction between the solution component under study and a receptor layer immobilised on the surface.

The direct optical methods that have been most generally employed for detecting biochemical reactions are the surface-plasmon resonance [1, 2], whose sensitivity can be substantially improved by using phase detection methods [3 – 5], and a variety of the waveguide methods ('a resonance mirror' [6], grating couplers [7], a planar Mach – Zehnder interferometer [8], etc.)

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Received 10 October 2000
Kvantovaya Elektronika **30** (12) 1099 – 1104 (2000)
Translated by M N Sapozhnikov

All these detection methods are based on the use of a near-surface electromagnetic wave (which exponentially decreases outward from a sensitive element inside the solution), whose effective refractive index n depends on the thickness of a biomolecular layer. However, this refractive index strongly changes with temperature (by 10^{-4} per 1°C), which hinders the applications of these methods and restricts the resolution in studies of liquids, although the threshold sensitivity can be very high ($\Delta n/n \approx 10^{-8}$ when phase detection methods are used [3, 4, 7, 8]).

This difficulty was overcome in some degree in the method of the so-called reflectometric interference spectroscopy [9]. In this method, a thin (fractions of the wavelength) transparent film is used with the refractive index that is close to that of a biomolecular layer deposited on its surface, the film and the biomolecular layer being treated as a single sensitive layer. This sensitive layer is illuminated by a broadband radiation from a tungsten incandescent lamp and the spectrum of reflected light is detected. This spectrum proves to be modulated due to the interference in the sensitive layer and allows one to measure the optical thickness of this layer, whose variation gives information on the reaction under study.

However, because of a small thickness of a sensitive layer, the method [9] gives a slowly varying distribution of the intensity in the spectrum being detected, which is described by a sinusoid whose period covers the spectral region exceeding the visible range. For this reason, the information signal strongly depends on the uncontrollable variations in the intensity in different parts of the spectrum. These variations are unavoidable because of the use of a high-power broadband radiation source (an incandescent lamp) subjected to the temperature drifts of a colour temperature and the spectral distribution of the lamp radiation intensity, parameters of a spectrometer, the spectral sensitivity of a photodiode array, etc.

In this paper, we proposed and successfully tested a simpler and more reliable method of the direct optical detection of reactions on a surface. This method provides the information signal that is independent of the refractive index of the solution and related temperature drifts, as well of the drift of the central wavelength of a radiation source.

2. Physical foundations of the method

The method we propose can be called a spectral-phase interference (SPI) method. The method is based on the fact that secondary light waves produced at the interfaces of a sensitive layer form the modulated intensity distribution in the

spectrum of the reflected or transmitted light, which depends on the phases of these waves. The phase of this spectral distribution represents an information signal.

As a sensitive layer, a sufficiently thick plane-parallel transparent plate is used of thickness of about tens or hundreds of micrometers, together with a biomolecular layer, which is immobilised on the plate surface. The reaction of this layer with the chosen component of the solution is the object of investigation. The plate and biomolecular layer have close refractive indices, so that the reflection of light at their interface can be neglected in the first-order approximation.

The scheme of the interference of light waves that have experienced partial reflection from different interfaces of the sensitive layer is shown in Fig. 1. The result of the interference naturally depends on the phase thickness of the sensitive layer.

During the reaction under study, some components of the solution add to the surface of the biomolecular layer, whereas the other ones separate from this layer. This leads to a change in the phase (optical) thickness of the biomolecular layer and in the layer sensitivity as a whole, resulting in a change in the phase difference between the interfering waves.

In our method based on the use of a broadband radiation source, the thickness of the sensitive layer is much greater than the coherence length of the incident radiation. Therefore, it is impossible to observe a single interference pattern. However, by separating sufficiently narrow spectral intervals from the reflected radiation, one can provide conditions at which the radiation coherence length in each interval will exceed the doubled optical thickness of the sensitive layer. In this case, in each interval the interference of secondary light waves will be observed, which appear at the interfaces of the sensitive layer, and the entire reflection spectrum will be modulated.

Thus, let a plane light wave be incident normally on the plate surface. Let the refractive indices of three media located in tandem be n_1, n_2, and n_3 (according Fig. 1). In the case of detection of biochemical reactions, medium *1* is air, medium *2* is a sensitive layer consisting of glass and a biomolecular layer, which have close refractive indices, and medium *3* is a biological medium being tested (usually, aqueous solution). We neglect the dispersion of the refractive indices. As we will show below, our method allows us to use radiation sources with narrow emission spectra within which the dispersion of the refractive index is negligible.

The coefficients of reflection from the interfaces between media *1* and *2* and between media *2* and *3* have the form [10]

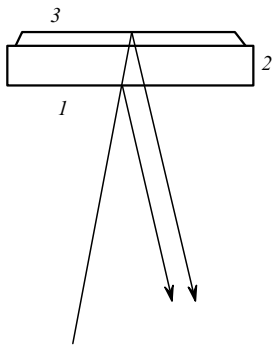

Figure 1. Scheme of the interference pattern formation: (*1*) Air; (*2*) transparent plate and biomolecular layer; (*3*) biological solution.

$$r_{12} = \frac{n_1 - n_2}{n_1 + n_2}, \tag{1}$$

$$r_{23} = \frac{n_2 - n_3}{n_2 + n_3}. \tag{2}$$

According to [10], the energy reflectivity of the sensitive layer is

$$R = \frac{r_{12}^2 + r_{23}^2 + 2r_{12}r_{23}\cos 2\beta}{1 + r_{12}^2 r_{23}^2 + 2r_{12}r_{23}\cos 2\beta}, \tag{3}$$

where $\beta = (2\pi/\lambda)dn_2 = (\omega/c)dn_2$ is the phase thickness of the sensitive layer; d is its geometrical thickness; λ and c are the wavelength and the speed of light in vacuum, respectively; and $\omega = 2\pi\nu$ is the circular frequency of light.

Expression (3) demonstrates the harmonic dependence of R on β, with the period $\beta = \pi$. The dependence of R on the frequency ν has extrema at $\sin 2\beta = 0$, i.e., at $\beta = m(\pi/2)$ or at ν satisfying the condition

$$\nu = \frac{mc}{4dn_2}, \tag{4}$$

where $m = 0, 1, 2, \ldots$. One can easily see that the function $R(\nu)$ has maxima at odd m and minima at even m if $(n_1 - n_2) \times (n_2 - n_3) < 0$ and vice versa, if $(n_1 - n_2)(n_2 - n_3) > 0$. In the method proposed here, the first case is realised, when medium *2* (a transparent plate and a biomolecular layer) has a greater optical density than media *1* and *3*.

Thus, the reflection spectrum is modulated by the function (3) with maxima and minima, which are equally spaced in frequency from each other. The frequency interval between adjacent maxima (minima) is

$$\Delta\nu = \frac{c}{2dn_2}. \tag{5}$$

Because the transmission T of a nonabsorbing sensitive layer is related to the reflection coefficient by the simple expression $T = 1 - R$, the transmission spectrum is analogous to the reflection spectrum, the only difference being that the reflection minima correspond to the transmission maxima, and vice versa. Therefore, one can use the detection of both reflected and transmitted light. The detection of the reflection spectrum is preferable because it imposes no limitations on absorption and transmission of light in solution.

It is obvious that the maximum contrast of the reflected light modulation is achieved for $r_{12} = -r_{21}$, when the waves reflected from two interfaces of the sensitive layer completely cancel each other at the frequencies satisfying the condition $\cos 2\beta = 1$, i.e., for even m. This condition can be provided by using as medium *1* the solution with the same refractive index as that for solution *3* or, if medium 1 is air, by using an antireflection coating at the interface between media *1* and *2*. However, a satisfactory contrast can be also obtained in the usual case, when medium 1 is air ($n_1 = 1.0$), medium *2* is glass ($n_2 = 1.5$), and medium *3* is water ($n_3 = 1.33$). Then, $r_{12} = -0.2$, $r_{21} = 0.06$, $R_{\min} = 0.020$, $R_{\max} = 0.067$, and the contrast is $(R_{\max} - R_{\min})/(R_{\max} + R_{\min}) = 0.54$.

Fig. 2 shows the reflection spectrum $R(\omega)$ calculated for a plane-parallel plate of thickness 150 μm illuminated by a radiation source with a 0.85-μm Gaussian emission band with the FWHM of 30 nm (which corresponds to a typical

superluminescence diode). One can see that the spectrum exhibits the periodicity in frequency and high contrast.

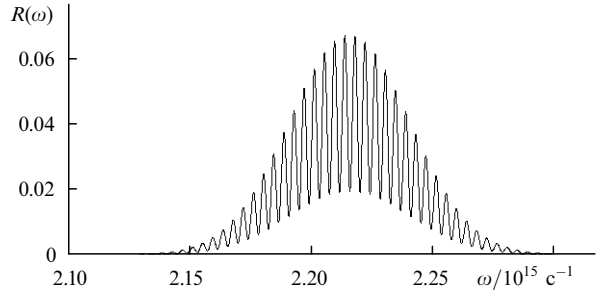

Figure 2. Calculated reflection spectrum of a plane-parallel glass plate.

As the thickness of the sensitive layer changed during the reaction under study, the interference pattern in the detected spectrum was displaced and its period change in accordance with expressions (4) and (5). When the layer thickness increased due to the attachment of molecules from solution to a biomolecular layer, the interference maxima and minima shifted to the red and the distance between them decreased, whereas in the case of decreasing layer thickness, the maxima and minima shifted to the blue and the distance between them increased.

In the method proposed, the thickness of a plate and of the entire sensitive layer considerably exceeds (by an order of magnitude and more) the wavelengths of the spectrum detected. For this reason, the spectrum exhibits the interference maxima and minima with high values of m [expression (4)] and the shift of maxima and minima caused by the reaction is much more pronounced than the change in the distance between them. Therefore, the information signal about the increase in the thickness during the reaction under study can be most simply measured from the shift of a 'comb' of the interference maxima and minima, i.e., from the phase of the distribution $R(\omega)$ modulating the spectrum.

It is obvious that to detect the phase of the distribution $R(\omega)$ with good accuracy, the spectral interval should contain many periods of this distribution. This requirement is satisfied for nonmonochromatic sources when the optical thickness of the sensitive layer is much greater than the coherence length of the source radiation. Therefore, the SPI method, unlike the method [9], allows one to use radiation sources with a narrow spectrum (light-emitting diodes, superluminescence diodes, etc.). Also, tunable lasers, in particular, semiconductor lasers can be used. Although their tuning range is usually very narrow, nevertheless, because of a great thickness of the sensitive layer, a sufficient number of the interference periods can be obtained in this range. The modification of the SPI method with the use of a monochromatic source scanned over the spectrum is especially promising for multichannel detection of biochemical reactions on a single glass substrate-biochip because this method does not require the use of a spectrometer in each individual channel. The characteristic size of a sensitive cell of such a biochip can be of the order of micrometer.

Because the information signal in this method is obtained namely from the phase of the interference pattern, it does not depend on the variation in the radiation source power and the detector sensitivity. The result of measurements only weakly depends on the spectral parameters of the radiation source and detector. The result also does not depend on variations in the refractive index of the solution under study because they change only the contrast of the interference pattern. All these features make the SPI method superior over other methods discussed above.

The SPI method was experimentally realised and successfully tested by detecting various biochemical reactions in real time.

3. Experiment

Fig. 3 shows a scheme of the experimental setup. Radiation from a laser diode *1*, operating in superluminescence regime at 0.85 μm (the FWHM of the emission band was about 30 nm and the power was less than 100 μW) was collimated with a lens *2* and was incident on a beamsplitter cube *3*. Then, a fraction of radiation was incident on a plane-parallel glass plate *4* of thickness 150 μm, which was used as substrate for the sensitive layer. The reflected radiation again entered a beamsplitter cube *3* and a fraction of it was coupled into a multimode fibre *6* with the help of a focusing lens *5*. This fibre had a core of diameter 50 μm, a numerical aperture of 0.22, and length from 1.5 to 150 m. The fibre provided a flexible coupling between a detector and a spectrometer. In addition, the fibre core served as an entrance aperture of a grating spectrometer.

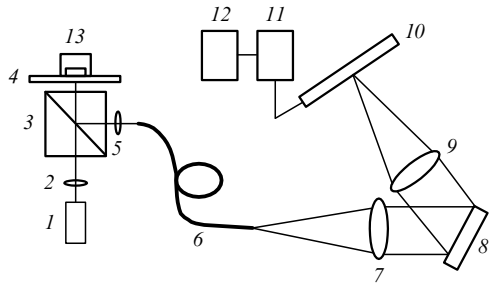

Figure 3. Scheme of the experiment: (*1*) Laser diode; (*2*, *5*) lenses; (*3*) beamsplitter cube; (*4*) glass plate; (*6*) fibre; (*7*, *9*) objectives; (*8*) diffraction grating; (*10*) CCD diode array; (*11*) analog-to-digital converter; (*12*) computer; (*13*) cell.

A spectrometer consisted of the first objective *7* with the focal length of 120 mm, a 600 lines/mm diffraction grating *8* of size 30 mm × 30 mm, the second objective *9*, which was similar to the first one, and a CCD array *10* containing 2048 pixels of size 14 μm × 14 μm. An output signal of the CCD array was digitised using the 12-digit analog-to-digital converter *11* and was fed into a personal computer *12* in a parallel code.

A cell *13* with the liquid under study was attached to the upper surface of a plate *4*. A cell *13* was used either in a continuous-flow regime or was adjusted to work using a pipet. In the first case, a continuos flow of the liquid through a cell was produced by a syringe pump of a Milichrome instrument controlled with a step motor.

After the entry of the digitised spectrum from an analog-to-digital converter to a processor, the following operations were performed: (1) the fast Fourier transform with the determination of a dominating harmonics; (2) sine- and cosine transformations (we denote the results of these transforma-

tions by S and C); (3) the phase determination from the expression $\varphi = -\arctan(S/C)$; and (4) the calculation of an increase in the plate thickness caused by the biochemical reaction from the expression

$$\Delta d = \frac{(\varphi - \varphi_0)\lambda}{4\pi n_2}, \tag{6}$$

where φ_0 is the initial phase fixed at the beginning of the experiment. The algorithm we used is valid when the phase changes only within one period of arc tangent. This is sufficient for detecting biochemical reactions when the maximum increment of the thickness of the sensitive plate does not exceed 200 nm.

The calculated values of Δd were used as output signals and were stored in a computer *12* as a sensogram, i.e., the dependence of the output signal on the running time of the experiment. The rate of the information output was 0.2 Hz, corresponding to averaging over 23 cycles of the data acquisition from a CCD array.

Before performing a biochemical experiment, we modified the surface of a glass plate *4* placed in a cell *13*. The glass surface was subjected to repeated chemical cleaning and then aminated and biotinylated. A scheme of the surface modification and of the subsequent biochemical bonding is presented in Fig. 4. In the initial state, before the beginning of detecting biochemical reactions, the glass had biotinyl groups on its surface. Then, the solution of protein streptavidin was circulated through cell *13*. The streptavidin molecules have four sites, which can specifically bind biotinyl groups. As a result, streptovidin molecules add to the surface of the biotinylated glass and form an additional layer. After the addition reaction, they acquire new sites, which can specifically bind biotinyl groups, so that the subsequent passing of the solution of biotinylated protein through a cell results in the bonding of this protein by streptovidin molecules on the surface.

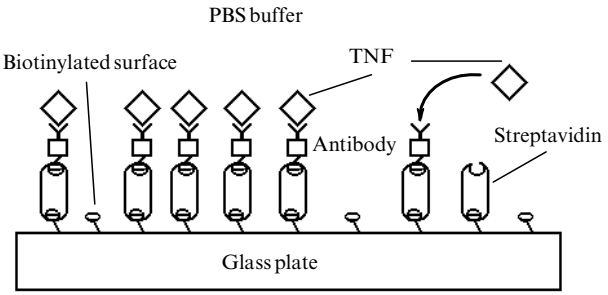

Figure 4. Scheme of biochemical bonding on a surface.

In the first series of experiments, antibodies that were specific to an antigen – a tumour necrosis factor (TNF) – were used as such protein. After addition of these antibodies, the surface is capable of specific bonding only TNF molecules. Such reactions are of special interest for immunodiagnostics of various diseases. We detected in our experiments both the direct reaction of specific addition of TNF to antibodies on the surface and the reverse reaction of the TNF separation due to dissociation of the bond between TNF and antibodies in an acid buffer at pH 2.0.

In the next series of experiments, after deposition of streptovidin molecules on a biotinylated glass surface, we passed through a cell the solution of biotinylated goat antibodies specific to the human immunoglobulin hIgG. Then, the cell was filled with the solution of immunoglobulin hIgG at different concentrations. We detected in real time all the above bonding reactions, as well as the surface regeneration and desorption of hIgG molecules in a buffer at pH 2.2.

4. Experimental results and discussion

Fig. 5 shows the experimental reflection spectrum of a plate *4*. It represents a periodic function with the envelope that is determined by the spectrum of a radiation source. The reflection spectrum is represented by dots corresponding to the output signals of an analog-to-digital converter. One can see that its character completely corresponds to the calculated spectrum (Fig. 2).

Preliminary experiments confirmed that the detected output signal Δd is independent of the refractive index of the liquid in a cell. In particular, the output signal did not changed upon the replacement of water by the phosphate-salt buffer PBS, which is conventionally used in biochemical experiments. In addition, we never observed jumps of the output signal caused by the change in the refractive index of the solution during consecutive biochemical reactions and cell rinsing at the moment of the solution replacement. This circumstance confirms an important advantage of our method over the known methods discussed above, in which jumps of the refractive index during the solution replacement results in abrupt jumps of the output signal and require a special correction.

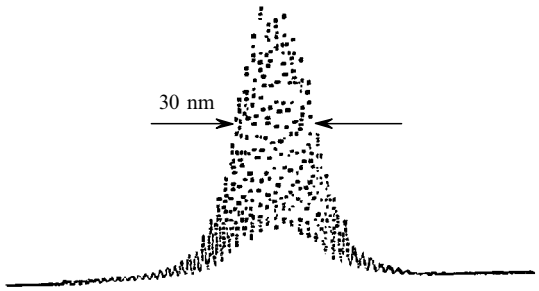

Figure 5. Experimental reflection spectrum of a photosensitive layer (the central wavelength is 0.85 μm).

Fig. 6 demonstrates the recording of the development of a number of consecutive biochemical reactions. The first of the detected reactions is bonding of streptovidin (region *1*) from its solution in the PBS buffer. Then, a surface was washed with the PBS buffer (region *2*). The cell volume in this experiment was 2.5 μlitre, the solution circulation rate was 5 μlitre min^{-1}, and the concentration of streptovidin in the PBS buffer was 10^{-6} mol litre^{-1}. The increment of the biomolecular layer thickness in the region 1 was 2.0 nm. The layer thickness did not change after rinsing with buffer.

The next reaction (region *3*) represented a selective addition of TNF-specific biotinylated antibodies. The concentration of antibodies was 10^{-7} mol litre^{-1}. Then, rinsing with buffer was performed (region *4*). At this stage, the total thickness of the added biomolecular layer (sandwich) was 4.0 nm. The third reaction consisted in the specific addition of TNF molecules from the solution at a concentration of

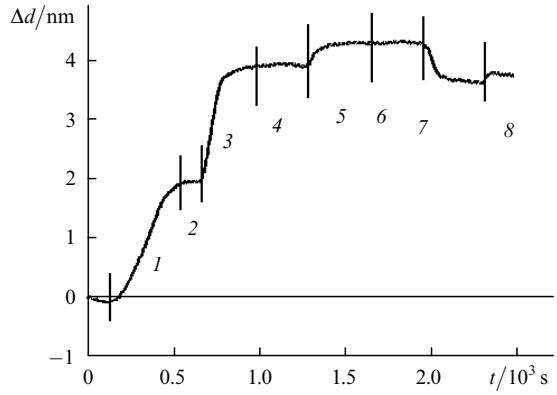

Figure 6. Sensogram of the course of consecutive biochemical reactions on the surface of a sensitive layer: (*1*) Bonding of streptavidin on a biotinylated glass surface; (*2*, *4*, *6*, *8*) rinsing with the PBS buffer; (*3*) bonding of the TNF-specific biotinylated antibodies; (*5*) bonding of TNF; (*7*) washing out of TNF in the acidic glycine buffer at pH 2.0.

10^{-6} mol litre^{-1} to antibodies (region *5*). Then, the surface was again washed with buffer (region *6*). The change in the biomolecular layer thickness caused by the TNF bonding was 0.6 nm, in good agreement with a smaller size of TNF protein molecules compared to that of antibodies.

In region *7*, TNF was washed out with the dissociation of specific bonds between antibodies and TNF in the glycine solution at a concentration of 0.1 mol litre^{-1} at pH 2.0. In region *8*, the cell was again filled with the PBS buffer solution. The sensogram in two last regions demonstrates that antibodies have remained finally on the surface, whereas TNF molecules have been washed out by the solution.

An increase in the output signal at the beginning of region 8 was reproduced from experiment to experiment and can be explained both by purely chemical and physical reasons. Thus, a change in the chemical composition and acidity (pH) of the solution can result in a change in the size of protein molecules, uncontrollable precipitation of impurities, etc. On the other hand, a more complicated model predicts that if the total thickness of added biolayers is large (of about the wavelength) and the difference between the refractive indices of biolayers and a substrate is considerable, the output signal weakly depends on the refractive index of the solution. A more detailed consideration of this question is beyond the scope of this paper.

Another series of experiments, whose typical sensogram is shown in Fig. 7, was performed in the following way. First, streptavidin was bound in a flow with the biotinylated glass surface. After rinsing with the PBS buffer, the solution of biotinylated goat antibodies specific with respect to the human immunoglobulin hIgG passed through the cell. Thus, antibodies specific to hIgG were immobilised on the surface of a sensitive element before recording of the sensogram shown in Fig. 7.

In regions *1*, *3*, and *5*, the PBS buffer solution containing 0.05 % of Twin 20 detergent passed through the cell. The solution circulation was performed at a constant rate of 15 μlitre min^{-1}. In region *2*, the immunoglobulin hIgG solution at a concentration of 1 μg mlitre^{-1} in the same buffer was added to the cell. The sensogram shows that this was accompanied by the increase in the adsorbed layer thickness by 0.75 nm. This is explained by a specific bonding reaction between hIgG and antibodies. The thickness of the adsorbed layer did not decrease after the subsequent circulation of the

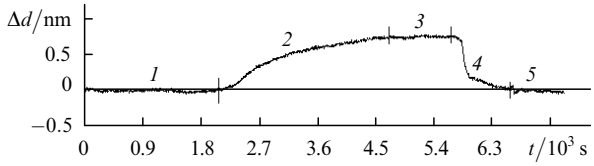

Figure 7. Sensogram of the course of adsorption and desorption of the human immunoglobulin hIgG on the surface of a sensitive layer containing the hIgG-specific biotinylated goat antibodies immobilised on the glass via the biotin-streptavidin bond: (*1*, *3*, *5*) Rinsing with the PBS buffer containing 0.05 % of Twin 20 detergent; (*2*) circulation of the solution of immunoglobulin hIgG at a concentration of 1 μg mlitre^{-1} in the same buffer at the rate 15 μlitre min^{-1}; (*4*) desorption of hIgG molecules during circulation of the buffer solution at pH 2.2 in the cell.

buffer solution through the cell (region *3*). The region *4* corresponds to the circulation of the acid buffer solution at pH 2.2. In this case, the desorption of hIgG molecules was observed. A series of control experiments with different biocomponents showed the absence of nonspecific bonding and the absence of a signal.

Thus, Fig. 7 shows the course of the reaction between protein and antibody and its reversibility. The noise level of our experimental setup (root-mean-square deviation) estimated from the sensogram was approximately 3 pm, which determines the resolution of the method. The signal drift of the setup during experiments did not exceed 15 pm h^{-1}. This drift occurs slowly and can be corrected using a special computer program or improving the experimental scheme, in particular, by employing two- or multichannel detection.

5. Conclusions

A new SPI method proposed for the real time detection and investigation of surface biochemical reactions is simpler and more reliable than other known methods. The method has the high sensitivity and resolution. In addition, the SPI method is devoid of the spurious sensitivity to drifts of the external parameters, such as the refractive index and temperature of solutions, the colour temperature of radiation sources, etc. This opens up broad possibilities for its further technical improvement, unlike other label-free methods (for example, the method based on the surface plasmon resonance), where the sensitivity threshold is mainly determined by the stabilisation of the solution temperature or the application of two- or multichannel detection schemes for the thermal drift compensation [5]. The SPI method can be successfully applied in medicine and ecology, for example, for testing samples for the presence of toxic or infection agents, detection of the DNA hybridisation, testing of pharmaceuticals, etc.

Acknowledgements. The authors thank A A Beloglazov, V M Nebusov, and S A Grachev for their help in the study. This work was partially supported by INTAS and the Russian Foundation for Basic Research.

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