REGISTRATION OF TULAREMIA BY SPR TECHNIQUE

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Abstract

Adsorption of antibodies to tularemia *Francisella tularensis* (F. name of the genus) on the surface of gold-plated glass chips and the subsequent reaction of antigen-antibody were studied by surface plasmon resonance (SPR) technique. It was found that these antibodies tend to polylayer adsorption on the gold surface. However, washing with a buffer solution leads to desorption of weakly associated molecules, and the monomolecular coating with the same parameters remains on the surface for all initial concentrations of antibody in the solution. The antibody monolayer characterized by surface protein concentration near 2.6 ng/mm^3. This value corresponds to the area of about 100 nm^2 per molecule of the immunoglobulin. This first adsorption layer adjacent to the surface irreversibly binds to it, which makes it possible to use such a system for subsequent diagnosis of tularemia microbes in solution. It was shown that rapid determination of the microbe in solution up to concentrations of 1.0 × 10^2 mcl / ml can be carried out using such biosensors using the SPR method. Based on the estimated area per molecule of the adsorbed antibody in the monolayer and the comparatively low signals of the antigen-antibody reaction, a conclusion was made about the lateral arrangement of the antibody molecules in the adsorption layer. The sensitivity of the proposed method can be improved by creating an oriented layer of antibody molecules, which promotes more effective interaction with the antigen.

Keywords: Biosensors, SPR, orientation of protein, antibodies, the adsorption of proteins, *Francisella tularensis (F. tularensis)*

1. INTRODUCTION

Recognition of antigens and antibodies is an important direction in the diagnosis of infectious diseases and specific indication of pathogenic agents in environmental objects, clinical material, food products, etc. One of the most dangerous infections for a person is tularemia. The susceptibility of people to tularemia is 100%. The causative agent of tularemia is the small bacterium *Francisella tularensis (F. tularensis),* which is characterized by high resistance in the environment, especially at low temperatures and high humidity. The spread of infection is hampered by the timely detection of its pest-spots. At present, a number of diagnostic methods are used to identify tularemia, the most famous of which are enzyme immunoassay (ELISA) and microgravimetric analysis [1-3]. These diagnostic methods are based on a highly selective biochemical "antigen-antibody" reaction. However, these methods have a number of significant drawbacks, including multistage and duration of the process, the need for additional labels, a sufficiently high concentration of antibody solutions (125 μg / ml) and antigen (10 ^{6} - 10^4 microbial cells per ml (mcl / ml))), which determine a large expenditure of expensive reagents.

One of the new promising methods for recording specific interactions is the surface plasmon resonance (SPR) method, which allows one to record with high sensitivity the binding of reagents to the sensor surface in real time and to characterize the energy of these interactions. This method is sensitive to changes occurring at distances of about 200 nm from the chip surface. During the deposition of the antibodies on the surface of the SPR sensor and subsequent interaction with the antigen, the optical properties (refractive index) of the near-surface layer change. As a result, the shift of the resonance curve of the SPR is recorded. This shift is proportional to the surface concentration of adsorbed substances that binds to the chip [4].

Obviously, the antibody molecules in the sensitive layer of a SPR chip should be properly oriented to increase the accuracy and efficiency of antigen detection. In the present work, a method for recognizing the pathogen of tularemia in a solution by the SPR method is proposed. Based on the evaluation of the surface concentration of antibodies and the efficiency of their subsequent interaction with the antigen, a conclusion is drawn about their spatial orientation in the adsorption layer.

2. MATERIALS AND METHODS

2.1. Methods of obtaining tularemia antigens and antibodies to them

Water-soluble tularemia antigens isolated complex method: water-salt extraction and disintegration of microorganisms: *F. tularensis Miura, F. tularensis 890-Az, F. tularensis 543/6, F. tularensis 503/840, F. tularensis 15 NIIEG,* grown in egg yolk medium Mac-Coy for 24 hours at 370 C and inactivated with chloroform. The extraction of antigenic complexes was performed in 2.5 % solution of sodium chloride. Disintegration was performed under high pressure in the X-press (Sweden) and ultrasonic method on the apparatus of UZDN - 2T (Russia) at a frequency of 22 kHz for 10 min. The degree of destruction of microbial cells was judged by the change in optical density, the amount of protein in the supernatant fluids and microscopically.

The used tularemia antiserum obtained by immunization of rabbits according to the scheme developed by I. S. Tyumentsevoj and E. N. Afanasiev with the immunomodulator feracrylum [5]. For isolation of immunoglobulins used method for the fractionation of protein mixtures using caprylic acid. In the obtained serum of more than 90% were immunoglobulin (Ig) class G.

2.2. The study of adsorption of antibodies on the gold surface of the SPR method

Adsorption of proteins on the surface of gold-coated glass slides was studied by the surface plasmon resonance technique using a Biosuplar 6 (Mivitec, Germany) SPR spectrometer equipped with a flat two-chamber flow cell (each chamber size was about 1 × 0.5 × 0.1 cm^3). Before carrying out the measurements, the surface of the SPR chips was thoroughly cleaned by ethanol in ultrasonic bath and washed with twice-distilled water. During the study, the solutions were pumped through both chambers of the measuring cell using a two-channel peristaltic pump (BT100-1F, LONGER Precision Pump, PRC) at a rate of 960 µl/min. A solution of phosphate buffered saline (PBS) (pH = 7.4) was passed through the cell until a stable signal was established. After this, a protein solution in the same buffer was introduced. Its adsorption on the chip surface was accompanied by a change in the optical parameters of the surface layer of the liquid, which led to an increase in the signal of the SPR spectrometer, measured in conventional units (CU). Solutions of antibodies and microbial cells were passed to the outlet for the first 1.5-2 minutes to establish the necessary concentration in the measuring cell. After that, the flow of liquid through the cell was circled. To study the reversibility of the protein-surface interaction the kinetics of protein desorption was studied in response to washing the system with the initial buffer solution. All measurements were carried out in a thermostatable box at a temperature of 25 ° C.

The surface concentration of the adsorbed proteins was estimated from the value of the saturated adsorption SPR signal. It is known that this value correlate linearly with the mass per unit area of the adsorbed protein [10] and for most proteins the change in signal for 1 RU for SPR spectrometer Biocore T100 corresponds to

adsorption of 1 pg/mm^2. We compared the responses of Biosuplar 6 and Biocore T100 devices to the same series of solutions of potassium chloride with different optical densities. It was found that the signal 1 CU on Biosuplar 6 corresponds to the signal 6.5 RU on Biocore T100. Thus, it was found that the value 1 CU on the Biosuplar 6 device corresponds to the adsorption of approximately 6.5 pg/mm^2 of protein.

3. RESULTS AND DISCUSSION\

To obtain a biosensor sensitive to *F. tularensis*, we investigated the binding of antibodies to this microbe to the surface of a gold-plated SPR chip in the concentration range from 5 μg/ml to 125 μg/ml (**Figure 1**). The maximum concentration of antibodies in this range (125 μg / ml) is typical for the determination of tularemia by enzyme-linked immunosorbent assay and microgravimetric analysis [1-3]. With such a high concentration of antibodies, adsorption on the surface of the PPR chip occurred most intensively: 400 CU in less than 20 minutes (**Figure 1**, curve 1). For lower concentrations, similar values of the adsorption signal were achieved for a longer incubation period (**Figure 1**, curves 2 and 3). Interestingly, almost never, even after 1 hour or more, the signal did not reach saturation. The growth of the adsorption signal did not stop. In addition, when washed with PBS buffer, the partial desorption of antibodies always ends when the PPR signal achieved approximately the same level near 390 CU. Accordingly, in relatively dilute protein solutions, desorption was practically not observed. Both these facts point to the tendency of antibodies to form a multilayer coating, which leaves after washing an irreversibly adsorbed monolayer on the surface. The monolayer is the same for all initial conditions (protein concentrations) of adsorption. Indeed, in multilayer coatings, the first adsorbed monolayer is usually most strongly bound to the surface, and subsequent layers are adsorbed reversibly [6,8]. Irreversible monomolecular adsorption of the antibodies makes it possible to use the resulting modified surface to recognize tularemia microbes in a solution based on the antigen-antibody reaction.

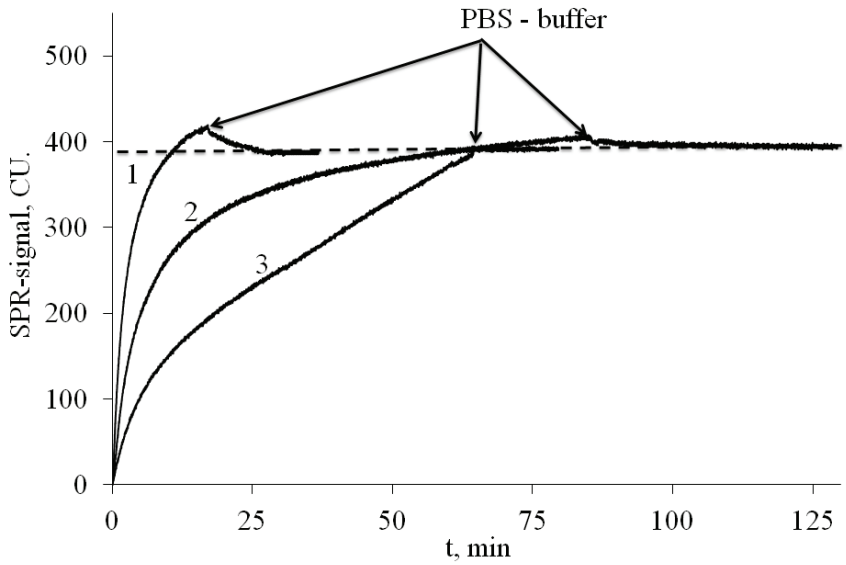

Figure 1 Kinetics of adsorption of antibodies on the surface of the SPR chip and subsequent desorption in response to flushing the buffer solution depending on their concentration. The dotted line denotes the level of the end of the desorption of antibodies from the surface of the SPR chip. The concentration of immunoglobulin IgG (antibody): curve 1 - 125 μg/ml, curve 2 - 42 μg/ml, curve 3 - 5 μg/ml.

Antibodies to tularemia are class G immunoglobulins. Their molecules with a mass 150 kDa are Y shape monomers with dimensions of 14.3x7.7x4.0 nm^3. These molecules consist of two light and two heavy chains that are pairwise connected by disulfide bonds (-S-S-) (**Figure 2**). Between heavy chains there is also a

disulfide bond. This is a "hinge region". This type of interpeptide conjunction gives the structure of the molecule dynamism, which makes it easy to change the conformation depending on the environment [7].

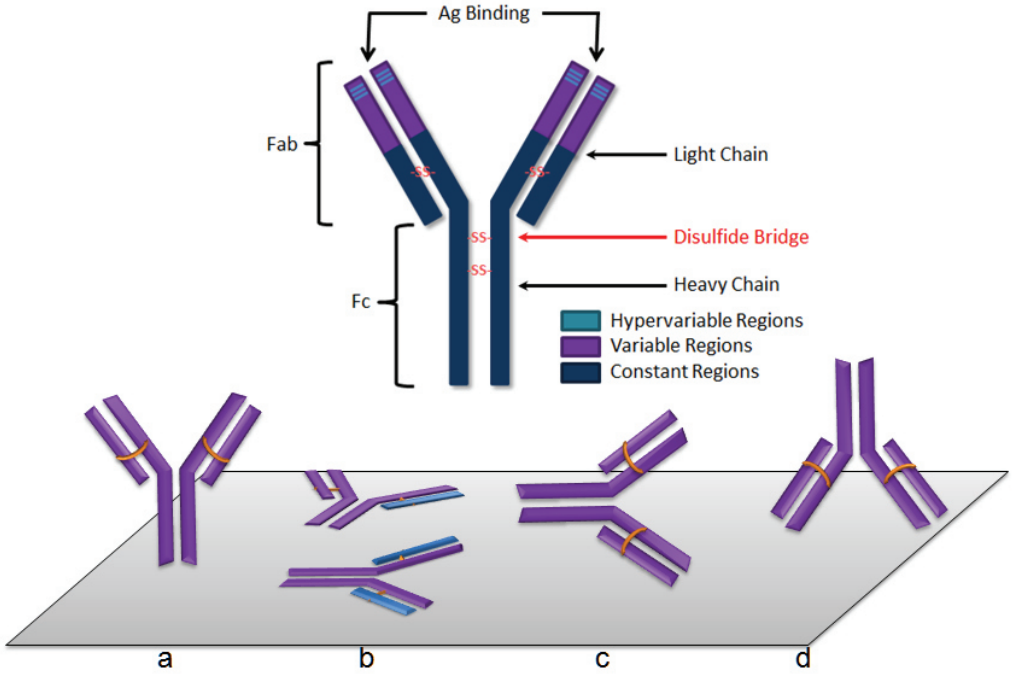

Figure 2 Schematic depiction of the IgG molecule and various variants of their location on the surface of the PPR chip: a, d - orthogonal, b - lateral arrangement of the molecule, c - "on the lateral sides", intermediate position

Immunoglobulin IgG molecules can be oriented in different ways on the surface when binding to it. Examples of their different arrangement are shown in **Figure 2** [7]. The most effective orientation for the antigen recognition is the orthogonal arrangement of the molecule, corresponding to case (a). With this orientation, the antigen-binding centres of the immunoglobulin are directed to the solution and are most accessible for interaction with the antigen. However, the binding with the surface in this case, obviously, should be weaker because of the small contact area. According to simple geometric estimations, one immunoglobulin molecule with this orientation should occupy near 30 nm^2 of the surface area. The same area will be occupied by one IgG molecule with a similar but inverted orientation (case (d)). In case (b), with the lateral arrangement of the molecule with the maximum interaction with the surface, the surface area per molecule will be maximal and will be near 110 nm^2. In the intermediate case (c) each adsorbed molecule will occupy near 60 nm^2.

Recalculation of the SPR signal corresponding to the monomolecular layer of antibodies (390 CE), allowed us to estimate surface concentration of the antibodies. The corresponding procedure is described above. This concentration was about 2.6 ng/mm^2. According to the molecular weight of this protein, this value corresponds to approximately ~ 10^{10} $molecules/mm^2$. In other words each antibody molecule occupies an area of about 100 nm^2 on the surface, which is close to the above estimate area for the lateral arrangement of molecules on the surface. According to the above estimates, with the maximum dense lateral placement, each antibody molecule should occupy 110 nm^2 of the surface area. Obviously, such an ideal dense coating of the sensor surface is hardly possible. Probably the results indicate that some of the antibody molecules are not located laterally and are therefore more accessible for an effective interaction with the antigen.

The following experiments confirmed the assumption of a relatively low activity of adsorbed antibodies as a result of their predominantly lateral orientation. In **Figure 3** shows a sensorogram reflecting the kinetics of the processes occurring during sequential adsorption of an immunoglobulin at a concentration of 5 μg/ml on the

surface of a SPR chip, washing out weakly bound molecules, and then introducing a sample of *F. tularensis* antigen.

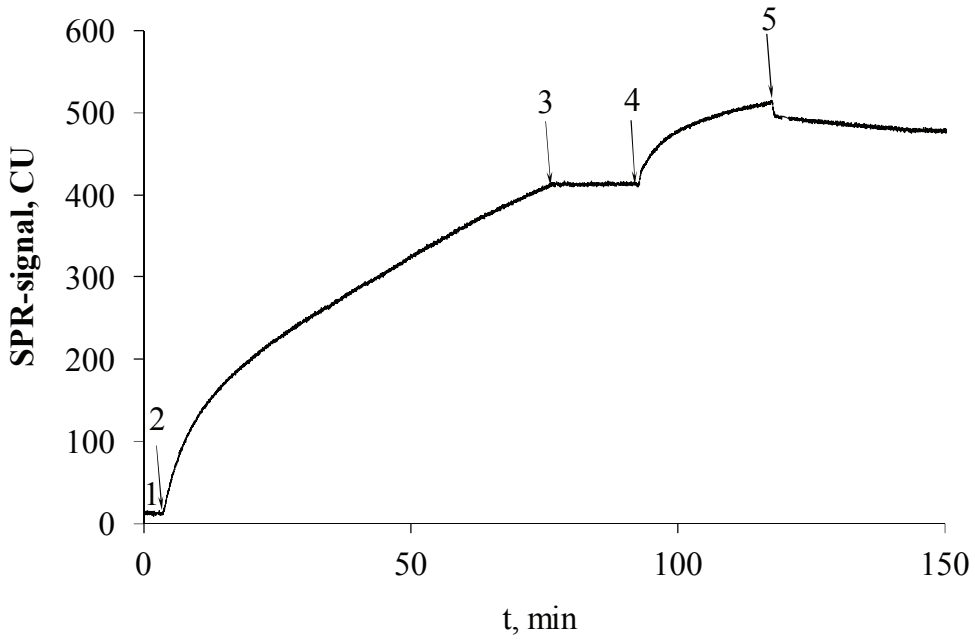

Figure 3 SPR sensogram sequential adsorption of antibodies and antigen. The numbers are shown: 1 - the baseline PBS-buffer, 2 - the introduction of IgG (antibodies) with a concentration of 5 μg/ml, 3, 5 - washing PBS-buffer, 4 - introduction of *F. tularensis* antigen in PBS buffer with a concentration of 10^2 mcl./ml

As in the experiments described above, after reaching a sufficiently high level of antibody adsorption on the surface of the SPR chip, the reversibly bound molecules were removed by pumping through the PBS buffer in the liquid circulation regime. The washing ended when the stable level was reached on the SPR sensogram. When a sample of F. tularensis antigen was injected at a concentration of 10^4 mcl / ml in PBS buffer, signal growth of SPR was observed, which indicate an "antigen-antibody" reaction. However, despite the considerable mass and size of the causative agent *F. tularensis* (0.2-0.7 x 0.7-1.7 μm) and its rather high concentration, the increase in the SPR signal was much less intense than during antibodies adsorption. Such a relatively weak signal may be due to the ineffective predominantly lateral orientation of the antibody molecules.

4. CONCLUSION

Thus, we have shown that solutions of antibodies with a concentration of 5 μg/ml can be used to create sensitive sensors for the determination of tularemia by the SPR method. The sensitivity of the received sensors allows rapid recognition of the microbe in solution up to concentrations of 1.0×10^2 mcl/ml. Predominantly lateral orientation of antibodies on the surface does not allow obtaining sufficiently high signals of antigen binding. The sensitivity of the proposed method can be increased by optimizing the conditions for adsorption of antibodies that promote their more efficient orthogonal orientation on the surface. More optimal orientation with greater availability of the antigen-active end of the immunoglobulin molecule can be achieved by improving the technique of their binding to the surface of the SPR chip. In particular, this result can be achieved by increasing the surface roughness, which helps to minimize the contact of the active regions of antibodies with the surface [9].

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