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OPTIMIZATION OF OPTICAL IMMUNE BIOSENSOR WORK AT THE EXPRESS DETERMINATION OF THE INSULIN SPECIFIC ANTIBODIES

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ABSTRACT

Summary: The detection of individual autoantibodies, including insulin-specific ones, in the blood of patients is essential at the diagnosis and treatment of diabetes. The existing traditional methods allow for the necessary analysis, but they require considerable time for its implementation, the availability of specialized stationary laboratories and are quite expensive. An alternative solution is to use analytical tools based on biosensor principles. This article presents the results of studies on the optimization of the work of the immune biosensor based on the surface plasmon resonance (SPR) at the analysis of anti-insulin immunoglobulin's (Ig). It has been found that the preliminary preparation of the transducer surface of the SPR with a monolayer of such polyelectrolyte as polyallylamine (PAA) hydrochloride is the best option. Because the sensitivity and specificity of the biosensor SPR is quite high when used on the transducer surface of an insulin monolayer at the identification of the diabetic patients, there is no need to form its branched layers, despite the fact that this significantly increases the sensitivity of the assay. The concentration of insulin to be immobilized on the surface of the transducer should be in the range of 250-500 μg/ml. The simplest and most appropriate for practice is the using assay that involves the direct interaction of insulin with specific serum antibodies. To avoid nonspecific reactions at the determination ot anti-insulin Ig the dilution of serum blood should be 10-100 times.

KAYWORDS: Diabetes, anti-insulin antibodies, biosensor, analysis optimization.

1. INTRODUCTION

Today, there are essentially two types of diabetes: insulin-dependent and insulin-independent, or type 1 and type 2 diabetes.[1, 2] The first is characterized by absolute or relative insulin deficiency, which is caused by an autoimmune process, which is accompanied by the progressive and selective damage of the pancreatic beta cells in the corresponding group of people. Moreover, the autoimmune process leading to diabetes begins very well in advance of the clinical signs. In the blood of patients, the titer of various auto antibodies to the islet cells, to insulin and to the glutamate decarboxylase can be detected.

Type 2 diabetes is a group of the heterogeneous carbohydrate metabolism disorders. There is a possibility that both the gene responsible for the insulin secretion and the gene contributing to the insulin resistance are involved in the pathogenetic process. A common defect in the glucose recognition system of the B cells or the peripheral tissues is likely, resulting in a decrease in the glucose transport or a decrease in the glucose-stimulating response of these cells. The risk of developing type 2 diabetes is dramatically increased if the parents or close relatives have such defect, as well as in obesity. Considering the phenomenon of the insulin resistance in type 2 diabetes, there is should pay attention to a group of substances called - insulin antagonists: contraceptive hormones (growth hormone, cortisol, thyrotropin, placental lactogen, prolactin, glucagon, antibodies to insulin receptors and some others.[3-5]

The presence of the insulin antibodies clearly indicates on the development of autoimmune disorders in the body. Therefore, the problem of the identification and quantitative determination of the specific antibodies is the great scientific and practical importance, because it allows to study the mechanism of development of various pathological forms of the pancreas, the knowledge of which will allow to individuate and improve the effectiveness of the insulin therapy. The presence of antibodies to insulin can interfere with the determination of its level in the blood and affect the results of glycemic control, prolonging the period of disintegration of this active agent.

There is needed dynamic monitoring of the concentration of this type of antibody in diabetic patients receiving various insulin preparations. An insulin antibody detection test can also be used to identify the risk groups for I and II types of diabetes. It should be noted that in most cases there is a need to use express (sometimes even result should be obtained immediately), highly sensitive and strictly specific methods. The last two requirements meet the classical methods of modern immuno-chemical analysis, such as radio-immune (RIA) and immuno-

enzyme (ELISA) methods. The last has been used to determine antibodies to glutamic acid decarboxylase.[6] Its sensitivity reached 20 ng/ml. However, speed of analysis of both the above mentioned methods is insufficient. To achive, it is only possible to provide this by the immuno-biosensor analysis, which has several advantages over other ones varieties, as it is simultaneously sensitive, simple, express, cheap and can be performed directly in the patient's bed.

There is information on the use of an ellipsometry-based immune biosensor to determine insulin concentration in patients's.[7] Such a biosensor is capable of controlling insulin levels in the range of 10 ng/ml to 100 µg/ml.

The main purpose of this work is to develop a new and simple immuno-chemical approach for testing antibodies against insulin based on the modern biosensoric principles, namely the effect of the surface plasmon resonance (SPR). The implementation of such an approach will allow specialists in the field of diabetes to implement a fundamentally new strategy for the diagnosis and study of the pathogenetic mechanisms of diabete, which is considered to be "the number one problem" in the endocrinology.

2. MATERIAL AND METHODS

The studies were performed on a "Plasmon SPR-4M" device developed at the Institute of Semiconductor Physics of the National Academy of Sciences of Ukraine. The principle of its work has been discussed in detail earlier.[8-13] The converter of the immune biosensor in the form of a glass plate with successively filled layers of chromium (3 nm) and gold (20 nm) was combined with the prism of the optical device by means of an the immersion fluid (polyphenyl ether) with a refractive index of 1.6.

Immunoglobulin G (IgG) and insulin (both from Sigma, USA) were used as the antigen. In the experiments it was used a solution of antigen at a concentration of 1 mg/ml in 1 mM Tris-HCl buffer, pH 8.2, containing 0.14 M sodium chloride (bufered physiological solution - BPhS). A 1% solution of the bovine serum albumin (BSA) obtained from Sigma was prepared using BPhS.

Dodecanthiol and polyelectrolytes (all from Sigma, USA) were used to modify the transducer surface. Chemical sorption of dodecanthiol was carried out by immersing a freshly prepared gold film at room temperature for 15 hours, after which the surface was washed with ethanol

and dried in a stream of pure air. The polyelectrolyte insoluble film on the gold surface of the glass plate was formed using two oppositive charged polymers: polyallylamine (PAA) hydrochloride (polycation) and (PSS) polystyrenesulfonate (polyanion). The glass plates were immersed in an aqueous solution of polyelectrolytes in the following sequence: PAA - PSS - PAA, resulting in a positive charge on the surface of the transducer. Polyelectrolytes were used at a concentration of 2 mg/ml at pH 2 (PAA) and pH 8 (PSS). Exposure time for each solution is 30 minutes at room temperature. The surface was washed each time with the distilled water.

Testing serum for the presence of the antibodies specific to insulin or IgG was performed as follows. Initially, the resonance angle was recorded when the distilled water was introduced into the measuring cell (10 µl volume). A solution of the specific antigen was then introduced into it, kept there for 20 min at room temperature. Then, measuring cell was washed with the BPhS, to remove excess antigen that was not adsorbed on the surface, and recorded a shift of the resonance angle. To prevent further nonspecific binding of the serum components on the transducer surface, a 1% BSA solution was introduced into the cell, kept there for 10 min. After that the measuring cell was washed with the BPhS and the instrument readings were recorded. Next, the measuring cell was filled with a solution of anti serum, sequentially reducing the dilution level from 1: 100 to 1: 200. The incubation time for each sample was about 10 min. Each time, the measuring cell was washed with a buffer containing 0.1% Tween-20 in the BPhS to remove unbound items and then recorded the immune sensor response.

3. RESULTS AND DISCUSSION

3.1. Developing effective insulin immobilization methods on the surface of a biosensor transducer

An important requirement for the biosensor is the stability and reproducibility of the results. Therefore, one of the main tasks in creating any type of biosensor, especially immune, is to ensure effective standardized immobilization of the selective biological material and optimize the functional parameters of the device.

Protein molecules can be adsorbed on the metal and silicon surfaces, but the efficiency of such a process is often not high enough, on the one hand, and on the other, active centers can be blocked during sorption, resulting in a significant loss of specific interactions on the transducer surface. In this case, the level of the nonspecific interactions may remain

unchanged, or even increase, and then the signal-background ratio will increase sharply, and the sensitivity of the immune biosensor will sharply decrease. To avoid these problems, the surface of the transducer, as mentioned above, is pre-treated with thiols or polyelectrolytes, which contributes to the standardization of its condition.[10-11] In addition, apply the intermediate layers of other molecules that help to the representation of the selective sites of the sensitive biological structures in the direction of a solution. In particular such structures are protein A of *Staphylococcus aureus*, or the corresponding lectins that interact with Fc fragments of the immunoglobulins, releasing their fragments for antigen binding.[12]

It is proved that at the modification of the transducer surface by thiols and polyelectrolytes the adsorption of the antigen is stable over time and is not destroyed when washing the measuring cell by the BPhS. Immobilization of the antigen is accompanied by a change in the resonance angle. In the case of polyelectrolytes, the amount of the adsorbed antigen was slightly larger, and the response of the biosensor was more stable and reproducible compared to the uncovered surface. The use of the dodecanethiol also helps to stabilize the immobilized protein layer and also increases the density of the antigens (both IgG and insulin) on the surface (Table).

Subsequent surface treatment with a 1% BSA solution showed no significant changes in the refractive angle. This means that the number of the free binding sites was minimal and the concentration of the antigen (insulin) optimal for the creation of a dense layer.

Table. The magnitude of the response of the PPR immune biosensor on the introduction of insulin into the measuring cell with the different types of the transducer surfaces.

Type of converter surface	Deviation of resonant angle, angle/sec
Crude	620±20
Processed by: polyelectrolytes	890±34
dodecanthiol	750±30

The number of the physically adsorbed biological molecules is limited by the surface area of the transducer, which is the subject to the optical registration. This limitation can be prevented by using compounds that are capable of forming branched structures on the surface and thus ensuring the location of the biological material in three-dimensional space. Thus, the amount of immobilized material can be increased, and thus further able to increase the sensitivity of the immune biosensor. Therefore, another scheme for the use of

polyelectrolyte's was proposed when polycations and polyanions were used not to cover the surface but to form a double branched layer of biomolecules.

In the special experiments the measuring cell at firstly was filled by distillated water and then its was washed by the solution of polyelectrolytes in the sequence PAA - PSS - PAA. The concentration of each polyelectrolyte was 1 mg/ml in distilled water, the incubation time was about 20 min. Then the rabbit antibodies to human Ig at the same concentration were introduced into the measuring cell, kept for 20 min and washed with by the BPhS. Then 0.5% glutaraldehyde was added to the meassuring cell. After incubation for 10 min, the polyelectrolytes were removed with 0.05 M HCl buffer, changing the pH from 4.0 to 8.0 and destroying the electrostatic bonds between IgG and polyelectrolytes. Thus, a double layer of antibodies is formed on the surface of the transducer due to covalent bonds between IgG molecules formed by glutaraldehyde. Then a solution of human IgG was added to the measuring cell in the concentration range from 1 ng/ml to 100 μg/ml in the BPhS and the immunosensor response was recorded. The displacement of the resonance angle was proportional to the concentration of human IgG in the solution.

It is shown that the preparation of the sensitive surface in this way can significantly increase the sensitivity, which in this case was less then 0,5 ng/ml, and the linear segment of the graph of the dependence of the SPR signal on the amount of human IgG in the solution is in the concentration range 0,5 - 200 ng/ml. The linear plot of the sensitivity of the immunosensor using a monolayer of antibodies was within 5 - 100 ng/ml (Fig. 1). Thus, it was found that the formation on the surface of the transducer multilayer (three-dimensional) branched structure of antibodies using polyelectrolytes and glutaraldehyde can significantly increase the sensitivity of the SPR immunosensor.

A similar scheme was used to immobilize insulin. It has been found that the formation of a double layer of insulin on the surface of the transducer significantly increases the sensitivity of the immune biosensor to specific antibodies. This is especially important in the study in the early stages of the disease, when the titer of specific antibodies is low. However, such complication of the process of testing serum samples in the development of immunosensory analysis is not advisable, since the main advantage of biosensor analysis is its expressivity That is, the main purpose of the work is to create a simple algorithm for screening patients with diabetes.

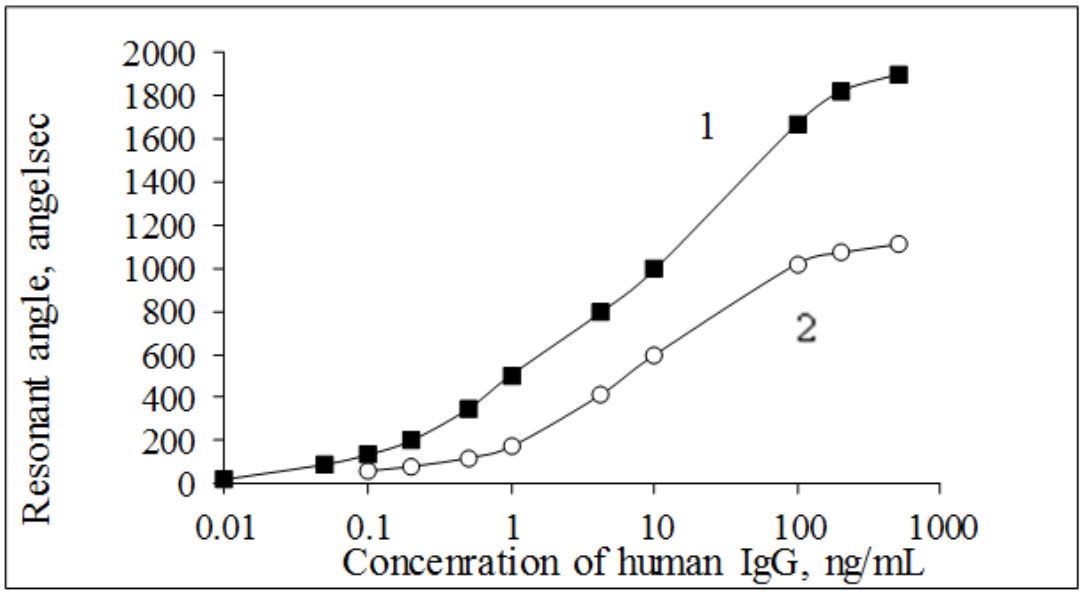

Fig.1: The response of the SPR biosensor to the introduction of a human IgG solution in the concentration range of 10 - 500 ng/ml using different variants of the sensitive surface: 1 - double layer of antibodies created by polyelectrolytes; 2 is a mono layer from one of them (PAA).

3.2. Elaboration of the basic analysis algorithm: selection of optimal concentrations of components of the immunochemical reaction.

Earlier[13-15] we established that for the detecting low molecular weight compounds it is necessary to develop specific algorithms of analysis, such as: "competitive", when the immobilized compound competes for binding sites on selective molecules with the one in the free state, or vice versa, when one of them is in the free state and other one is in the conjugated state with another high molecular weight substance. Both of them compete for binding sites in the antibodies immobilized on the transducer surface. Another method of analysis is related to such an algorithm of its performing, when the immobilized antibodies interact firstly with a low-molecular substance and then with its conjugate with the some high-molecular compounds. It is so-called "saturation" algorithm. Finally, the simplest analysis algorithm is when the antigen or antibodies are immobilized on the surface of the transducer and they interact directly with the corresponding structures that are in the controllable solution.

We conducted studies to determine the most optimal algorithm for analyzing the level of insulin-specific antibodies in the blood of patients with diabetes. According to the results obtained, it is found that the most acceptable is the last of the above algorithms, namely, the

direct determination of anti-insulin antibodies when insulin is immobilized on the surface of the optical transducer. It was found that the optimal concentrations of insulin for its immobilization on the surface of the transducer are 250-500 µg/ml (Fig. 2).

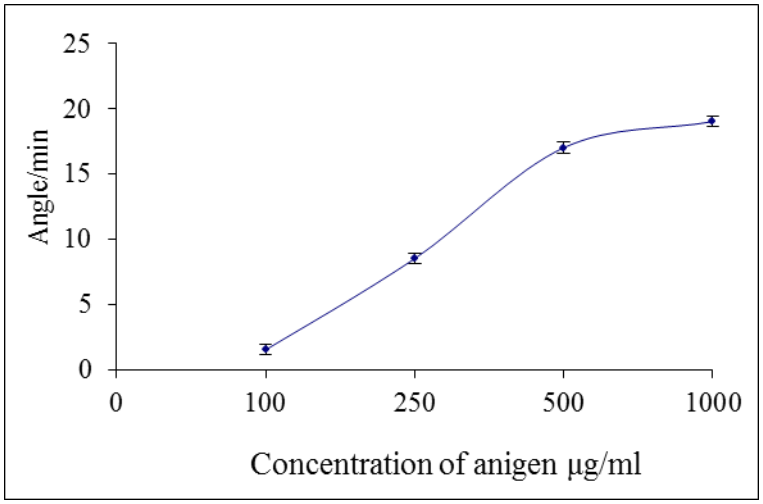

Fig. 2: The magnitude of the shift of the resonant angle of the immune biosensor at the different concentrations of antigen taken for immobilization on the surface of the transducer.

3.3. Sample preparation.

Taking into attention that the analyzed substance will be blood there is very important to know it is possible to take whole blood or only plasma must be used. Given that the analysed sample will be in the form of blood, it is very important to know whether it is possible to take a whole blood or use plasma only. It turned out that whole blood can be used to determine the level of autoantibodies to insulin, although the presence of erythrocytes in it, especially due to their spontaneous hemolysis, significantly reduces the sensitivity of the analysis. Therefore, the use of a whole blood sample for analysis may be in the case of fast screening investigation. However, it is more appropriate to perform analysis with blood samples after pre-precipitation of erythrocytes and dilution of plasma not less than 10 and not more than 100 times by the BPhS.

4. CONCLUSION

The main conditions of the work of the immune biosensor based on the SPR at the determination of anti-insulin Ig level at the diabetes were characterized. It was stated that the immobilization of selective biological material on the surface of the SPR converter, pre-coated with various chemical agents (thiols, polyelectrolytes), was provided the formation of

the functionally stable unified sensing elements. The use of polyelectrolytes for surface modification of the transducer of an immune biosensor based on SPR is the most expedient, inexpensive and simple. Because the sensitivity and specificity of the biosensor SPR is quite high when used on the transducer surface of insulin monolayer at the identification of the diabetic patients, there is no need to form its branched layers, despite the fact that this significantly increases the sensitivity of the assay.

The concentrations of insulin for its immobilization on the surface of the transducer should be in frame of 250-500 µg/ml. To avoid nonspecific reactions at the determination to anti-insulin Ig the dilution of serum blood should be 10-100. The simplest and most appropriate for practice is the using assay that involves the direct interaction of insulin with specific serum antibodies.

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