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Study on interactions of human IgG with immobilized anti-IgG or recombinant Staphylococcal protein A using surface plasmon resonance spectrometry

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Aim. Comparison of the IgG-binding activity of recombinant Staphylococcal protein A with introduced C-terminal cysteine residue (SPA-Cys) or goat anti-human IgG antibodies (anti-IgG) after their immobilization on a gold sensor surface of surface plasmon resonance (SPR) spectrometer. **Methods.** SPA-Cys or anti-IgG were immobilized on a gold sensor surface to form two variants of a bioselective element of the immunosensor. SPR spectrometry was used for the detection of IgG-binding activity of the immobilized proteins. **Results.** The SPR sensor response to the immobilization of anti-IgG was more than two times higher than that to the immobilization of SPA-Cys. However, there is almost the double advantage for SPA-Cys in the number of immobilized molecules. Moreover, the bioselective element of the immunosensor based on SPA-Cys showed a much better capability of binding IgG than the bioselective element based on anti-IgG. **Conclusions.** Comparison of immobilization of SPA-Cys or anti-IgG on the sensor surface of SPR spectrometer, and the interactions of immobilized proteins with human IgG demonstrated obvious advantages of SPA-Cys.

Keywords: antibodies, recombinant Staphylococcal protein A, protein immobilization, immunosensor, surface plasmon resonance.

Introduction

The successful development of approaches and analytical tools of biotechnology will enable to upgrade substantially the quality of life by improving the methods of biomedical diagnostics, molecular engineering, environmental monitoring, food analysis, drug discovery, *etc.* For this purpose, in many cases, the biosensors could be helpful. Their design feature is the combination of a bioselective element that specifically reacts with an analyte and a physical transducer that transforms the result of biological process into the electrical signal suitable for further processing and characterization [1, 2]. The use of variety of immune components is very attractive in this area of research. The immunosensors demonstrate high selectivity through the use of immune molecules, simple operation, simple sample preparation and high

sensitivity [3]. Furthermore, the biosensors based on the phenomenon of surface plasmon resonance (SPR) are applied to the label-free and real time detection of various intermolecular interactions. SPR spectrometer registers the changes of the refractive index of a thin layer (~ 200 nm) of medium, adjacent to the sensor surface. Immobilization of biomacromolecules and interaction of immobilized components with their partner molecules alter the refractive index of this layer and cause the SPR response [4]. An important step to achieve high efficiency of the immunosensor is the preservation of the functional activity of immune components during their immobilization on the sensor surface. However, during the immobilization of antibodies on the surface of physical transducers their antigen-binding activity is usually much lower in comparison with the activity of the same antibodies in solution. The main reasons for this are believed to be random orientation of antibodies on the sensor surface and steric hindrance caused by the influence of the surface of a solid substrate [5]. To prevent this, it is necessary to create an intermediate layer, which would include the immunoglobulin-binding proteins, such as Staphylococcal protein A (SPA). It is known that SPA selectively binds Fc-fragment of antibody, leaving available antigen-binding sites [6]. The immunoglobulin-binding region of SPA does not contain cysteine residues [7, 8], so the immobilization of SPA is possible only by physical sorption on the sensor surface, which is not always reliable. Genetically engineered introduction into the recombinant SPA of the cysteine residue, which interacts with the gold sensor surface through exposed SH-group, improves the reliability of SPA immobilization preserving its functional properties. In the Institute of Molecular Biology and Genetics NAS of Ukraine, the original genetically engineered construct was created and the recombinant Staphylococcal protein A with the introduced C-terminal cysteine residue (SPA-Cys) was obtained [9].

Species-specific antibodies, such as goat anti-human IgG antibodies (anti-IgG), are more traditional immunoglobulin-binding proteins, which could also be immobilized on a sensor surface as an intermediate layer for further immobilization of IgG.

The aim of the present work is the comparison of IgG-binding activity of SPA-Cys or goat anti-human IgG antibodies (anti-IgG) after their immobilization on a gold sensor surface of the SPR spectrometer.

Materials and Methods

NaCl, KH_2PO_4, SDS, Bromophenol Blue and goat anti-human IgG antibodies were purchased from "Sigma" (USA), human IgG – from "Serotec" (USA), Na_2HPO_4 and glycerol – from "Applichem" (Germany), Coomassie Brilliant Blue R250 and Unstained Protein Molecular Weight Marker SM0431 – from "Fermentas" (Lithuania), Tris-HCl buffer solution (pH 8.0) – from "Helicon" (Russia), milk proteins (Milk Powder "Fluka", Switzerland), other reagents and solvents were obtained from "UkrOrgSyntez" (Ukraine). Sodium phosphate saline buffer solution (PBS), which includes 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4, citrate saline buffer solution (SSC), which includes 15 mM sodium citrate and 150 mM NaCl, pH 7.2, and 100 mM carbonate buffer solution (CB), pH 9.5 were used.

Human IgG was obtained also by affinity chromatography using SPA-Cys-modified silica as described in [9]. The synthesis and purification of recombinant SPA-Cys were described in [9]. The homogeneity of the proteins used was checked by 13% SDS-PAGE.

The SPR spectrometer ("Plasmon SPR-4m") was used to study the protein-protein interactions (the device and corresponding software have been developed at the Lashkaryov Institute of Semiconductor Physics NAS of Ukraine). This computer controlled optoelectronic spectrometer uses the optical phenomenon of SPR in Kretschmann configuration. A thin layer (50 nm) of gold, which was deposited on a glass plate, is used as a sensitive element of the SPR spectrometer. Just before the experiment, the gold surface of the glass plate was purified by incubation in a mixture of "piranha" (a mixture of 30% H_2O_2 and concentrated H_2SO_4 in 1:3 ratio; WARNING:

"piranha" reacts violently with organic compounds and must be handled with extreme care) for 2 minutes. Then the plate repeatedly washed with water and dried in air. The treated glass plate is mounted on the prism of the device using the immersion liquid that has the same refraction index as the prism and the glass plate. The gold surface serves as a bottom of a flow measuring cell (~20 μl). A silicone rubber ring serves as the side walls. A plexiglass cover contains input and output pipes, which the buffer solution and investigated samples pass through [10]. The flow rate of liquid (usually 40 μl/min) is controlled by peristaltic pump «Ismatec» (Switzerland).

At the beginning of the experiment the measuring cell was washed with the working buffer solution (PBS, in some cases – CB or SSC), to obtain a stable signal of the device – the baseline. For immobilization of the proteins 120 μl of 1 μM protein solution in PBS, CB or SSC was injected into the measuring cell. After 25 min-incubation of the sample (the pump was switched off) a flow of the working buffer solution was used to remove the excess of the proteins that were not immobilized.

To prevent non-specific adsorption of studied components on the surface sites, which are left uncovered with the immobilized protein, a sensor surface was passivated with milk proteins. For this purpose, 120 μl of 200 μg/ml milk proteins in the working buffer solution (PBS) were injected into the measuring cell, then incubated at the pump switched off for 20 min, after that the surface was washed with PBS until stabilization of the sensor signal [11].

On the main stage of the experiment 120 μl of solutions of different concentrations of IgG in PBS were injected into measuring cell, incubated at the pump switched off for 10 min, then the cell was washed with PBS until stabilization of the sensor signal.

For regeneration of a bioselective element (destruction of bonds between the immobilized protein and IgG as well as a removal of the latter), 120 μl 40 mM citrate buffer (pH 2.5) was injected into the measuring cell, then the cell was washed with PBS until stabilization of the sensor signal [12].

Results and Discussion

In this paper the following proteins were investigated: recombinant Staphylococcal protein A with the introduced C-terminal cysteine residue (SPA-Cys), goat antibodies against human immunoglobulin (anti-IgG), human IgG obtained from "Serotec", and human IgG obtained by affinity sorbent. All of them are represented on the electrophoregram after electrophoresis in 13 % SDS-PAG (Fig. 1). The only one band, which moved a little faster than a marker protein of 35 kDa, is clearly seen on the lane 1. This is consistent with the mobility of SPA-Cys (34.5 kDa). All preparations of antibodies have two major bands corresponding to heavy and light chains of IgG. The presence of additional minor band (~ 33 kDa) in the "Serotec" preparation of human IgG can be explained by the presence of some degradation products or residual serum proteins.

Fig. 2 shows the SPR sensogram representing the immobilization of SPA-Cys on the gold sensor surface. After prolonged washing of the measuring cell by PBS and stabilization of sensor signal the difference between the signal before injection of 120μl of 1 μM SPA-Cys and after washing with PBS was almost 0,14 angular degrees (Fig. 2). This value represents the number of reliably immobilized SPA-Cys

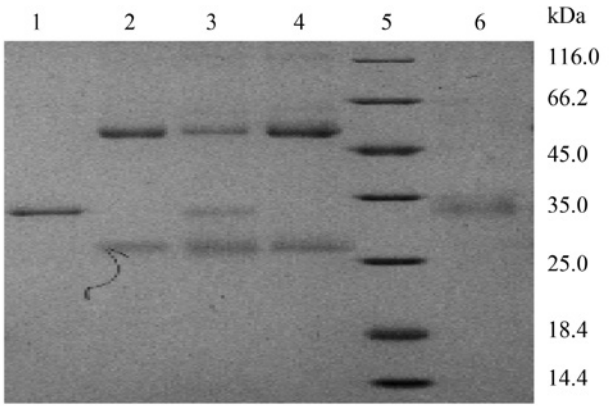

Fig. 1. The electrophoregram of the proteins in 13 % SDS-PAG. 1 – SPA-Cys, 2 – anti-IgG, 3 – "Serotec" human IgG, 4 – human IgG obtained by affinity sorbent (used for comparison), 5 – a set of molecular weight markers, 6 – milk proteins. The amount of a protein, which was injected into each lane, is ~ 1 μg.

molecules. According to the conversion factor of SPR response into the value of the surface density of immobilized protein [13], it is 1.35 ng/mm^2 (Table). Given the molecular weight of SPA-Cys (34.5 kDa), one can calculate that on average about 2.4 molecules of SPA-Cys were immobilized for every 100 nm^2. It does not indicate the formation of a dense monolayer of proteins on the sensor surface. So, after the immobilization of SPA-Cys the free sites for the non-specific sorption on the gold surface were blocked with the injection of a solution of 200 μg/ml milk proteins in PBS.

After immobilization of SPA-Cys and passivation of the gold sensor surface the interactions of immobilized SPA-Cys with IgG were investigated. The level of the SPR sensor response was shown to be directly proportional to the concentration of IgG, at least in the range of 10–40 μg/ml (Fig. 3). This figure also illustrates a successful regeneration of the bioselective element. After treatment of the sensor surface with a solution of 40 mM citrate buffer (pH 2.5) [12] the SPR signal returned back almost to the values that preceded the injection of the sample. It demonstrates the effective destruction of the bonds between immobilized SPA-Cys and IgG and the removal of the latter. The subsequent injection of new samples of IgG showed that the regeneration did not affect the level of immunoglobulin-binding activity of the immobilized SPA-Cys. Thus, it becomes possible to reuse the bioselective element of the immunosensor based on SPA-Cys.

At first, the immobilization of another immunoglobulin-binding protein, namely, goat antibodies against human immunoglobulin G (anti-IgG) was performed under the same conditions as those during the immobilization of SPA-Cys (1 μM of protein in PBS). The sensor response after immobilization of anti-IgG was more than two times higher than the response we received after immobilization of SPA-Cys (Fig. 2, Fig. 5, Table). After blocking the non-specific sorption sites with milk proteins the injection of IgG solutions of concentration from 10 to 40 μg/ml showed that the sensor registered the interactions of immobilized anti-IgG and IgG, but the level of SPR sensor response practically does not depend on the IgG concentration (Fig. 4). This may indicate a rapid saturation of all available antigen-binding sites of anti-IgG. In addition, the values of the SPR sensor response during the interaction of IgG with immobilized anti-IgG is significantly lower than during the interaction with the immobilized SPA-Cys (Fig. 4).

According to the conversion of the SPR response into the value of the surface density of immobilized protein [13], the values of the surface density were calculated for the immobilized SPA-Cys and anti-IgG, as well as for human IgG, which interacted with the above mentioned immobilized proteins (Table).

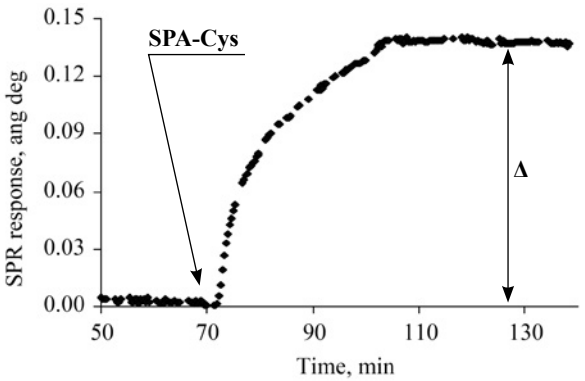

Fig. 2. The SPR sensogram representing the immobilization of SPA-Cys (120 μl of 1 μM SPA-Cys was injected) on the gold sensor surface. *Δ* – the sensor response that represents the number of reliably immobilized SPA-Cys molecules.

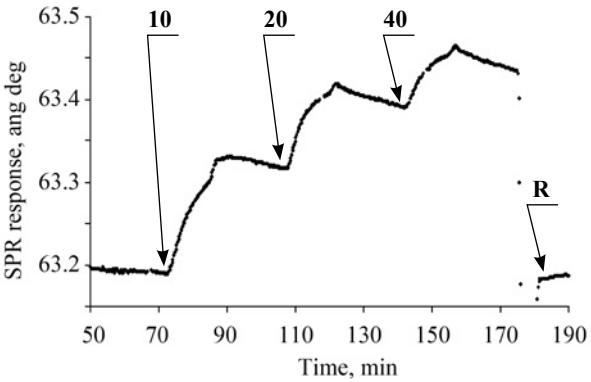

Fig. 3. SPR sensogram representing the interactions of the SPA-Cys bioselective element with 120 μl of 10, 20 and 40 μg/ml IgG. *R* – the base line after regeneration of the bioselective element.

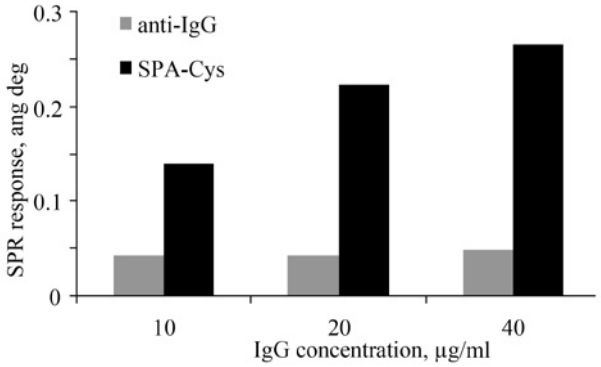

Fig. 4. Comparison histogram of the SPR sensor responses at the interaction of the bioselective element based on SPA-Cys or anti-IgG with 120µl of 10, 20 and 40 µg/ml IgG

Given the molecular weight of SPA-Cys (34.5 kDa) and IgG (150 kDa), we can also calculate the number of immobilized molecules per 100 nm^2 of the sensor surface.

Although the sensor response after the immobilization of anti-IgG was more than twice higher than the response we received after the immobilization of SPA-Cys, the obtained values of the surface density expressed in molecules per 100 nm^2 show almost two-fold advantage of SPA-Cys. Further calculations showed a clear advantage of SPA-Cys over anti-IgG in binding human IgG, too. Regarding the efficiency of two prepared bioselective elements for IgG binding near 5-fold prevalence of that based on SPA-Cys is observed. And the last line in Table certainly indicates a much better accessibility of binding sites of the immobilized SPA-Cys compared with those of the immobilized anti-IgG: only one-sixth of all immobilized molecules of anti-IgG bind with IgG (at the injection of 40 µg/ml IgG) and almost every second molecule of the immobilized SPA-Cys does it.

It should be noted that the increased sensor response on the IgG addition to the immobilized SPA-Cys can be also explained by vertical orientation of molecule-dipole, which increases the interaction of the molecules with the plasmon polariton electromagnetic field, which in its turn evokes some additional shift of the SPR curve toward larger angles [14]. However, IgG molecule should not be regarded as a rigid rod, and it is not known how much the orientation of IgG molecule at the interaction with immobilized SPA-Cys differs from that at the interaction with immobilized anti-IgG. Therefore, it is quite difficult to determine to what extent the above explanation is applicable to this case.

It is well-known that at the immobilization of antibodies on the surface of immunological plate during ELISA a carbonate buffer is used. So, to look for more favorable conditions for the immobilization of anti-IgG on the gold sensor surface 100 mM carbonate buffer (pH 9.5) and SSC buffer solution (pH 7.2) were applied. Unfortunately, as shown in Fig. 5, the application of these buffer solutions does not lead to an increased level of the immobilized anti-IgG.

The application of CB and SSC buffer solutions at the anti-IgG immobilization did not also improve the level of interactions (in PBS) of the immobilized anti-IgG with human IgG: the binding level is in accordance with the level of anti-IgG immobilization (Fig. 6). It should again be noted, that in most cases there is no clear dependence of the sensor response of the bioselective anti-IgG element on the concentration of IgG. It may be associated with a relatively

Table. **The surface density of the immunoglobulin-binding proteins immobilized on the sensor surface, and the number of interacting IgG molecules.**

Parameters \ Immobilized protein	SPA-Cys	Anti-IgG
Surface density of immobilized protein, ng/mm^2	1.35	2.98
Number of molecules of immobilized IgG-binding proteins per 100 nm^2	2.36	1.20
Surface density of IgG, which interacted with immobilized proteins, ng/mm^2	2.67	0.49
Number of IgG molecules, which interacted with immobilized proteins, per 100 nm^2	1.07	0.20
The ratio of bound IgG molecules to the number of molecules of IgG-binding protein	0.45	0.17

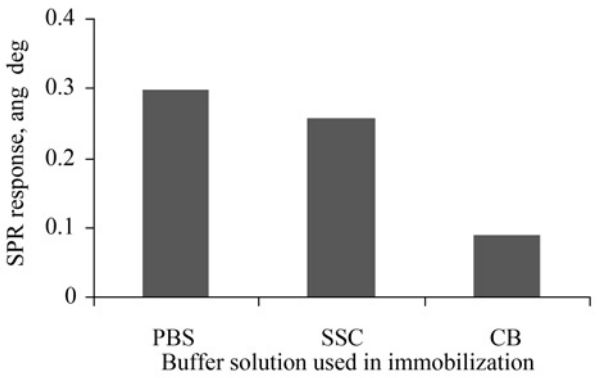

Fig. 5. Histogram showing the dependence of the level of immobilized anti-IgG on a gold sensor surface on the composition of the buffer solutions at the immobilization

small number of available antigen-binding sites of the immobilized anti-IgG, almost all of which become occupied by IgG at the injection of the smallest concentration of the latter.

Conclusions

Thus, the study of the immobilization of the recombinant SPA-Cys or the goat anti-human IgG antibodies on the gold sensor surface, and their interactions with IgG demonstrated the significant advantages of SPA-Cys. Although the level of the sensor response at the immobilization of anti-IgG was higher compared to that of SPA-Cys, but given the difference in the molecular weights of two proteins, there is almost twofold advantage of SPA-Cys in the number of immobilized molecules. As a result, after further injection of IgG solutions into the measuring cell the bioselective element of immunosensor based on SPA-Cys demonstrates a significantly higher ability to bind IgG, than bioselective element based on anti-IgG.

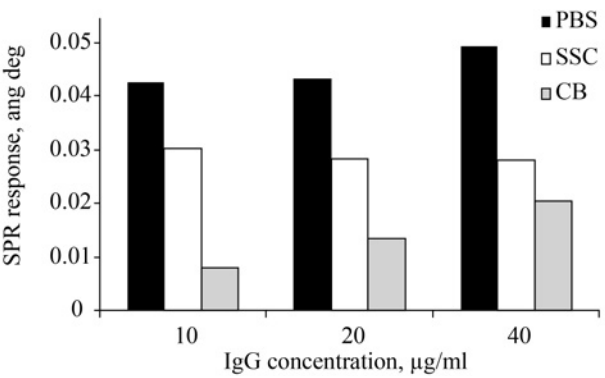

Fig. 6. Comparison histogram of the values of SPR sensor response at the interactions of the bioselective element based on anti-IgG immobilized on a gold sensor surface in buffer solutions PBS, SSC and CB with 120 µl of 10, 20 and 40 µg/ml IgG.

Obviously, the bacterial synthesis of recombinant SPA-Cys is much more convenient and profitable than obtaining antibodies through the immunization of animals. A consideration of bioethical issues also inclines in favor of SPA-Cys application. It also needs to take into account the fact that SPA binds different classes of immunoglobulins of many organisms (human, rabbit, mouse, *etc.*), therefore its application can create so-called "universal" immunosensor platform, while the specificity of anti-IgG is much narrower. All these considerations together with our results clearly indicate the good prospects of the recombinant protein SPA-Cys in the scientific research and for the practical applications in the immunosensors.

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Вивчення взаємодії IgG людини з іммобілізованими анти-IgG або рекомбінантним білком А *Staphylococcus aureus* за допомогою спектрометрії поверхневого плазмонного резонансу

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Мета. Порівняння імуноглобулін-зв'язувальної активності козячих антитіл проти IgG людини (анти-IgG) або рекомбінантного білка А *Staphylococcus aureus* з С-кінцевим залишком цистеїну (SPA-Cys) після їх іммобілізації на золотій сенсорній поверхні спектрометра поверхневого плазмонного резонансу (ППР). **Методи.** SPA-Cys або анти-IgG були іммобілізовані на золотій сенсорній поверхні для формування двох варіантів біоселективного елементу імуносенсора. Дослідження IgG-зв'язувальної активності іммобілізованих білків проводили за допомогою спектрометрії ППР. **Результати.** Сенсорний відгук при іммобілізації анти-IgG виявився більш ніж удвічі вищим за відгук, отриманий при іммобілізації SPA-Cys. Однак по кількості іммобілізованих молекул – майже двократна перевага за SPA-Cys. Крім того, біоселективний елемент імуносенсора на основі SPA-Cys значно краще зв'язує IgG, ніж біоселективний елемент на основі анти-IgG. **Висновки.** Дослідження іммобілізації SPA-Cys або анти-IgG на сенсорній поверхні спектрометра ППР, а також взаємодії іммобілізованих білків з IgG продемонструвало очевидні переваги SPA-Cys.

Ключові слова: антитіла, рекомбінантний білок А *Staphylococcus aureus*, іммобілізація білка, імуносенсор, поверхневий плазмонний резонанс.

Изучение взаимодействия IgG человека с иммобилизованными анти-IgG или рекомбинантным белком А *Staphylococcus aureus* с помощью спектрометрии поверхностного плазмонного резонанса

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Цель. Сравнение иммуноглобулин-связующей активности козьих антител против IgG человека (анти-IgG) или рекомбинантного белка А *Staphylococcus aureus* с С-концевым остатком цистеина (SPA-Cys) после их иммобилизации на золотой сенсорной поверхности спектрометра поверхностного плазмонного резонанса (ППР). **Методы.** SPA-Cys или анти-IgG были иммобилизованы на золотой сенсорной поверхности для формирования двух вариантов биоселективного элемента иммуносенсора. Исследование IgG-связующей активности иммобилизованных белков проводили с помощью спектрометрии ППР. **Результаты.** Сенсорный отклик при иммобилизации анти-IgG оказался более чем вдвое выше отклика, полученного при иммобилизации SPA-Cys. Однако по количеству иммобилизованных молекул – почти двукратное преимущество за SPA-Cys. Кроме того, биоселективный элемент иммуносенсора на основе SPA-Cys значительно лучше связывает IgG, чем биоселективный элемент на основе анти-IgG. **Выводы.** Исследование иммобилизации SPA-Cys или анти-IgG на сенсорной поверхности спектрометра ППР, а также взаимодействия иммобилизованных белков с IgG продемонстрировало очевидные преимущества SPA-Cys.

Ключевые слова: антитела, рекомбинантный белок А *Staphylococcus aureus*, иммобилизация белка, иммуносенсор, поверхностный плазмонный резонанс.

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