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Study on efficiency of oriented immobilization of antibodies on the SPR sensor surface using Staphylococcal protein A or its recombinant analogue

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Aim. Comparison of IgG-binding activity of Staphylococcal protein A (SPA) and recombinant SPA with specially introduced C-terminal cysteine residue (SPA-Cys) after their immobilization on a gold sensor surface of the surface plasmon resonance (SPR) spectrometer. **Methods.** SPA or SPA-Cys was immobilized on a gold sensor surface to form two variants of bioselective elements of biosensor. SPR spectrometry was used for detection of IgG-binding activity of the immobilized proteins. **Results.** The SPR sensor response to the immobilization of SPA was more than three times less than that to immobilization of SPA-Cys. SPA-Cys demonstrates also almost 4-fold advantage in the number of immobilized molecules. Moreover, the bioselective element based on SPA-Cys showed a much better capability of binding IgG than the bioselective element based on SPA. **Conclusions.** The study of the processes of immobilization of SPA or SPA-Cys on the sensor surface of SPR spectrometer, and the interactions of immobilized proteins with human IgG demonstrated obvious advantages of recombinant protein A.

K e y w o r d s: antibodies, recombinant Staphylococcal protein A, protein immobilization, surface plasmon resonance.

Introduction

Over the last decades there has been a growing interest in biosensor technologies for research, medical diagnostics, environmental monitoring and food analysis purposes [1]. Biosensor is a device that uses specific biochemical reactions to detect chemical compounds converting a result of the biological process into the electric signal suit-

able for further processing and characterization [2].

In comparison with the conventional analytical techniques, the biosensors offer a number of advantages, such as easy, fast, real-time, sensitive, and specific measurements [3]. Optical biosensors based on the surface plasmon resonance (SPR) allow the registration of intermolecular interactions in real-time and label-free analyses. The refractive index of a thin layer (~200 nm) of medium, adjacent to the sensor surface changes during the immobilization of biomacromolecules and their interaction with the partner molecules. This change causes the SPR response that is registered by the biosensor [4].

Utilization of immune molecules allows the achievement of high specificity and sensitivity of the created biosensor. An important step in developing a highly reliable biosensor is the immobilization of biomacromolecules on the sensor surface. Unfortunately, physical adsorption of antibodies on the solid surface results in their random orientation, so a part of active sites of immobilized antibodies or sometimes even most of them are unavailable for antigen binding [5]. One of the ways to solve this problem is to create an intermediate layer of proteins capable of providing the oriented immobilization of antibodies by binding their Fc-fragment. One of such immunoglobulin-binding proteins is Staphylococcal protein A (SPA) [6]. Due to the fact that the immunoglobulin-binding region of SPA does not contain cysteine residues [7], its immobilization on the gold sensor surface is possible only by physical adsorption, which is not always reliable. Genetically engineered introduction of one cysteine residue into recombinant SPA, which interacts with the gold sensor surface through exposed SH-group, improves reliability of the SPA immobilization and accessibility of IgG-binding sites [8]. In the Institute of Molecular Biology and Genetics of the NAS of Ukraine, an original genetically engineered construct was created and a recombinant Staphylococcal protein A with specially introduced C-terminal cysteine residue (SPA-Cys) was obtained [9].

The aim of present work is the comparison of IgG-binding activity of Staphylococcal protein A (SPA) and recombinant Staphylococcal protein A with specially introduced C-terminal cysteine residue (SPA-Cys) after their immobilization on a gold sensor surface of the SPR spectrometer.

Materials and Methods

NaCl, KH_2PO_4, p-nitrophenyl phosphate, bovine serum albumin (BSA) and Staphylococcal protein A (SPA) were purchased from "Sigma-Aldrich" (USA), Na_2HPO_4 – from "Applichem" (Germany), milk proteins (Milk Powder "Fluka", Switzerland), Tween 20 – from "Helicon" (Russia), other reagents and solvents were obtained from "UkrOrgSyntez" (Ukraine).

Human IgG was obtained by affinity chromatography using SPA-Cys-modified silica as described in [9]. Synthesis and purification of recombinant Staphylococcal protein A with specially introduced C-terminal cysteine residue (SPA-Cys) were described in [9]. The genetically engineered fusion protein containing five IgG-binding domains of SPA and the bacterial alkaline phosphatase with enhanced catalytic properties (SPA-BAPmut) were obtained as described in [10].

1. Competitive ELISA

The wells of the immunological plate were coated overnight at 4 °C with 100 μL of 4 μg/ml IgG in 100 mM carbonate buffer, pH 9, and then washed with PBS containing 0.1 % Tween 20 (PBST). The surface of the wells was blocked to prevent nonspecific sorption with 150 μl of 2 mg/ml skim milk powder solution in PBS. After 1-hour incubation at 37 °C the plates were washed with PBST. The mixtures of 50 μl of 4 μg/ml SPA-BAPmut with 50 μl of PBS, 50 μl of SPA-Cys or 50 μl of SPA solutions of different concentrations were added to the wells. After 1-hour incubation at 37 °C the plates were washed with PBST and the buffer solution for alkaline phosphatase, which included 100 mM tris-HCl, 140 mM NaCl, 15 mM $MgSO_4$, pH 9.5, and 100 μl of substrate solution (p-nitrophenyl phosphate in buffer solution for alkaline phosphatase), was added. After incubation for 30 min at room temperature, the color development was stopped with 1 M NaOH (50 μL per well) and the optical density was measured at 405 nm using the micro plate reader "Titertek Multiskan MCC/340" (Germany).

2. SPR spectrometric analysis of protein-protein interactions

The glass plates with a thin layer of gold were cleaned and mounted on the spectrometer prism as described in [11]. SPR analysis was performed using a measuring flow-cell of the spectrometer "Plasmon-4m" (the device and corresponding software have been developed at the Lashkaryov Institute of Semiconductor Physics of the NAS of Ukraine) and a peristaltic pump "Ismatec" (Switzerland) at the pump speed of ~40 μl/min. The full procedure of SPR analysis is described in [12]. At first the measuring flow-cell was thoroughly washed by working buffer solution (PBS) to stabilize the SPR signal. Then 120 μl of a sample were injected and incubated with the pump switched off for 30 min for the protein immobilization on the sensor surface, and for 10 min for the interactions of immobilized components with IgG. After that the measuring flow-cell was washed by PBS again until a stable SPR signal was obtained. To distinguish an actual sensor response caused by the interactions between a sample and the sensor surface or preliminary immobilized components from the signal caused by the random fluctuations of medium, it is necessary to wash the flow-cell before and after each sample with the same buffer solution, and only then to determine a value of the SPR response.

Results and Discussion

Before applying Staphylococcal protein A (SPA) and recombinant Staphylococcal protein A with specially introduced C-terminal cysteine residue (SPA-Cys) in the biosensor analysis, their immunoglobulin-binding activity was tested using the competitive ELISA. The SPA and SPA-Cys solutions of different concentrations and a fixed concentration of the fusion protein containing IgG-binding domains of SPA and the bacterial alkaline phosphatase with enhanced catalytic properties (SPA-BAPmut) were added to the immobilized human IgG. SPA or SPA-Cys competes with SPA-BAPmut for binding immobilized IgG, therefore, the values of optical density are in inverse dependence on the competing protein concentration (Fig. 1). It can be concluded that there is a sufficient specific interaction of both

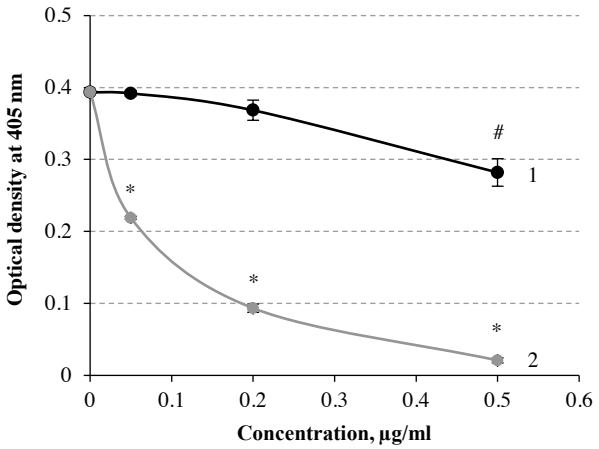

Fig. 1. The competitive ELISA for testing the IgG-binding activity. The graph of dependency of results at optical density 405 nm on the SPA (1) and its recombinant analogue SPA-Cys (2) concentrations using the SPA-BAPmut. *, # - p <0.01 and p <0.05 compared to the negative control: interaction of the immobilized IgG with the SPA-BAPmut.

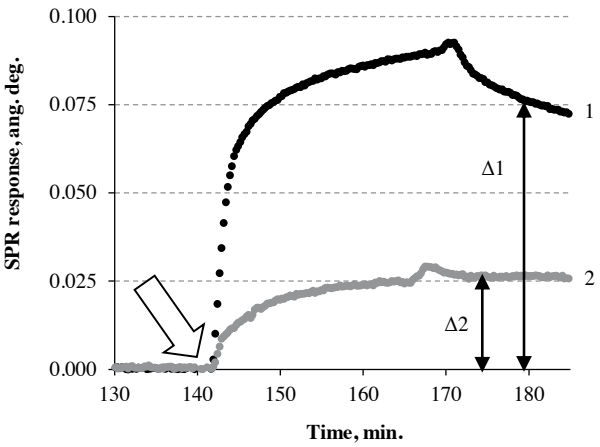

Fig. 2. The SPR sensograms representing the processes of SPA-Cys (1) and SPA (2) immobilization on the gold sensor surface. An arrow represents the injection of 120 µl of 1 µM solution of either protein. Δ1 and Δ2 – sensor responses that represent the number of reliably immobilized SPA-Cys and SPA molecules, respectively.

immunoglobulin-binding proteins with immobilized IgG, noting that SPA-Cys has a better ability to interact with IgG than SPA. This can be explained either by the lower activity of SPA, or by its lower stability during storage at + 4 ° C.

The SPR sensograms representing the processes of immobilization of SPA or SPA-Cys on the gold surface of SPR biosensor are shown in Fig. 2. The difference between the signal before injection of 120µl of 1 µM solution of either protein into the measuring cell and after washing it with PBS was measured (Fig. 2, Δ1 and Δ2). This value represents the number of reliably immobilized protein molecules. According to the conversion factor of SPR response into the value of the surface density of immobilized protein [13], it was ~ 0.3 ng/mm^2 and ~ 0.9 ng/mm^2 for SPA and SPA-Cys, respectively, indicating a ~3 times higher surface density of the immobilized SPA-Cys.

The molecular mass of the SPA is somewhat higher than the molecular mass of the SPA-Cys (42 and 34.5 kDa, respectively), because SPA contains C-terminal part used for attachment to the bacterial cell wall, which was removed from the recombinant SPA-Cys. Based on the molecular masses of SPA and SPA-Cys, it was calculated that on average 0.4 molecules of SPA or 1.5 molecules of SPA-Cys were immobilized on every 100 nm^2 of the sensor surface. The calculation of minimum radius of a molecule (R_{min}) based on its molecular mass (M) is described by the formula [14]:

$$R_{min} = 0.066 \times M^{1/3}$$

Using this formula, it can be calculated that ~ 8 nm^2 or ~ 27 nm^2 out of every 100 nm^2 of the sensor surface were occupied by the immobilized SPA or SPA-Cys, respectively. These numbers do not indicate the formation of a dense monolayer of proteins on the sensor surface. So, after immobilization of either SPA or SPA-Cys the free sites of the non-specific sorption on the gold surface were blocked with the injection of 200 μg/ml milk proteins solution in PBS.

To test, whether the molecules of SPA or SPA-Cys retain their immunoglobulin-binding properties after the immobilization process, the human IgG solution was injected into the measuring flow-cell. Both SPA (Fig. 3, A, black) and SPA-Cys (Fig. 3, B, black) demonstrated a satisfactory immunoglobulin-binding activity during the interaction with 50 μg/ml IgG without a noteworthy decrease of the signal after a prolonged wash with PBS. At the same time, the injection of 50 μg/ml BSA does not cause noticeable changes in the sensor response in both cases (Fig. 3, gray). So, the created bioselective elements of the SPR biosensor based on SPA or SPA-Cys demonstrated a high selectivity.

For the further use of the prepared bioselective elements, IgG molecules should be removed from the surface by disruption of the affinity links between the immobilized proteins and IgG. In this work, the 40 mM sodium citrate buffer solution (pH 2.5) was used for this purpose [15]. After treating the sensor surface with this solution, the sensor signals came back to the values that preceded the IgG injection, and the subsequent injections of new IgG samples showed the same level of sensor response, so the re-use of both bioselective elements is possible.

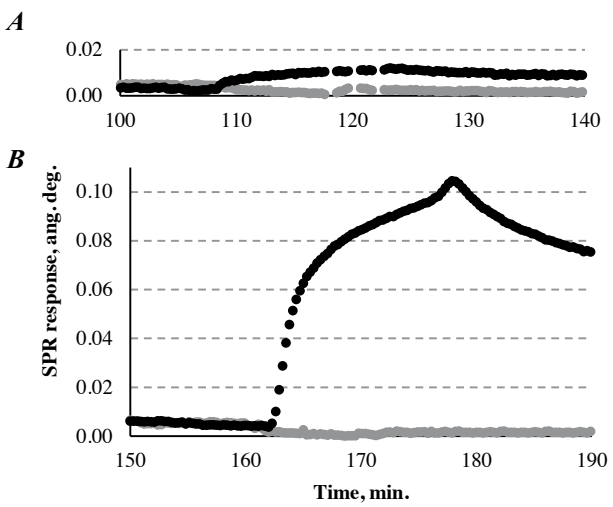

Fig. 3. The SPR sensograms representing the interactions of the bioselective elements based on the immobilized on the gold sensor surface SPA (A) and SPA-Cys (B) with 50 μg/ml solutions of BSA (gray) and IgG (black).

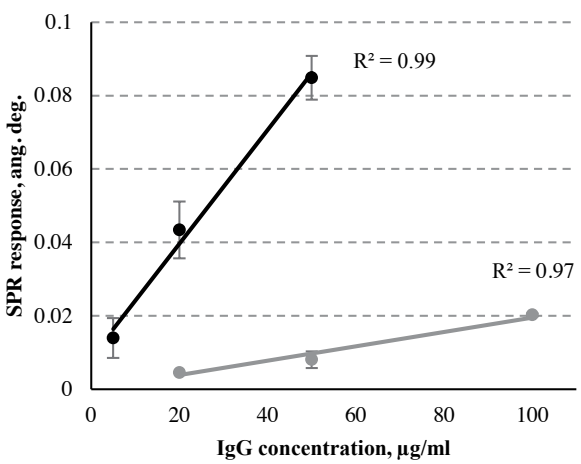

Fig. 4. Graph of the dependency of the sensor responses of bioselective elements based on SPA-Cys (black) and SPA (gray) on the concentration of the injected IgG solutions in the ranges 5-50 and 20-100 μg/ml, respectively.

The level of the SPR sensor response was shown to be directly proportional to the concentration of IgG in both cases, at least in the range of 5–50 μg/ml for SPA-Cys (Fig. 4, black) and 20–100 μg/ml for SPA (Fig. 4, gray). As it can be seen from Fig. 4, the sensor responses to the introduction of 50 μg/ml IgG solution in PBS are almost 11 times greater in favor of the SPA-Cys-based bioselective element.

The calculated values of the surface density of SPA, SPA-Cys and human IgG, which interacted with one of the immobilized proteins during the injection of 50 μg/ml solution, as well as the number of immobilized molecules per 100 nm^2 of the sensor surface for SPA, SPA-Cys and IgG given its molecular mass (150 kDa), are presented in the Table 1. The ratio of bound IgG molecules to the number of molecules of IgG-binding protein in the case of SPA-Cys immobilization was 2.8 times greater than in the case of SPA. Table 1 shows a clear advantage of the SPA-Cys over SPA.

By definition, lifetime of the bioselective element (tL) is the storage or operational time necessary for the sensor response, within the linear concentration range, to decrease by 50 % (tL50) [16]. The sensor response of bioselective element based on SPA at the injections of 100 μg/ml IgG was measured over a period. As can be seen on Fig. 5, 50 % reduction in the sensor response occurs on the 13th day after SPA immobilization, so tL50 of this bioselective element is 13 days. The tL50 value of the bioselective element based on the SPA-Cys that was determined by the same way is 11 days.

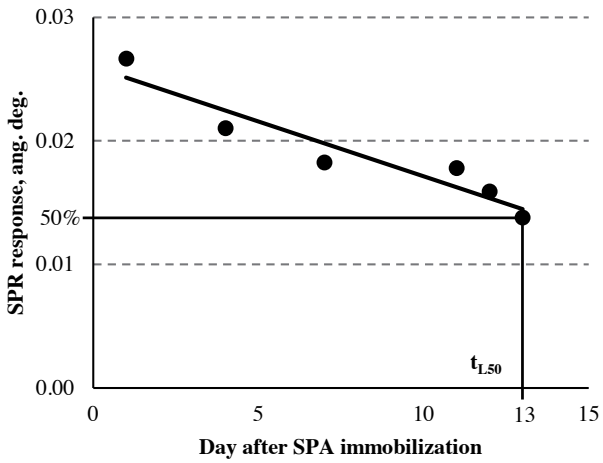

Fig. 5. Graph of the dependency of the average SPR sensor responses of the bioselective element based on SPA at the injections of 100 μg/ml IgG, on time after SPA immobilization. Storage and operation were done in PBS at room temperature.

Table 1. **The surface density of immunoglobulin-binding proteins immobilized on the sensor surface, and the number of molecules of IgG, which interacted with immobilized proteins**

Parameters \ Immobilized protein	SPA	SPA-Cys
Surface density of immobilized protein, ng/mm^2	0.3	0.9
Number of molecules of immobilized molecules per 100 nm^2	0.4	1.5
Surface density of IgG, which interacted with immobilized proteins during the injection of 50 μg/ml IgG, ng/mm^2	0.08	0.85
Number of IgG molecules, which interacted with immobilized proteins, per 100 nm^2	0.03	0.34
The ratio of bound IgG molecules to the number of molecules of IgG-binding proteins	**0.075**	**0.22**

Conclusions

The study of the processes of immobilization of the Staphylococcal protein A (SPA) or the recombinant Staphylococcal protein A with specially introduced C-terminal cysteine residue (SPA-Cys) on the gold sensor surface, and their interactions with IgG demonstrated significant advantages of SPA-Cys. The presence of Cys residue and the absence of hydrophobic domain responsible for attachment to the cell wall in the C-terminal part of SPA-Cys allow its oriented immobilization and benefit the availability of its Fc-binding domains. Both bioselective elements demonstrated sufficient selectivity during interactions with non-specific BSA and almost the same value of lifetime. The immobilization level, the accessibility of binding sites and the ability to bind IgG of the immobilized SPA-Cys were compared with those of the immobilized SPA and the results showed undeniable advantage of using SPA-Cys in scientific research and for practical applications in the formation of an intermediate layer for the oriented immobilization of antibodies.

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Дослідження ефективності орієнтованої іммобілізації антитіл на поверхні сенсора ППР за допомогою білка А стафілокока або його рекомбінантного аналога

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Мета. Порівняння імуноглобулін-зв'язувальної активності білка А стафілокока (SPA) або рекомбінантного білка А стафілокока зі спеціально введеним С-кінцевим залишком цистеїну (SPA-Cys) після їх іммобілізації на золотій сенсорній поверхні спектрометра поверхневого плазмонного резонансу (ППР). **Методи.** SPA або SPA-Cys були іммобілізовані на золотій сенсорній поверхні для формування двох варіантів біоселективних елементів біосенсора. Дослідження IgG-зв'язувальної активності іммобілізованих білків проводили за допомогою спектрометрії ППР. **Результати.** Сенсорний відгук при іммобілізації SPA виявився більш ніж втричі меншим за відгук, отриманий при іммобілізації SPA-Cys. Також, по кількості іммобілізованих молекул – майже чотирикратна перевага за SPA-Cys. Крім того, біоселективний елемент на основі SPA-Cys значно краще зв'язує IgG, ніж біоселективний елемент на основі SPA. **Висновки.** Дослідження процесів іммобілізації SPA або SPA-Cys на сенсорній поверхні спектрометра ППР, а також взаємодії іммобілізованих білків з IgG, продемонструвало очевидні переваги рекомбінантного білка А.

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

Исследование эффективности ориентированной иммобилизации антител на поверхности сенсора ППР с помощью белка А стафилококка или его рекомбинантного аналога

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Цель. Сравнение иммуноглобулин-связывающей активности белка А стафилококка (SPA) или рекомбинантного белка А стафилококка со специально введенным С-концевым остатком цистеина (SPA-Cys) после их иммобилизации на золотой сенсорной поверхности спектрометра поверхностного плазмонного резонанса (ППР). **Методы.** SPA или SPA-Cys были иммобилизованы на золотой сенсорной поверхности для формирования двух вариантов биоселективных элементов биосенсора. Исследование IgG-связывающей активности иммобилизованных белков проводили с помощью спектрометрии ППР. **Результаты.** Сенсорный отклик при иммобилизации SPA оказался более чем втрое меньше отклика, полученного при иммобилизации SPA-Cys. Также, по количеству иммобилизованных молекул – почти четырехкратное преимущество за SPA-Cys. Кроме того, биоселективный элемент на основе SPA-Cys значительно лучше связывает IgG, чем биоселективный элемент на основе SPA. **Выводы.** Исследование процессов иммобилизации SPA или SPA-Cys на сенсорной поверхности спектрометра ППР, а также взаимодействия иммобилизованных белков с IgG, продемонстрировало очевидные преимущества рекомбинантного белка А.

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

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