Method Article

Method of kinetic characterization of immunoreagents for development of express high-sensitive assays for detection of ochratoxin A and heart fatty acids binding protein

Alexey V. Orlov\textsuperscript{a,*}, Juri A. Malkerov\textsuperscript{a,b}, Denis O. Novichikhin\textsuperscript{a,b}, Alexei I. Nikitin\textsuperscript{c}, Gennadiy M. Sorokin\textsuperscript{d}, Maxim L. Antopolsky\textsuperscript{a}, Sergey L. Znoyko\textsuperscript{a}

\textsuperscript{a}Prokhorov General Physics Institute of the Russian Academy of Sciences, Moscow, Russia
\textsuperscript{b}National Research Nuclear University MEPhI (Moscow Engineering Physics Institute), 31 Kashirskoe shosse, 115409 Moscow, Russia
\textsuperscript{c}Volga branch of Moscow Automobile and Road State Technical University, Cheboksary 428000, Russia
\textsuperscript{d}Chuvash State University, 15 Moskovskij Ave., Cheboksary 428015, Russia

A B S T R A C T

Development of rapid and sensitive immunoassays is a task of great importance in a variety of fields ranging from clinical practice and urgent diagnostics to food quality control and environmental monitoring. High attention of researches is paid to methods of screening, selection, and kinetic characterization of antibodies that enable fast, specific, and effective formation of immunocomplexes. Herein, we present a method for direct investigation of kinetics of immunoreagents during developments of express high sensitive lateral flow assays. As model biomolecules to be detected, the following substances were tested: ochratoxin A (OTA), which is one of the most dangerous mycotoxins naturally present in many vegetable raw materials; and heart fatty acids binding protein (hFABP), which is a cardiac marker used in differential diagnosis of acute myocardial infarction. The kinetic constants of association (k\textsubscript{on}) and dissociation (k\textsubscript{off}) with monoclonal antibodies are determined along with the corresponding equilibrium constants (K\textsubscript{A} and K\textsubscript{D}). The obtained values are as follows: for the anti-OTA antibodies - \textit{k}\textsubscript{on} = 4.54\times10^3 \text{ M}^{-1}\text{s}^{-1}; k\textsubscript{off} = 3.32\times10^{-4} \text{ s}^{-1}; K\textsubscript{A} = 1.37\times10^7 \text{ M}^{-1}; K\textsubscript{D} = 7.31\times10^{-8} \text{ M}; and for the anti-hFABP antibodies - \textit{k}\textsubscript{on} = 7.28\times10^3 \text{ M}^{-1}\text{s}^{-1}; k\textsubscript{off} = 1.97\times10^{-4} \text{ s}^{-1}; K\textsubscript{A} = 3.70\times10^7 \text{ M}^{-1}; K\textsubscript{D} = 2.70\times10^{-8} \text{ M}. The proposed method can be employed in combination with the immunochromatographic assays based on magnetic biolabels.

- Investigation of immunoreagent kinetics for development of express high sensitive lateral flow assays
- Kinetic characterization of monoclonal antibodies against OTA and hFABP for their rapid and sensitive detection

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* Corresponding author.
E-mail address: alexey.orlov@kapella.gpi.ru (A.V. Orlov).

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• Both kinetic and equilibrium constants of association and dissociation are determined

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A R T I C L E  I N F O
Method name: Kinetic characterization of immunoreagents for development of express high-sensitive assays
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Specifications table

| Subject Area:          | Chemistry                      |
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| More specific subject area: | Development of immunoanalytical methods for detection of biomolecules |
| Method name:           | Kinetic characterization of immunoreagents for development of express high-sensitive assays |
| Name and reference of original method: | Spectral-phase and spectral-correlation interferometry [1,2] |
| Resource availability: | N/A                            |

Method details

Materials

Bovine serum albumin (BSA), (3-Aminopropyl)triethoxysilane (APTES), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), dimethylformamide (DMF), succinic anhydride were purchased from Sigma-Aldrich (Germany). Sulfuric acid, hydrochloric acid, 2-propanol and hydrogen peroxide were purchased from Chimmed (Russian Federation). Monoclonal antibodies against Ochratoxin A and BSA-OTA conjugates were obtained from IL Test Puschino Ltd. (Russian Federation). Heart fatty acids binding protein and corresponding monoclonal antibodies were obtained from HyTest Russia (Russian Federation).

Spectral interferometry instrument

An instrument (Fig. 1) employed in this research for label-free measuring the kinetic characteristics is based on the spectral interferometry (SI) approaches [1–3]. The device recorded in real-time alterations in the thickness of a layer of biomolecules on its sensor chip [1,2,4]. The instrument provided results in metrical units because its optical scheme used as a light source a superluminescent diode, which had the central wavelength of 850 nm and the spectral width about 50 nm. During the measurement, the instrument analyzes an interference pattern between the beam reflected from the bottom surface of a sensor chip and that reflected from an upper surface of the molecular layer.

Cleaning the surface of a microscope cover glass plate

A single-used sensor chip is a microscope cover glass plate (Fig. 2) with a molecular layer immobilized on its surface. For fabrication of such sensor chips, their glass surface was first cleaned as follows. The glass plates were washed with methanol and treated with a mixture of 30% hydrogen peroxide with 95% sulfuric acid (1:3) for 40 min at 70 °C. After cooling down, the plates were washed three times with triple distilled water and twice with methanol.

Introducing free alkylamino groups to the glass surface

The cleaned microscope cover glass plates were put into a 3% solution of (3-Aminopropyl) triethoxysilane (APTES) in methanol and kept overnight at room temperature (RT). Then, they were
Fig. 1. Photograph of the instrument based on spectral interferometry approaches for label-free measurements of kinetics.

Fig. 2. Photograph of a microscope cover glass plate used for fabrication of the single-used sensor chips.

washed three times with propanol-2 and dried. The aminated cover glasses were stored at RT before use [5,6].

Carboxylation of the glass plate surface

The aminated cover glass plates were washed three times with DMF, covered with a 15-mmol solution of succinic anhydride in DMF, and incubated for 2 h at RT. Finally, the modified microscope cover glass plates were thrice washed with DMF, dried, and stored at RT before use [7,8].

Immobilization of a molecular target onto carboxylated glass surface

The final modification of the glass plate surface was made as follows: 6% EDC solution in 0.1M MES buffer was applied to the carboxylated glass surface and kept for 15 min, then the surface was
washed with PBS buffer. After that, a solution of OTA-BSA or hFABP (50 μg/mL in PBS) was applied, and the plate was incubated for 1 h at RT. Then the surface of the fabricated sensor chip was washed with PBS, exposed to 1 mL of 0.1M TRIS buffer for 30 min at RT, three times washed with water and dried.

Investigation of association and dissociation of antibodies

A solution of BSA (10 mg/mL in PBS) was pumped inside the SI instrument along the sensor chip with attached OTA-BSA or hFABP for 15 min. Then, the solution was changed for anti-OTA or anti-hFABP antibodies (25 μg/mL in PBS with BSA 10 mg/mL). This solution was pumped until the biolayer thickness reached a plateau. After that, a BSA solution (10 mg/mL in PBS) was pumped along the sensor chip for the next 1.5 h. The final step was necessary to initiate the dissociation of antigen-antibody [5,9,10]. During the procedure, the flow rate of solutions pumped along the glass sensor chip was constant – 6.7 μL/min.

Calculation of kinetic constants

The kinetic constants of interactions between the antigen on the sensor chip surface and free antibodies in a solution were calculated using a standard model for determination of kinetic constants [11–14]. For the calculations, we used the sensograms (temporal dependences of SI signal) recorded in real-time by the SI instrument.

The fitting equation for the sensogram fragment corresponding to the antibody interaction with the antigen on the sensor chip surface:

\[ R(t) = R_{\text{max}} \left( 1 - e^{-\left( k_{\text{on}} + k_{\text{off}} \right) t} \right) \]

where \( R(t) \) - is a temporal dependence of thickness of the molecular layer on the sensor chip surface, \( R_{\text{max}} \) – maximal increase of thickness at the stage of antigen-antibody interaction, \( k_{\text{on}} \) – kinetic constant of association, \( k_{\text{off}} \) – kinetic constant of dissociation.

The fitting equation for the sensogram fragment during desorption of the antibodies from the antigen on the sensor chip surface:

\[ R(t) = R_{\text{max}} \cdot e^{-k_{\text{off}} t} \]

Equilibrium constants were calculated as the ratio of the corresponding kinetic constants [15].

Method validation

As model biomolecules to be detected, the following substances were tested: ochratoxin A, which is one of the most dangerous mycotoxins naturally present in many vegetable raw materials [16–18]; and heart fatty acids binding protein, which is a cardiac marker used in differential diagnosis of acute myocardial infarction [19–21]. Fig. 3 exhibits the results of employment of the described above method for characterization of monoclonal antibodies to OTA and hFABP. As can be seen from the figures, the sensogram fragments related to both association and dissociation stages match well to the proposed theoretical model. The correlation between the experimental and theoretical data is not less than 95%.

The calculated values of the kinetic constants of association (\( k_{\text{on}} \)) and dissociation (\( k_{\text{off}} \)) for monoclonal antibodies to OTA and hFABP, as well as the corresponding equilibrium constants (\( K_A \) and \( K_D \)) are shown in Table 1.

The presented here method was used for characterization and selection of immunoreagents for development of express high-sensitive detection of ochratoxin A in food by a lateral flow immunoassay based on magnetic biolabels [22]. Besides, the method can be used in developments of other rapid immunoassays, for example, point-of-care immunochromatographic tests based on magnetic particles [23–25] for differential diagnosis of acute myocardial infarction.
Fig. 3. Fitting the recorded sensogram fragments that correspond the processes of association and dissociation of antibodies to OTA and hFABP.

Table 1
Calculated values of kinetic and equilibrium constants for monoclonal antibodies to OTA and hFABP.

| Complex               | $k_{on}$, M$^{-1}$s$^{-1}$ | $k_{off}$, s$^{-1}$ | $K_D$, M   | $K_A$, M$^{-1}$ |
|-----------------------|-----------------------------|---------------------|--------------|-----------------|
| mAb – OTA-BSA         | $4.54 \times 10^3$         | $3.32 \times 10^{-4}$ | $7.31 \times 10^{-8}$ | $1.37 \times 10^7$ |
| mAb – hFABP           | $7.28 \times 10^3$         | $1.97 \times 10^{-4}$ | $2.70 \times 10^{-8}$ | $3.70 \times 10^7$ |

Data availability

No data was used for the research described in the article.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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