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A new real-time method for investigation of affinity properties and binding kinetics of magnetic nanoparticles

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ABSTRACT

A method for quantitative investigation of affinity constants of receptors immobilized on magnetic nanoparticles (MP) is developed based on spectral correlation interferometry (SCI). The SCI records with a picometer resolution the thickness changes of a layer of molecules or nanoparticles due to a biochemical reaction on a cover slip, averaged over the sensing area. The method is compatible with other types of sensing surfaces employed in biosensing. The measured values of kinetic association constants of magnetic nanoparticles are 4 orders of magnitude higher than those of molecular antibody association with antigen. The developed method also suggests highly sensitive detection of antigens in a wide dynamic range. The limit of detection of 92 pg/ml has been demonstrated for prostate-specific antigen (PSA) with 50-nm MP employed as labels, which produce 3-order amplification of the SCI signals. The calibration curve features high sensitivity (slope) of 3-fold signal raise per 10-fold increase of PSA concentration within 4-order dynamic range, which is an attractive compromise for precise quantitative and highly sensitive immunoassay. The proposed biosensing technique offers inexpensive disposable sensor chips of cover slips and represents an economically sound alternative to traditional immunoassays for disease diagnostics, detection of pathogens in food and environmental monitoring.

1. Introduction

Lately, considerable efforts have been put into research on biofunctionalized magnetic particles intended for diagnostics and therapy of diseases, targeted drug delivery, biocomputing and biosensing, construction of biological tissues, etc. [1–5]. The nanoparticles are generally functionalized by immobilization on their surface of various biomolecules such as peptides, antibodies, lectins, and DNA [5–8]. These biomolecules furnish the nanoparticles with additional properties, in particular, the ability to specifically bind with targets. The affinity properties and binding kinetics during interaction with targets and non-target molecules are among the key parameters for characterization of the nanoparticles [9,10].

Currently, the kinetic properties of macromolecules and nanoparticles are commonly studied by label-free methods based on the surface plasmon resonance (SPR) [11–14]. Using these methods it was shown, in particular, that the affinity of multivalent (multi-point) interaction of the nanoparticles with targets can be several orders of magnitude higher than the interaction between individual macromolecules [11,15]. Bigger particles or clusters are often considered preferable to provide higher affinity and better limit of detection (LOD). However, in the case of planar biochips, the bigger particles may cause a decrease of the slope of calibration curve in logarithmic scale, which represents the sensitivity of the analyte concentration measurements [16]. Therefore, the proper balance between LOD and sensitivity (slope) should be found for particular applications of nanoparticles with planar biochips. The cost of SPR biochip consumables that carry precisely deposited gold films may become a limitation factor for a comprehensive study of kinetic properties of the nanoparticles. In addition, the SPR-based methods have substantial limitation for deposition of films with thickness sufficient for mimicking other surfaces, e.g. glass and plastic ones, which are the most common in biosensing, as such films usually disturb the resonance much stronger than studied fine biointeractions.

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Among the recent techniques, real-time recording of the kinetics of protein interactions and binding properties of magnetic nanoparticles is realized by high-density sensor arrays based on giant magneto-resistance (GMR) [17]. This approach demonstrates a very attractive LOD of tens zeptomoles of solute while recording interactions with antigens of MP functionalized by antibodies. However, this approach based on registration of magnetic properties of MP cannot register the interaction kinetics of the same agents in molecular form, which is important for control of affinity changes while conjugation of proteins on the surface, as well as for finding the MP valence, i.e. the number of active molecules on a single particle. In this work, we propose a method for real-time investigation of affinity, binding kinetics of molecular agents and functionalized MP on transparent plates such as inexpensive microscopic cover slips and for a study of biosensing parameters of related dose–response curves. This direct optical method is based on the spectral correlation interferometry (SCI) and can operate on the surfaces without coatings or with the thin films typical for other biosensing chips. The method has been demonstrated by experimental investigation of the kinetic characteristics of MP used in the final stages of a sandwich immunoassay for the total form of prostate-specific antigen (t-PSA).

2. Materials and methods

2.1. Reagents

We used prostate-specific antigen, monoclonal antibody to PSA, clone M612165 and conjugates of monoclonal antibody to PSA, clone M612166, with biotin (Fitzgerald Industries, USA); (3-Glycidyloxypropyl)trimethoxysilane (Aldrich, USA); magnetic nanoparticles conjugated with streptavidin (Miltenyi Biotec, Germany). Other reagents were at least of analytical grade.

2.2. Method of spectral correlation interferometry

The optical scheme of the SCI method [18–20] employs two Fabry–Perot interferometers and a wide-band radiation source. One of the interferometers has its inter-mirror distance periodically scanned by a piezoelectric actuator. The solutions under test are pumped along a surface of a glass cover slip, which serves simultaneously as a sensor chip and the second interferometer. The interference, which is produced by a reference beam reflected from the bottom surface of the slip and a beam reflected from the biolayer/water interface, depends upon the optical thickness of the combination of the slip with the biolayer (see Fig. 1) [19]. The change of this optical thickness Δdis measured by a phase change of a correlation signals from photodetectors or a CCD-camera.

2.3. Modification of glass surface

Glass surface was epoxilated according to the technique developed for detection of cardiac troponin [21] and metabolite of pyrethroids [22]. Briefly, glass cover slips washed by methanol were incubated in 1:3 solution of 30% hydrogen peroxide and 95% sulfuric acid for 40 min at 70 °C with further thrice washing with tridistilled water and twice washing with methanol. Then the glass slips were incubated for 16 h under room temperature in 5% solution of (3-glycidyloxypropyl)trimethoxysilane in methanol. After that, the slips were thrice washed by isopropyl alcohol and kept in a drying oven for 1 h at 105 °C. Before experiments, the glass slips were stored at room temperature. The antibody was immobilized directly in a reaction cell of a biosensor by passing along the glass surface of antibody solution of 25 μg/ml in PBS.

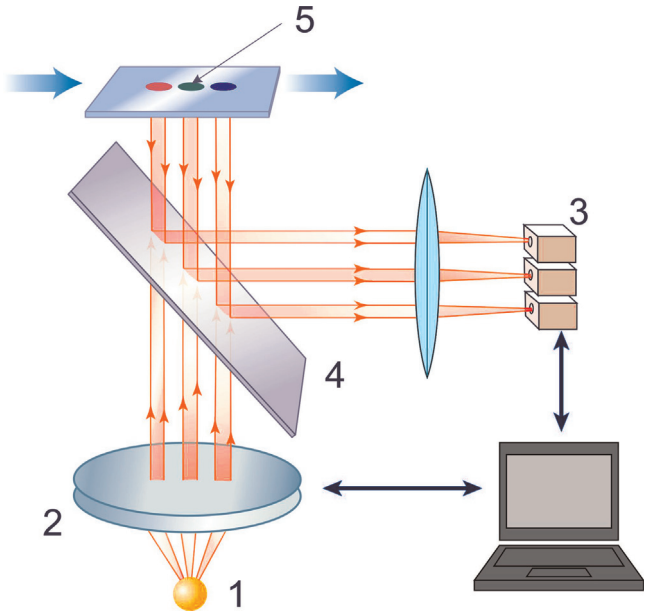

Fig. 1. Optical scheme of the spectral correlation interferometry: 1 – radiation from a superluminescent diode, 2 – scanned Fabry–Perot interferometer, 3 – photodetectors, 4 – beam splitter, 5 – biochemical reactions on the surface of a cover slip.

2.4. Determination of kinetic parameters

The kinetic parameters were determined based on the 1:1 kinetic model of association [23], which describes interaction of receptor A with ligand B as follows:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} AB \tag{1}$$

Since our method records an average increase of the biolayer thickness on a glass slip, the signal is proportional to the number of complexes AB formed on the surface. The kinetics of the complex formation can be described by the equation:

$$\frac{d[AB]}{dt} = k_{on}[A][B] - k_{off}[AB] \tag{2}$$

The quantity of receptors immobilized on the surface is constant in time. Therefore, the concentration of unoccupied receptor is proportional to $(R_{max} - R)$, where signal R_{max} corresponds to the saturation. Then, the signal change kinetics is described as

$$\frac{dR}{dt} = k_{on}(R_{max} - R)[A] - k_{off}R \tag{3}$$

By integrating this equation, we receive:

$$R = R_{max}(1 - e^{-k_{ob}t}) \tag{4}$$

where

$$k_{ob} = k_{off} + k_{on}[A] \tag{5}$$

The kinetic characteristics were determined by fitting the experimentally obtained sensograms of binding with Eq. (4), in which R_{max} and k_{on} varied as parameters.

3. Results and discussion

3.1. Model system

As a model target for investigation of kinetic characteristics of MP, we chose the total form of prostate specific antigen (t-PSA), which is an informative biochemical marker used for diagnostics

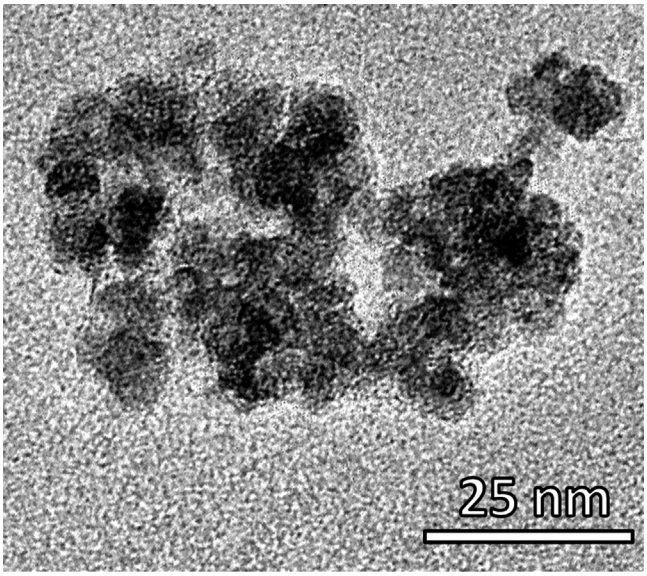

Fig. 2. Transmission electron image of MP conjugated with streptavidin.

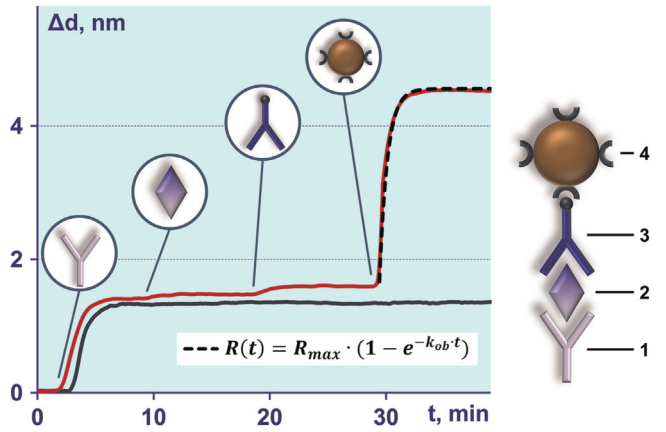

Fig. 3. Sandwich immunoassay for t-PSA: on the left – sensograms demonstrating all assay steps in absence of t-PSA (lower blue curve) and in presence of 1 μg/ml t-PSA (upper red curve); on the right – scheme of sandwich assay: 1 – capture antibody, 2 – antigen, 3 – biotinylated tracer antibody, 4 – magnetic nanoparticle coated by streptavidin. Dash line represents the curve fitting by Eq. 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and therapy control of hyperplasia, adenoma or prostate cancer [24–26]. Pathological anomalies of prostate gland structure usually cause drastic increase of t-PSA in blood, which can be registered in serum assays [27,28]. In most cases, t-PSA detection is not particularly demanding for high sensitivity of assays because its serum levels in healthy males may be as high as 4 ng/ml. However, even minimal t-PSA concentration in serum of patients after radical prostatectomy, X-ray or gland therapy, can indicate to the disease relapse [29–31]. To reveal and monitor t-PSA in these conditions, highly sensitive and precise quantitative methods are actively being developed nowadays.

3.2. Investigation of kinetic characteristics of MP

In the experiments, we used commercially available magnetic nanoparticles (Miltenyi Biotec, Germany). The particles of ~50-nm hydrodynamic size represent multiple iron oxide crystallites cores coated by a polysaccharide polymer. The particles are functionalized with bioreceptor molecules. Fig. 2 shows the image of these MP obtained with a transmission electron microscope JEOL JEM-2100 (accelerating voltage 200 kV).

The kinetic constants of interacting agents are measured by processing of the SCI sensograms, which are time dependences Δd(t) of binding reactions, using the equilibrium association model described in Section 2.4 of Materials and methods. The representative sensograms for all stages of a sandwich immunoassay with using MP as the labels for detection of t-PSA are shown in Fig. 3. The sensogram analysis yielded the following kinetic association constants: antigen binding – $(6.4 \pm 1.3) \times 10^4\ M^{-1}\ s^{-1}$, detecting antibodies binding – $(2.1 \pm 0.4) \times 10^4\ M^{-1}\ s^{-1}$, and magnetic particle binding – $(7.1 \pm 2.1) \times 10^8\ M^{-1}\ s^{-1}$. Accordingly, the kinetic constant for MP association is ~4 orders of magnitude higher than those for molecular entities. This difference is especially prominent taking into account that the observed association constant of each subsequent step is lower that the true constant due to partial dissociation of complexes of the previous steps. So high kinetic affinity of MP as compared to molecular antibodies and antigens can be due to the higher affinity of streptavidin to biotin (conjugated to MP) than that of monoclonal antibodies to antigen. Another possible and, perhaps more important, factor (also suggested earlier [21]) is the MP multivalency (each MP has a substantial quantity of active binding sites for the complementary ligand).

We should note that the most widely used biosensors, which allow investigation of kinetic parameters of nanoparticles, are based on SPR effect, which can only be observed on the surface of conducting gold and silver films. Since MP affinity characteristics may depend on interface surface chemistry used for immobilization of the affinity ligand, translation of the SPR results to other experimental setups may be tricky. The proposed method, though, allows deposition onto the cover slips of a wide variety of partially transparent films (polymer [32,33], carbon, etc.) or interface layers, i.e., it allows registration of molecular interactions directly on the glass surfaces as well as on other surface types typical for MP applications. This important advantage of the SCI method allows its employment for development of wide range of various platforms and substantially extends research opportunities for investigation of nanoparticles. The information regarding MP behavior and kinetic data obtained in the proposed setup can be easily transferred to a wide variety of experimental setups. For instance, the proposed method allows translation of all results onto label-based biosensoric systems, e.g., those employing highly-sensitive magnetic labels detection with compact electronic devices based on magnetic particle quantification (MPQ) by their non-linear magnetization [34–36]. Such magnetization of MP leds to frequency mixing which is very powerful tool allowed to observe very subtle magnetic phenomena in the presence of strong noise [37]. The MPQ is able interogate the magnetic labels outside the induction coils [38], for example, on the surface of simple disposable magnetic biochip having only the immunoreagents and MP on a substrate.

3.3. Signal amplification

The developed method, being of special interest for investigation and kinetic characterization of interaction of nanoparticles with molecules, offers exciting practical prospects. In particular, the developed optomagnetic biosensor based on the SCI with MP employment demonstrates high sensitivity and wide dynamic range for detection of antigens. Fig. 4 shows the representative dependences of changes of biolayer thickness Δd during MP passing stage on t-PSA concentration. The limit of t-PSA detection (LOD) determined by 2σ criterion was 92 pg/ml. At the same time,

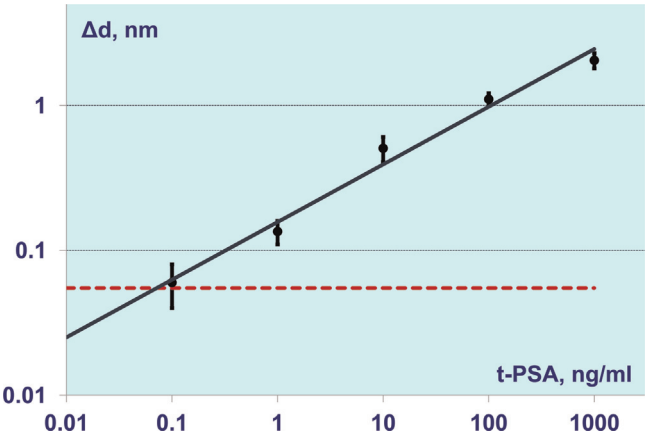

Fig. 4. Calibration curve for t-PSA detection with MP employed as labels: changes of biolayer thickness Δd depending on t-PSA concentration in log–log scale. Each data point is an average of three experiments and the error bars represent one standard deviation. Horizontal red dash line represents value that exceeds the negative control values by two standard deviations of negative control in the absence of the antigen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the label-free mode, when t-PSA is detected without signal amplification, i.e. directly at the stage of analyte passing, the LOD of 110 ng/ml was observed. Therefore, employment of MP improves the LOD of the assay by 3 orders of magnitude by signal amplification. A significantly higher biolayer thickness increase Δd at the stage of MP passing as compared to the stages of antigen and detecting antibody binding within the whole range of t-PSA concentrations is due to the substantially larger size of magnetic particles. The achieved t-PSA limit of detection of 92 pg/ml is clinically relevant for diagnostics of prostate cancer relapse [29,30]. The linear dynamic range of ~4 orders of magnitude (with respect to t-PSA concentration), allows quantitative determination of t-PSA levels of up to ~1000 ng/ml, which is relevant to the late stages of prostate cancer.

It should be noted that using of gold and latex particles in our experiments was less effective for signal amplification due to high nonspecific binding with glass sensor surface. At the same time using of small 50-nm MP leads to almost zero nonspecific binding. The absence of background signal could provide the increase of sensitivity by several amplification steps. In addition to low nonspecific binding MP carry the unique properties that can be used to further improvement of proposed optomagnetic biosensing method by acceleration of immunochemical reactions by magnetic stirring (rotation of MP chains by a magnetic field), enrichment of the sample with antigens by magnetic separation, decreasing of nonspecific binding of labels with the surface by removal of the loosely bound particles by a magnetic field ("magnetic washing" of the unbounded labels), etc.

To the best of our knowledge, the developed method offers higher sensitivity (slope of the calibration curve in logarithmic scale) than the majority of biosensors based on MP registration on plain surface. As can be seen from Fig. 4, the detectable signal (y-axis) increases by two orders of magnitude when t-PSA concentration changes from 0.1 ng/ml to 1000 ng/ml (x-axis), and the curve behaves linearly within the whole concentration range in log-log scale. Hence, the signal increases by a factor of $k \approx 3$ for every order of the concentration change. It should be noted that some flat biosensors that employ bigger 0.3–2.8 μm magnetic beads (MB) provided much better LOD, but at the cost of lower sensitivities (slopes) of calibration curves and lower ranges of detectable signals. For example, a remarkable LOD ~1 fg/ml was demonstrated by fluidic force discrimination assay and counting 2.8 μm magnetic beads by optical microscopy [39,40]. Although that method demonstrates wide dynamic range of more than 10 orders of magnitude (x-axis of analyte concentration), the 4-fold growth of signal (y-axis) is observed, so sensitivity (slope of calibration curve) is much lower: the recorded signal increases by factor of $k = 1.15$ per every order of concentration increase. The decrease of the bead size to 1 μm resulted in increasing the $k \leq 1.7$ for the "active assay" with electrophoretic collection of toxins on the surface [41] or for PSA detection by SPR and aggregates of 1 μm MB [42]. Taking into account the signal deviations, some authors report that the assay "is suitable only for a qualitative analysis giving positive signal" [41].

Another type of biosensors used MP on the flat surface is based on GMR [43]. New GMR structures protected by ultrathin 50-nm passivation layer were successfully used with small 50 nm MP [44] and demonstrated LOD which is 1000 times more sensitive than ELISA. Such GMR biochip with smaller MP featured higher $k = 2$-fold signal increase per 10-fold increase of antigen concentration [45].

Probably bigger magnetic particles cause a decrease of the slope of calibration curve due to steric hindrance on flat surface and nonspecific binding. Thus for particular applications of planar biochips the proper balance of assay characteristics such as LOD, sensitivity and dynamic range could be adjusted by changing size of magnetic particles.

As compared to the technologies mentioned above, higher sensitivity of the developed method may allow more accurate and reproducible assays for biomolecule detection for disease diagnostics, environmental monitoring, pathogen registration in food products as well as for investigation of affinity properties and binding kinetics of molecules and nanoparticles on various types of surfaces.

4. Conclusion

Here we propose a novel SCI-based method for real-time optical registration of kinetic interaction of 50-nm nanoparticles with biomolecules on a flat glass surface. As the demonstration of this method, the kinetic association constants were measured for every stage of the sandwich immunoassay for PSA detection, i.e. for antibody–antigen interaction in a molecular form, as well as for interactions of nanoparticles. The magnetic nanoparticles exhibited kinetic association constants of 4 orders of magnitude higher than those of molecular entities. Moreover, employment of the magnetic particles amplified the SCI-signals for a sandwich immunoassay resulting in more than 1000-fold enhancement of PSA limit of detection. In such setup, we achieved an attractive combination of a medically relevant LOD (92 pg/ml), high sensitivity (the detected signal increases ~3-fold for every order of concentration increase), and wide linear dynamic range (4 orders of magnitude of concentration). The results of kinetic characterization of nanoparticles and investigation of calibration curves behavior can be directly translated onto other biosensing platforms based on nanolabels.

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