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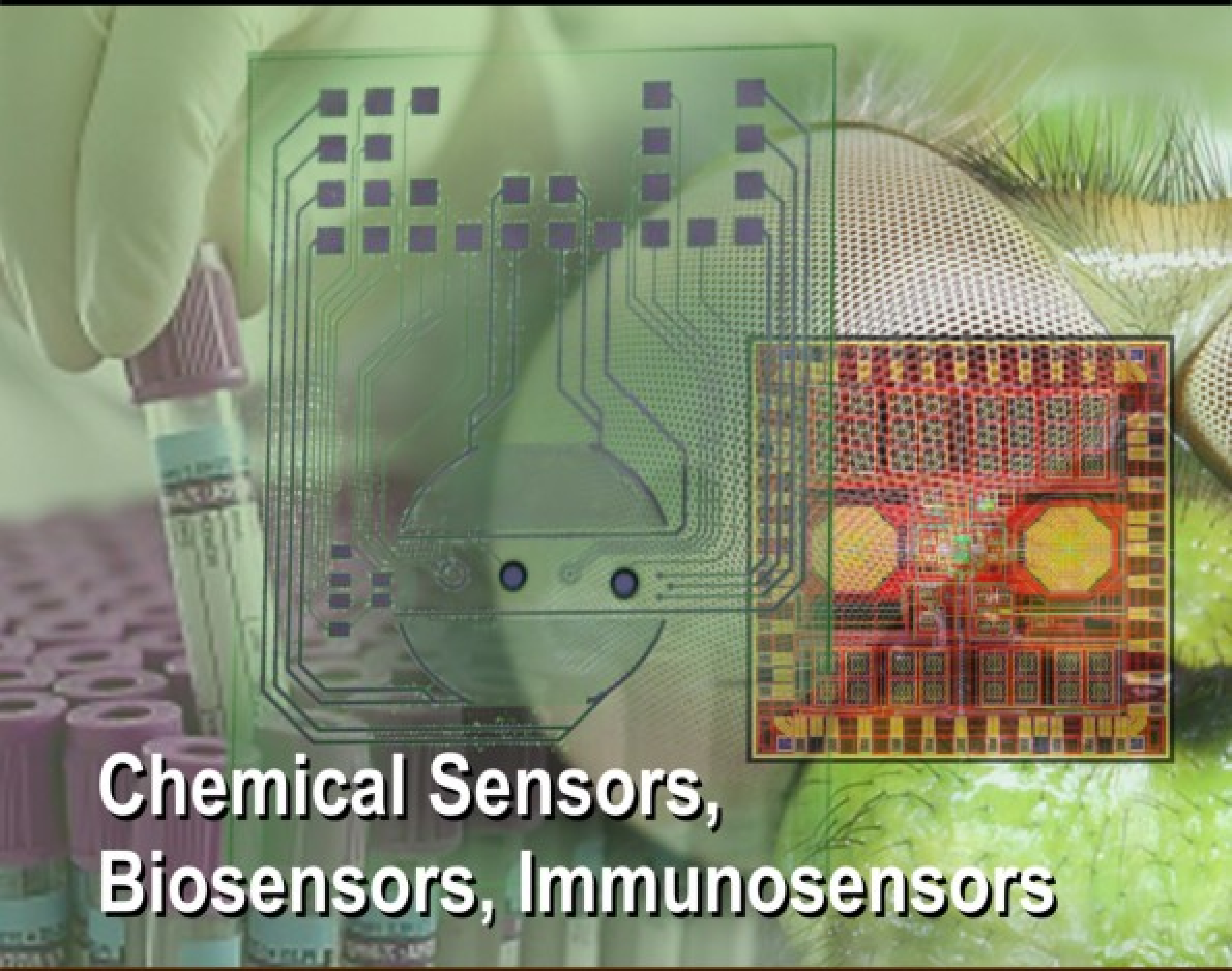

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SENSORDEVICES 2010:

The First International Conference on Sensor Device Technologies and Applications

July 18 - 25, 2010 - Venice, Italy

IARIA

The inaugural event SENSORDEVICES 2010, The First International Conference on Sensor Device Technologies and Applications, initiates a series of events focusing on sensor devices themselves, the technology-capturing style of sensors, special technologies, signal control and interfaces, and particularly sensors-oriented applications. The evolution of the nano- and microtechnologies, nanomaterials, and the new business services make the sensor device industry and research on sensor-themselves very challenging.

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- Sensor device technologies
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Important dates

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Notification: March 25, 2010
Registration: April 15, 2010
Camera ready: April 20, 2010

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- Microelectronics
- Electronics technologies
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Detection of a BSA-Biotin-Conjugate by a Novel Immunosensor

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Abstract: We describe a new kind of biosensor for the electro-magnetic sensing of analytes such as antibodies. In the present study the molecules bovine serum albumin (BSA) and the corresponding antibody (Anti-BSA) as well as biotin and NeutrAvidin are used as model systems. The analyte molecules are labelled with superparamagnetic microbeads and detected by sensor transducers with biofunctionalised microelectrodes. It is shown that a carboxyl-modified dextran (CMD) layer is well suited as an immobilization matrix for ligands on the microelectrode surface. The CMD layer offers a suitable and stable surface for binding of the antibodies and also suppresses unspecific binding of protein/antibody coated microbeads to the electroactive platinum surface. *Copyright© 2010 IFSA.*

Keywords: Microbeads, Superparamagnetic, Microelectrode, Intermolecular forces, Sandwich assay

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1. Introduction

Biosensors and particularly immunosensors based on different techniques and their response to specific molecules have been reported in recent experiments [1-5]. All these sensors utilize the very specific molecular recognition between ligands and receptors in biological systems. They can be used for diagnostic analysis and bioprocess monitoring [6] as well as for the characterization of intermolecular bindings [7-8]. The direct measurement of intermolecular forces for example between antibody-antigen pairs [9], complementary DNA strands and biotin-avidin pairs [10-11] has been mainly performed by atomic force microscopy (AFM) in recent years. However, such measurements are rather complex when performed in physiological solutions and simultaneous measurements of intermolecular forces on many individual molecules are not possible.

Such parallelized measurement can be performed using magnetic or electro-magnetic biosensors, as previously been reported by some groups [12-20]. In most of such experiments the analyte molecule is labelled with a magnetic label which is detected by the sensor by magnetic means. In this paper we describe a novel electro-magnetic biosensor that can detect superparamagnetic microbeads by electrical means. Compared to magnetic biosensors the electrical detection method is even simpler. In contrast, the biofunctionalisation of the transducer surface is more sophisticated. To ensure a high sensitivity of the sensor it is necessary that the biomolecules do only bind at predefined positions. When present, the analyte molecules should only bind directly at the functionalized microelectrodes and not at any other position on the sensor surface. In the following we will describe a method for the successful immobilisation of antibodies at the electrodes and present first results obtained with the biosensor.

2. Material and Methods

2.1. Reagents

2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-(3-Dimethylaminopropyl)-*N*´-ethylcarbodiimide hydrochloride (EDC), sodium acetate, sodium azide, *N*-hydroxysuccinimide (NHS), Tween 20 were obtained from Fluka (Germany). NeutrAvidin and Biotin binding protein, biotin-LC-hydrazide were obtained from Pierce (USA), monoclonal anti-bovine serum albumin antibody produced in mouse, bovine serum albumin (BSA) and chicken serum albumin (CSA) were obtained from Sigma (Germany), superparamagnetic Dynabeads M-450 were bought from Dynal (Hamburg, Germany).

2.2. Coating Latex Particles

In all experiments we used polystyrene microbeads 4.5 µm in size with epoxides as reactive groups for covalent immobilization. These microbeads contain superparamagnetic material and can therefore be magnetized by an external magnetic field. Proteins were immobilized onto the microparticles using a modified procedure from Kubitschko et al. [21]. 100 µl of the microbead suspension were taken from the original solution and washed several times with 0.1 M phosphate buffer. The washed microbeads were then resuspended into 100 µl of phosphate buffer. For immobilization, anti-BSA antibody was dissolved in DMSO and added to the particle solution. The microbeads were incubated for 20 hours at 4°C. After that, the antibody functionalized microbeads were washed three times with PBS, pH 7.4.

2.3. Electrode-Surface Modification and Protein Immobilization

The platinum electrodes of the transducer chip were cleaned by cyclic voltammetry in 1 M KCl solution. Subsequently the microelectrodes were coated with a 500 kDa carboxymethyldextran in brush-confirmation to minimize unspecific adsorption to the sensor chip surface. The dextran coated microelectrodes were then incubated in 20 µl freshly prepared solution of 0.5 M NHS, 0.5 M MES, pH 5.0 and 20 mM EDC to form amine reactive N-hydroxcsuccinimidyl ester. The activation mixture was incubated for 10 min at room temperature on an orbital shaker. NeutrAvidin was diluted in 2 mM sodium acetate, pH 4.5. The obtained concentration of the NeutrAvidin solution was 10 µg/ml. For covalent immobilization via amide bonds the sensor chip was incubated in the protein solution for 10 min. Unreacted NHS-esters of the activated CMD-layer were quenched with a 0.5 M ethanolamine solution (pH 8.5) for 20 min. Afterwards the chip was washed twice with PBS.

2.4. Sensor Concept

The sensor principle has been described in detail already in a previous paper [20]. It is based on the generation of an electrochemical current by biofunctionalized microelectrodes in solution and the detection of electrical current changes occurring when a biomolecule with an attached microbead label binds to or unbinds from such a microelectrode. The transducers we use have arrays of many platinum microelectrodes each having a diameter of 3 µm. After the biochemical functionalization of the microbeads and the transducer the measurement can be carried out. To distinguish whether the biomolecules (and thus the microparticles) are bound to the electrode specifically or unspecifically, we monitor the electrical current at a single microelectrode while we apply a constantly increasing magnetic force on the microparticles until the intermolecular bond is broken. Once the specifically or unspecifically bound analyte molecule becomes unbound the molecule and its microbead label is removed from the electrode by the external magnetic force. This causes an instantaneous increase of the electrical current from a low level to a high level. The force, at which the removal of the analyte molecule occurs, yields information about the nature of the binding. Thus, specific analyte binding can be distinguished from unspecific adhesion by the electro-magnetic biosensor.

2.5. Biochemical Functionalization of the Microelectrodes of the Immunosensor

One important part of this work was the biochemical functionalization of the microelectrodes. We used a carboxymethyl-modified-dextran (CMD) with a molecular weight of 500 kDa to form an immobilization matrix between the platinum surface and the immobilized protein. The usage of CMD layers for the immobilization of proteins has been described previously in different works [21-22]. Such a CMD layer fulfils two functions. It enables biomolecules such as antibodies or receptor proteins to bind covalently to the matrix and at the same time it leads to a reduction of unspecific adsorption of molecules due to the hydrophilic character of the dextran matrix. For our sensor system a sandwich-assay was established to detect a model analyte. For this application both the microelectrode surface and the microbead surface have to be functionalized.

3. Results and Discussion

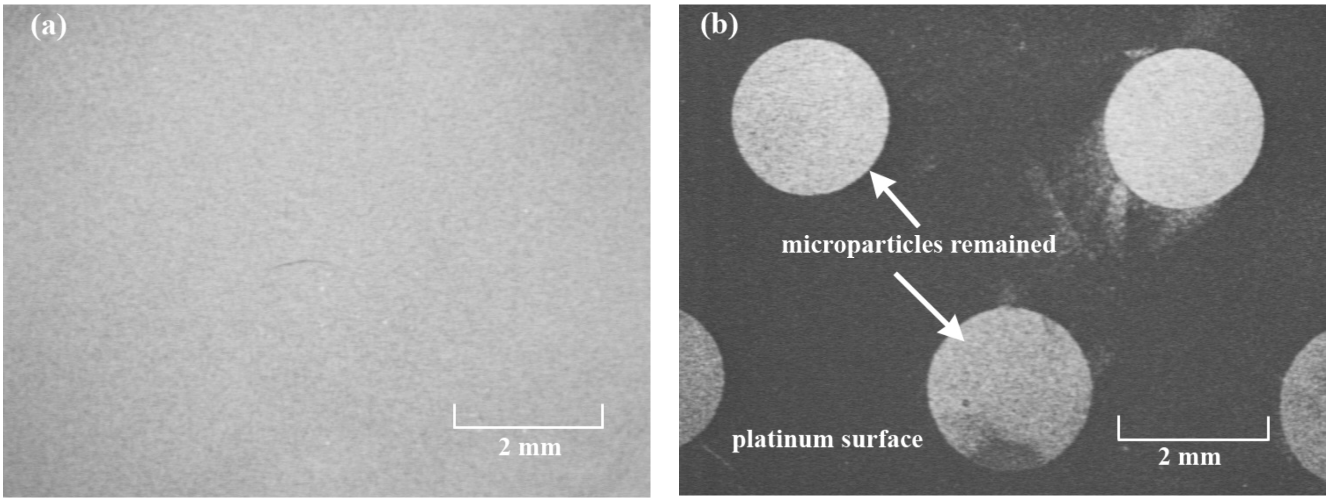
3.1. Immobilization Method

The specific immobilization of biological compounds (antibodies, proteins, DNA) on the sensor surface is a critical process. Immunoassays often use antibodies that have simply been adsorbed onto a solid substrate e.g. on microtiter plate wells. However, in the case of our sensor the resulting binding to the substrate would not be strong enough to withstand the magnetic forces used for the detection of specifically captured analytes. For this kind of immunosensor the capture molecules must be strongly attached, leaving the ligand-receptor bond as the weakest in the sandwich assay.

Furthermore, attachment of antibodies or ligands to substrates usually results in a loss of biological affinity due to inaccessibility for the analyte or structural changes e.g. in the region of the paratope of an antibody. A well designed covalent immobilization strategy can ensure that a great fraction of proteins remains fully functional. Thus the sensitivity of the immunoassay can be improved as well as the regeneration of the sensor for multiple measurements. Therefore the surface modification by carboxymethyl-modified-dextran which enabled a high yield of immobilized ligands in previous experiments [23-24] was investigated.

Before we started to functionalize the microelectrode chips we first investigated our sandwich assay on platinum coated glass wafers modified with a carboxymethyl-modified-dextran-layer (Fig. 1) to prove the surface derivatization strategy and the immobilization concept.

To assure that the results obtained with these macrochips were fully transferable to the micropatterned transducer chips both kinds of chips were run through the same fabrication processes. This means e.g. that the platinum surface shown in Fig. 1 was temporarily covered with a silicon nitride layer and has suffered reactive etching to remove this layer before the immobilisation experiments started.



(a) (b)

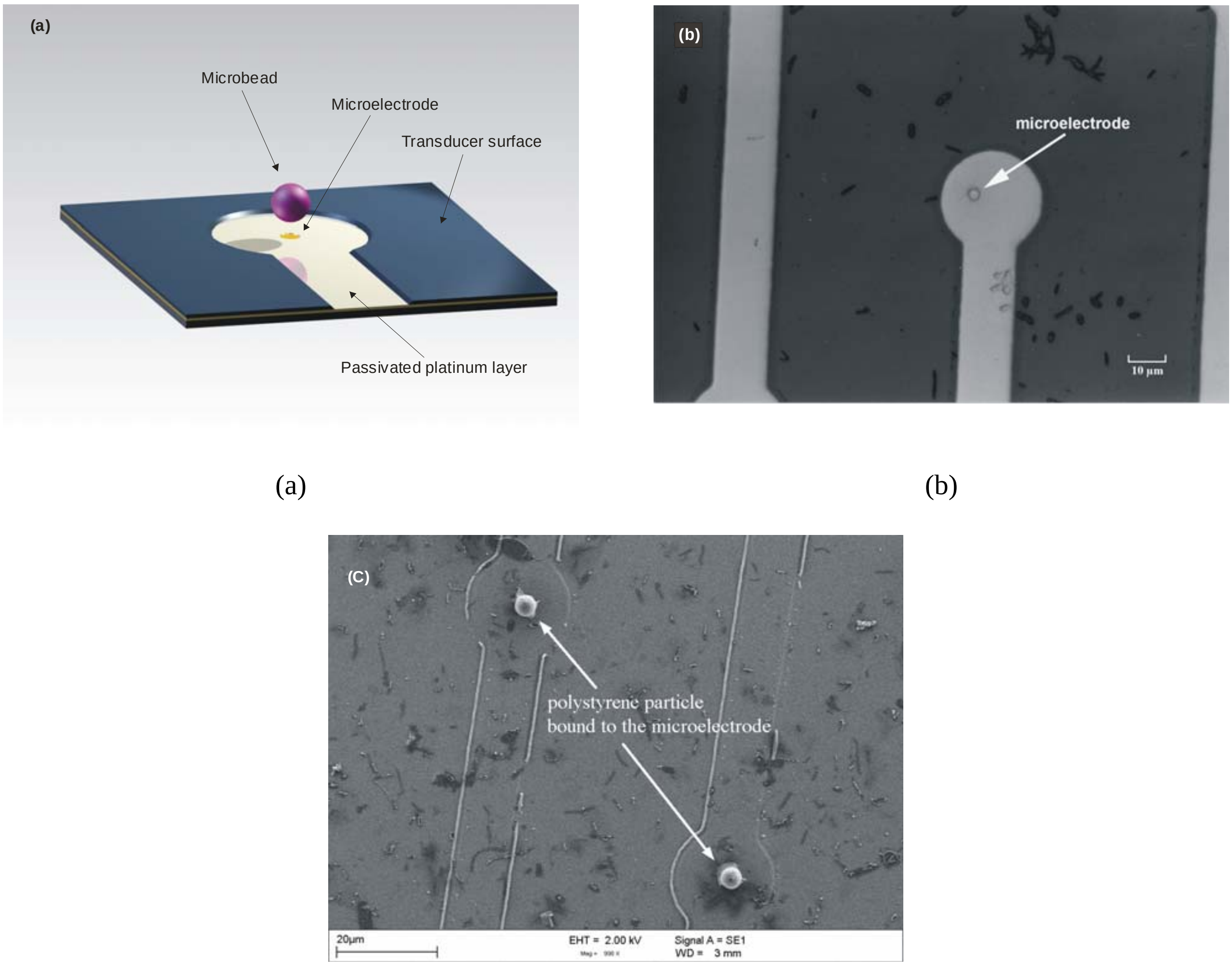
Fig. 1. Separation of specific from unspecific binding: Platinum surface with CMD coating demonstrating the hydrophilic character of the dextran layer to prevent unspecific binding (a) CMD-coated platinum surface fully covered with anti-BSA functionalized microbeads. The bright colour is caused by the huge number of microbeads. Before the particles were added some small areas (diameter = 2 mm) on the surface were functionalized with NeutrAvidin and the whole surface was subsequently incubated with a BSA-biotin conjugate; (b) After appliance of weak magnetic force only microbeads specifically bound to the activated areas remain (bright areas). Non-specifically bound particles are removed.

At first NeutrAvidin was covalently bound to certain areas of the modified platinum surface by spotting small droplets onto the surface. The whole chip was then incubated in a solution containing BSA-biotin conjugate as the analyte and afterwards one monolayer of anti-BSA-antibody modified microbeads was added to the functionalized surface and allowed to react with the BSA-biotin conjugate. Finally a very weak magnetic force was applied to remove unbound microbeads.

The result is shown in Fig. 1b. Although the whole surface was in contact with both the analyte and the subsequently added microbead solution, only small spots with microparticles remain after the appliance of the weak magnetic force.

The particles do only bind to the areas where the NeutrAvidin was immobilized. This experiment also demonstrates the hydrophilic character of the CMD layer which greatly prevents unspecific binding of analyte molecules to the not-activated part of the transducer surface. Only the particles bound via the antibody-antigen binding remain on the transducer after applying the magnetic forces. The CMD used for the hydrogel matrix contains one carboxylic group per anhydroglucose unit. In combination with the brush-like structure of the CMD-chains on the surface this results in more than one covalently bond of NeutrAvidin to the matrix which is strong enough to withstand the magnetic forces [25].

The next step was to transfer this technique to the micro-patterned transducer chip used for the sensor. This chip has an array of 100 microelectrodes and each of it has to be made sensitive by attaching capture molecules. At the same time the area between the array electrodes should be chemically inert to prevent unspecific adsorption of sample molecules. These transducers were also coated with the carboxymethyl-modified-dextran. After this modification the transducer surface was functionalized with NeutrAvidin. As described before the chip was then incubated in a solution containing BSA-biotin-conjugate. Subsequently one monolayer of anti-BSA-antibody functionalized microbeads was added to the chip. Then the chip was washed twice with PBS buffer and twice with water. The result is shown in Fig. 2.



(a) (b)

(c)

Fig. 2. Images showing NeutrAvidin functionalized microelectrodes incubated in BSA-biotin before and after adding anti-BSA-antibody modified particles to the sensor chip. (a) Schematic view of a section of the microelectrode array. One single microelectrode is shown together with a microbead that is close to attaching the electrode; (b) Microscopic image showing a passivated platinum conductor with one microelectrode upon it. Microbeads are not present; (c) A Scanning electron microscope (SEM) image of the sensor chip taken after adding functionalized microbeads and a subsequent washing step. Two microelectrodes are shown, each covered with one microbead. The two microbeads are bound to the microelectrodes via the described assay.

Fig. 2 a shows a scheme and Fig. 2b shows a microscopic image of a section of the electrode array. This picture shows one single microelectrode before adding microbeads to the chip. The platinum structure seen on the glass substrate is covered with insulating silicon nitride and the small hole in the insulator represents the active electrode surface.

In Fig. 2c a scanning electron microscope (SEM) image shows a part of the transducer surface after adding a monolayer of functionalized microbeads to the immunoassay chip and the subsequent washing step. Only two microbeads can be seen on the transducer surface. Each of the microbeads is indeed bound to one of the two microelectrodes and not to the passivation layer made of silicon nitride. Thus, the selective functionalization of the small electrodes was successful.

3.2. Sandwich Assay

The investigation of the sensor was mainly focused on the immobilization of the compounds of the sandwich assay. To optimize both the immobilization conditions of NeutrAvidin to the surface and the binding conditions of the analyte, we studied the assay by Surface Plasmon Resonance spectrometry (SPR) under various conditions. Surface Plasmon Resonance spectroscopy is a sensitive optical technique that can be used to study adsorption or binding reactions on noble metal films. Based on measuring the change in refractive index near to the metal surface, for example caused by the binding of analyte to an immobilized ligand, a real-time and label-free measurement is possible to determine e.g. binding constants and equilibrium state of a biological reaction. The surface plasmon resonance effect is based on the interaction between electromagnetic waves of incident light and free electrons in a conducting surface layer. When light is reflected at the interface of two dielectric media, total internal reflection will take place above the so-called critical angle. At the same time, the light will generate an electromagnetic field, called evanescent field, which has maximum intensity at the surface of the dielectricum. If a noble metal layer is present in the evanescent field, this field can be enhanced and the free electrons on the outer surface can resonate with the evanescent field. This phenomenon is called surface plasmon resonance. If resonance conditions are fulfilled e.g. by variation of the angle of incoming light, this light will be transferred to surface plasmon waves and a decrease of reflected intensity can be observed. The angle at which this decrease occurs is called the resonance angle. Changes of the refractive index, respectively the dielectric constant, near to the sensor surface, result therefore in a change of the resonant angle (m°). For biological interaction analysis (BIA) it is state of the art to coat the noble metal surface with carboxy modified dextran polymers (CMD) in three-dimensional structure (brush-confirmation) to prevent biomolecules from unspecific adsorption to the surface and to allow a directed and covalent immobilization of the ligand.

For our particular investigation SPR - sensorchips with CMD500 with a brush like surface structure were used. The dextran layer of the SPR-chips is equal to that of the coated microelectrodes. Experimental SPR-results for the sandwich assay are shown in Fig. 3.

After establishing stable baselines with PBS and 2 mM sodium acetate buffer (pH 5.0) the activation step with 0.5 M NHS, 0.5 M MES and 0.5M EDC, pH 5.5 was carried out for 7 min. Subsequently the immobilization was performed by injecting 20 µg/ml NeutrAvidin in 2 mM sodium acetate buffer (pH 5.0). The slope after the injection of NeutrAvidin is ascribed to the electrostatic pre-concentration effect of the negatively charged CMD-matrix and the over all positive net charge of NeutrAvidin in immobilizing buffer. The following surface treatment with ethanolamine quenches all remaining reactive succinimidyl esters. The strong increase of the baseline in the sensorgram in cases of injection of activation solution and quenching solution is caused by the change in refractive indices of these solutions.

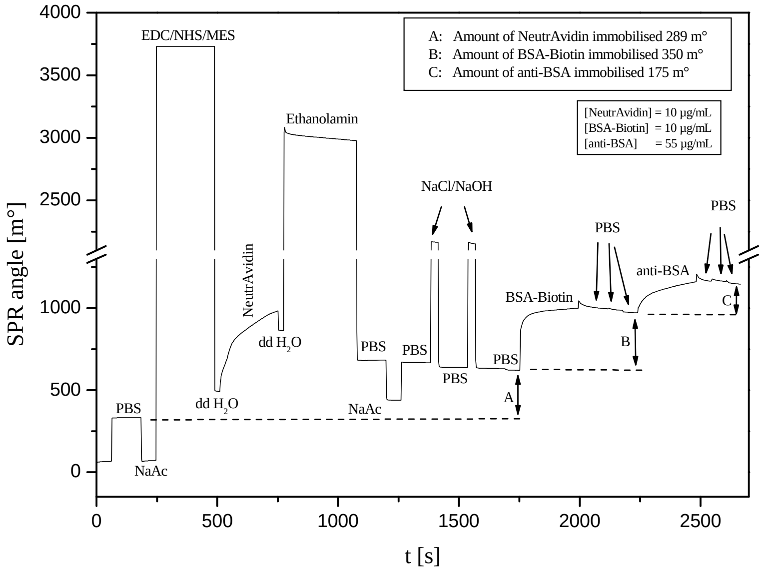


Fig. 3. Characterization of sandwich assay by SPR-measurements. The amount of Immobilized protein is characterized by the increase of the signal relative to the specific baseline (A, B and C).

In Fig. 3 the difference between the baseline signal before and after immobilization (A) is a measure of the amount of immobilized NeutrAvidin. After the immobilization the biological interaction analysis with BSA-biotin conjugate as the analyte and subsequent anti-BSA-antibody (coated magnetic microspheres) as the detector agent can be carried out in PBS.

The two association curves with following dissociation phases b) of BSA-biotin conjugate and c) of anti-BSA-antibody (both in PBS) show clearly binding to their ligands.

In case of BSA-biotin-conjugate association to immobilized NeutrAvidin a rapid binding ($K_a=10^{15}$ M^{-1}) to the ligand could be observed. Saturation of free binding sites was reached after approx. 300 s due to the fact that sterical hindrance from bound BSA-biotin conjugate in the top of the hydrogel caused a diffusion limitation from analyte to NeutrAvidin immobilized close to the sensor surface. The dissociation phase shows no significant dissociation.

After two injections of PBS the antibody against BSA was added in a concentration of 55 µg/ml in PBS. The association curve differs significantly from that of biotinylated BSA. After 500 s of incubation the endpoint of the assay was not reached which shows the lower association constant in contrast to the NeutrAvidin-biotin system. A significant dissociation was also not observable.

These SPR experiments have proved the feasibility of the sandwich assay for our sensor concept.

The optimized immobilization strategy on the brush-like CMD-hydrogel with its low unspecific adsorption of proteins under physiological buffer conditions as well as the use of biological components with different but high association constants (K_A) in the assay are the main factors to assure that the specificity is high enough and the bond between functionalized magnetic particle and analyte is the predetermined breaking point.

After the successful operation of the sandwich assay has been proved with the SPR detector the results were transferred to the microelectrode sensor. With the knowledge of the reaction conditions from the SPR-experiments we have functionalized both the sensor chip surface and the microbead surfaces. For these experiments we have functionalized the sensor with NeutrAvidin according to the optimized immobilization protocol. The superparamagnetic microbeads were functionalized with anti-BSA-antibody according to manufactures protocol [26]. After mounting a sensor chip into a small measurement cell it was incubated for 10 min with a solution containing the analyte molecules. Afterwards this solution was replaced by a buffer solution containing electroactive compounds and subsequently the electrochemical measurements were started. After a certain time functionalized microbeads were injected into the measurement cell and allowed to settle on the chip surface. Those microbeads that came into contact with a microelectrode could be bound via the antibody-antigen binding. As described in a previous paper the electrochemical current drops to a lower level when a microbead attaches a microelectrode [20].

After certain incubation time is was proved if antibody-antigen recognition had indeed occurred. For this purpose a very low magnetic force was switched on and steadily increased until the microbeads were removed from the electrodes. Several of such experiments were performed with different analyte concentrations including negative controls as well. Starting at analyte concentrations of 100 ng/ml we have lowered the analyte concentration to values of 10 ng/ml, 1 ng/ml and finally to only 10 pg/ml. Typical results obtained from measurement with analyte and without analyte present are plotted in Fig. 4.

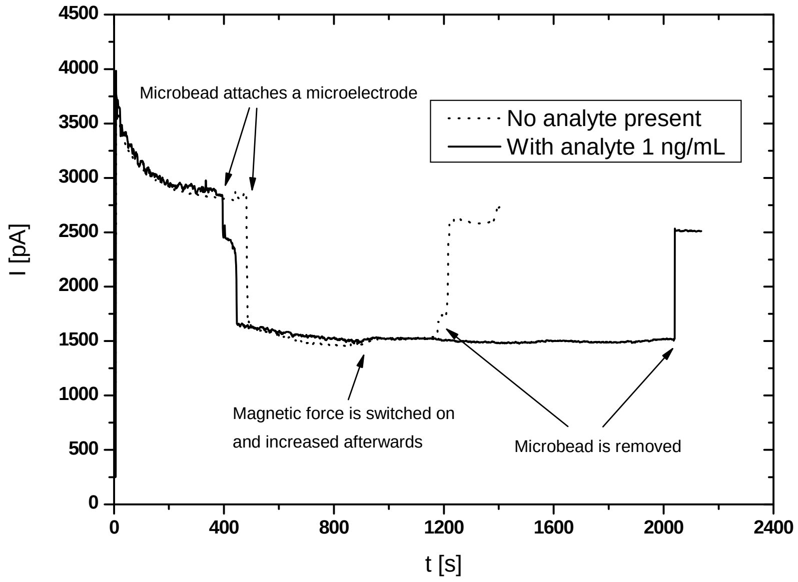


Fig. 4. Electrical current of a single microelectrode for absence or presence (1ng/ml) of analyte molecules. After appliance of the magnetic force at t=900s the force is increased non-linear (exponential-like) with increasing time.

For simplification the electrical signal of only one single microelectrode is shown. In fact one has to detect the current of many microelectrodes to get significant results.

When a microbead attaches a microelectrode the current drops from a value of 2900 pA to around 1500 pA. After an incubation time of 400 s the magnetic field was switched on (t=900 s) and steadily increased (non-linear increase).

Without analyte molecules (BSA-biotin conjugate) present, the microparticle is removed from the microelectrode already at a very low magnetic force (current jump at t = 1176 s). By contrast, nearly a hundred fold higher force is necessary to remove the microbead when a concentration of 1 ng/ml analyte is present (current jump at t = 2050 s). Measurements with different analyte concentrations revealed similar results, however, the force difference for non-specific binding (no analyte present) and specific binding is lower at lower analyte concentrations. Below a concentration of 10 pg/ml BSA-biotin it was not possible to differentiate unspecific and specific binding with our sensor.

To verify whether the sensor can differentiate between complementary and non-complementary analyte, we exchanged the analyte molecule. Instead of BSA-biotin we used CSA-biotin which should not bind specifically to the anti-BSA functionalized microbeads.

Several of such measurements with CSA-biotin revealed extremely low binding forces between CSA and anti-BSA (data not shown). Shortly after a very low magnetic force was enabled, the microbeads were removed from the microelectrodes. Furthermore, the variation of the measured binding forces was very low, which is similar to the case when no analyte is present. This shows that CSA was indeed not bound to the anti-BSA functionalized microbeads. Additionally it reveals that unspecific adsorption of molecules which is a frequent cause for false sensor results in many different types of sensor systems seems not to be a big problem when CMD coated microelectrodes are used. The experiments prove that the sensor system is sensitive only for the specific analyte (BSA).

4. Conclusion

In this work we describe a novel electro-magnetic biosensor using superparamagnetic microbeads as labels for the analyte molecules. Although the development of the sensor is still in its early stage, the sensor promises an easy way to detect the presence of analytes of low concentrations. To prove this we prepared a sandwich assay to detect biotinylated BSA as a model analyte. It has been shown that carboxyl-modified dextran can be used as an immobilization matrix for ligands on the microelectrode surface and that the CMD layer offers a suitable and stable surface for binding force measurements with our sensor system. Furthermore, the CMD layer prevents unspecific binding of antibody coated microbeads to the electroactive platinum surface. Both aspects are essential for a sensor system within a complex sample matrix, e. g. in clinical or food analytics or for a system which is reusable. By using the carboxylated-dextran layer the functionalized microbeads can be bound to the microelectrodes specifically by antibody-antigen-binding. Distinguishing between complementary and non-complementary analytes is easily possible by applying a certain magnetic force on the microbead labels.

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