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Voltammetric Detection of Ovalbumin at Screen-Printed Electrodes in Combination with Immunomagnetic Particles

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Abstract: Possibility of determination of ovalbumin as a model protein based on ELISA principle is reported in this contribution. Model system consisted of anti-ovalbumin IgG (primary antibody), ovalbumin and anti-ovalbumin-HRP (secondary antibody). After the addition of substrate (hydrogen peroxide), current decrease induced by its conversion by HRP has been detected by linear sweep voltammetry. Superparamagnetic microspheres were used for covalent immobilization of primary antibodies, which enables better antigen recognition compared to common sorption onto electrode surface.

Keywords: Voltammetric detection; Biosensor; Ovalbumin; Magnetic microspheres; Screen-printed electrodes; ELISA.

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Introduction

Nowadays, the detection of low concentrations of biologically important compounds in complex biological material is of great importance. Additionally, there is a continuous push to miniaturize and integrate as many laboratory instrumentation pieces as possible to one tube.

Electrochemical immunosensors become attractive due to good availability, simple use, fast analysis, low detection limits and possibility of miniaturization [1-4].

Combination of immunosensors with magnetic particles can prevent the poor regeneration and reproducibility (important factors in clinical and biomedical applications) in direct antibodies sorption onto electrode surface commonly used in this area of applications [5]. Furthermore, sensitivity of immunosensor in determination of low antigen amount could be increased by oriented immobilization resulting lower steric barriers for antigen recognition [6]. Although the immunosensors could be regenerated either by immunocomplex degradation using chaotropic agents or glycine buffer (pH 2,5) [7] or by complete elimination of the whole immunocomplex from electrode surface [8], both methods could negatively affect further use.

The use of microspheres represents relatively new option in immunoanalysis where magnetic particles are used as a supports for immobilization of primary antibodies or as a labels of secondary antibodies [9]. Magnetic particles of micrometer size enable highly efficient separations of target biomolecules (enzymes, proteins, antibodies) due to their large specific surface area for covalent binding and narrow size distribution ensuring homogeneous properties [10-14]. Magnetic electrochemical biosensors offer also possibility of analyte preconcentration, separation and accumulation on the surface of transducer by magnetic field [15]. Commonly used transducers are amperometric, potentiometric or conductometric [16].

This work deals with construction of electrochemical immunosensor for detection of ovalbumin which is based on Enzyme-Linked ImmunoSorbent Assay (ELISA) principle based on formation of specific immunocomplex between primary antibody and determined protein (antigen). Afterwards, the formation of immunocomplex is visualized by secondary antibody (enzymatically labeled) added to the immunocomplex. After addition of specific substrate the intensity of acquired signal is electrochemically monitored. In this work, the examined system consisted of polyclonal rabbit anti-ovalbumin IgG (primary antibody), protein ovalbumin (determined antigen) and secondary rabbit anti-ovalbumin IgG labelled with horseradish peroxidase.

All above mentioned advantages of combination of magnetic microspheres could be increased with the use of the screen-printed electrodes. Screen-printed electrodes (SPEs) avoid some of the common problems of classical solid electrodes, such as memory effects. Extensive range of forms of modification of SPEs opens a great field of applications for these electrodes. SPEs produced in large quantities by the printing of different inks on various polymer or ceramic supports are particularly advantageous for their relatively high sensitivity, defined electrochemical properties, portable size and low cost [17].

In this contribution, commercially available screen-printed three-electrode sensors comprising of the reference Ag/AgCl electrode and platinum auxiliary and working electrodes were used for sensing the electroactive product formed after enzymatic conversion of appropriate substrate.

Experimental

Chemicals

2-Morpholinoethane-1-sulfonic acid (MES), TWEEN 20*,* horseradish peroxidase (HRP), chicken egg albumin (ovalbumin, OVA) and bovine serum albumin (BSA) were from *Sigma-Aldrich* (St. Louis, USA). Affinity purified rabbit anti-ovalbumin (anti-OVA) IgG and rabbit anti-ovalbumin HRP labeled anti-ovalbumin (anti-OVA-HRP) were from *Patricell Ltd.* (Nottingham, UK) and all other chemicals were supplied by *Penta* (Chrudim, Czech Republic) and used without further purification.

Magnetic Particles

SiMAG-PGL with -CHO functional groups (1 μm diameter) were obtained from *Chemicell GmbH*, (Berlin, Germany), PGMA with -COOH functional groups (5 μm diameter) as well as HPM-07-S-M64 with $-SO_3^-$ functional groups (3,9 μm diameter) were newly developed at the *Institute of Macromolecular Chemistry, Academy of Science* (Prague, Czech Republic).

Apparatus

Compact electrochemical sensor interface PalmSens with PSTrace 2.1 software (*Palm Instrument BV*, Houten, Netherlands) has been used. Measurements were performed with commercially available miniaturized screen-printed sensors. Finally, two types of electrodes were elected, namely sensors with platinum working, Ag/AgCl reference and platinum auxiliary electrode produced by *BVT Technologies* (Brno, Czech Republic) and by *BST* (Berlin, Germany).

Immobilization of Primary Anti-Ovalbumin Antibodies on Magnetic Particles

For immobilization of primary antibodies, 1 mg of magnetic particles was five times washed with 0.1 M MES buffer pH 5.0. PGMA magnetic particles were incubated with 7.5 mg EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) in 500 μl MES buffer 10 min at room temperature under mild shaking.

After washing, the solution of primary anti-ovalbumin IgG (100 μg) in MES buffer (1 ml) was added. The immobilization proceeded over night at 4°C under mild shaking. In case of SiMAG-PGL and HPM-07-S-M64, autoreactivity of surface functional groups was utilized, hence after washing of 1 mg of particles five times with 0.1 M MES buffer pH 5.0, the solution of primary anti-ovalbumin IgG (100 μg) in MES buffer (1 ml) was added with subsequent 3 hour incubation at room temperature. After immobilization, all particles were washed five times with 0.1 M MES buffer pH 5.0 and non-specifically adsorbed molecules were removed with 0.05% trifluoroacetic acid (TFA; 200 μl) at room temperature for 5 min. The suspension was then washed five times with 0.1 M MES buffer pH 5.0. The immobilization efficiency was estimated by electrophoretic separation in polyacrylamide gel (SDS-PAGE) according to the standard protocol.

Electrophoresis was performed on a linear 12% SDS/polyacrylamide gel 0.75 mm thick. Samples were mixed with Laemmli sample buffer (1:1 v/v) and boiled at 100 °C for 2 min. SDS-PAGE proceeded in a Mini-PROTEAN electrophoresis cell (*Bio-Rad*, Philadelphia, USA) at 180 V with Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0,1 % w/w SDS). Gels were stained by silver staining method [2].

Electrochemical Detection

Measurement of Hydrogen Peroxide. Solutions of hydrogen peroxide were prepared in concentration range 1; 5; 10; 15; 20 and 25 mg l^{-1}. Each concentration was measured by one screen-printed sensor. The sensor was immersed into 800 μl of solution of hydrogen peroxide solution and then the current value at maximum peak potential was measured in every 5^{th} minute by anodic scan via linear sweep voltammetry in potential range 0 – 1 V, with potential step 5 mV, equilibration time 2 s and scan rate 0.1 V s^{-1}. The resulting graph was interpreted by software PalmSens PC.

Antigen Detection. Magnetic particles with immobilized primary anti-ovalbumin IgG (20 μg of anti-OVA) were five times washed with 0.1 M MES buffer pH 5.0 and solution of antigen ovalbumin (OVA) of appropriate concentration (1-60 μg) prepared in 0.1 M phosphate buffer pH 7.0 (500 μl) was added. After incubation at room temperature for 45 min under mild shaking, particles were five times washed with 0.1 M phosphate buffer pH 7.3 and 200 μl of the solution of secondary antibodies (anti-ovalbumin IgG/HRP) diluted 1:20 000 with 0.1 M carbonate buffer pH 9.49, 0.1 % BSA, and 0.05 % Tween 20 was added. The suspension was incubated at 37 °C for 1 hour under mild shaking. Non-specifically adsorbed secondary antibodies were washed five times with distilled water, then 800 μl of 1 mg l^{-1} hydrogen peroxide (diluted with 0.1 M phosphate buffer pH 7.3 with 0.15 M NaCl) was added and the current decrease in time (15 min totally) was measured by screen-printed sensor (BST) on a PalmSens interface [2].

Results and Discussion

Electrochemical Sensors

In this contribution, preparation of magnetically active electrochemical immunosensor for ovalbumin detection based on ELISA principle (Fig.1) is mentioned. Due to the necessity of analysis in sample amounts or volumes as low as possible, screen-printed three-electrode sensors comprised of working (Pt), reference (Ag/AgCl) and auxiliary (Pt) electrode were used. These types of sensors enabling the measurement in volume of sample less than 1 ml. Two types of commercially available sensor were tested, from which BST ones were the most suitable since they are characterized by a stable electrochemical signal for all individual electrodes and with minimal fluctuations in current response among them.

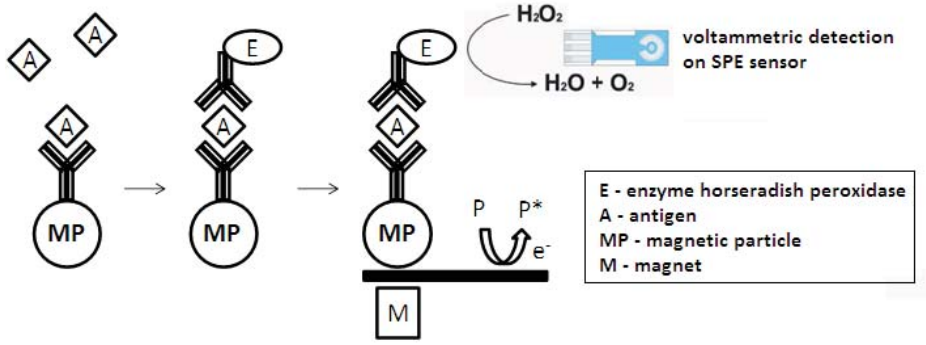

Fig. 1: *Arrangement of the system for ovalbumin detection based on ELISA principle in combination with electrochemical detection.*

Essential parameters of analysis as a selection of convenient SPEs sensors, the most suitable detection technique and other measurement conditions (buffer, pH) were tested by the measurement of substrate hydrogen peroxide calibration also due to fact that used secondary antibodies are labeled with horseradish peroxidase (HRP). Substrate concentrations were selected in range 1 – 25 mg l^{-1}; 1, 5, 10, 15, 20 and 25 mg l^{-1} and measured by linear sweep voltammetry (Fig. 2a) on screen-printed sensors with Pt working electrode made by BVT and BST using PalmSens potentiostat.

Evaluation of obtained curves has been done by reading the current value (μA) at a maximum peak potential resulting in appropriate calibration shown in Fig. 2b.

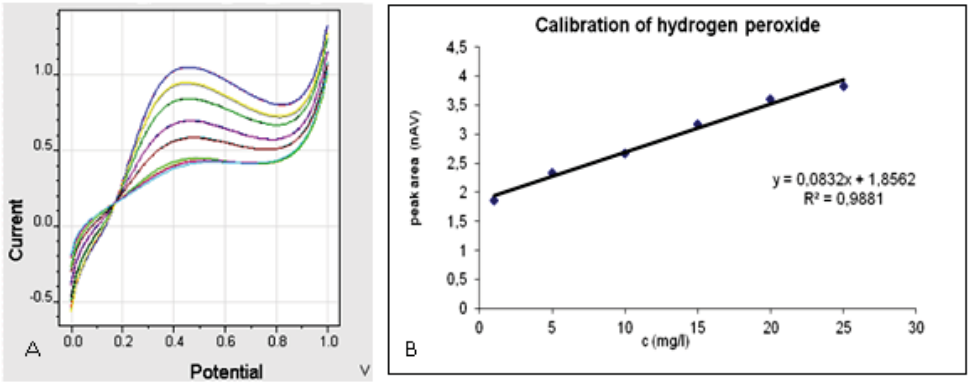

Fig. 2: **A**) *LSV voltammograms of hydrogen peroxide*. 1 – 25 mg l^{-1} H_2O_2 in 0.1 M phosphate buffer pH 7.3 with 0.15 M NaCl; BST sensor Pt-Ag/AgCl-Pt; LSV, potential range 0 – 1 V, scan rate 0.1 V s^{-1}. **B**) *Calibration curve of hydrogen peroxide detection*.

During the utilization of BVT sensors, low reproducibility and operational stability was observed in comparison with BST sensors. Therefore, BST sensors were determined to be suitable for our purpose.

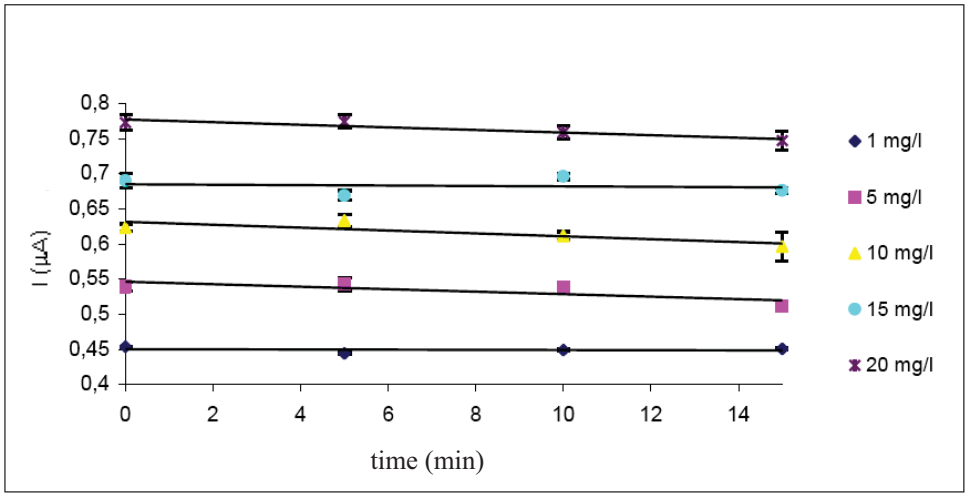

Fig. 3: *Operational stability of screen-printed sensors*. Evaluation of peak currents from LSV; 1-20 mg l^{-1} H_2O_2 in 0,1 M phosphate buffer pH 7.3 with 0.15 M NaCl; BVT sensor Pt-Ag/AgCl-Pt.

The best stability of electrochemical signal was observed for the lowest concentration of hydrogen peroxide 1 mg.l^{-1}. There is an apparent decrease of current value in time at higher concentrations.

Finally, the target system for ovalbumin detection was based on immune complex formation anti-ovalbumin – ovalbumin – anti-ovalbumin^{HRP}. In the last step, HRP consumes the added hydrogen peroxide (substrate) and its decrease is subsequently detected electrochemically. For higher sensitivity of antigen recognition, primary antibodies anti-OVA IgG were covalently bound onto the magnetic microspheres instead of pure adsorption to the electrodes used for detection. Today, various kinds of magnetic particles are commercially available, but they do not always meet all the demands needed for the specific application. One of the most important parameters is exact composition of particles and especially superparamagnetic core, embedded in the polymer shell. Substrate is then protected from the contact with iron oxides and possible interference from electrocatalytic oxidation of hydrogen peroxide induced by the presence of oxides on surface are suppressed.

For immobilization of primary anti-ovalbumin IgG, the three kinds of magnetic particles were used; one commercial SiMAG-PGL with autoreactive aldehyde groups on the surface and two types of newly developed magnetic particles, namely polyglycidyl-methacrylate particles (PGMA) with carboxylic groups and hypercrosslinked polystyrene particles (HPSM-M4-SO_3^-) with sulfo groups. Autoreactivity of surface active groups (SiMAG-PGL and HPSM-M4-SO_3^-) was employed for the formation of covalent bond. In case of PGMA particles, standard carbodiimide method was applied according to the instructions of the producer.

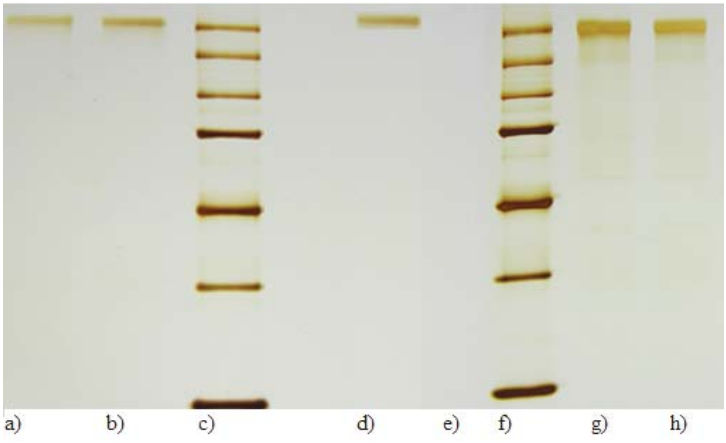

Fig. 3: *SDS-PAGE of anti-OVA antibodies immobilized onto magnetic particles*. **a**) PGMA supernatant after immobilization; **b**) original anti-OVA IgG; **c**) molecular marker (10 – 250 kDa); **d**) original anti-OVA IgG; **e**) HPM-07-S-M64 supernatant after immobilization; **f**) molecular marker (10 – 250 kDa); **g**) original anti-OVA IgG; **h**) SiMAG-PGL supernatant after immobilization.

The exact protocol for immobilization is mentioned in experimental part. The efficiency of immobilization was estimated by SDS-PAGE (Fig. 3). The most effective immobilization was onto HPM-07-S-M64 magnetic particles, which were therefore used for the construction of the whole immunosensor. The efficiency of antibody immobilization was estimated to 98 % (Fig.3).

Prepared immunosorbent was used for isolation of antigen ovalbumin with subsequent detection by secondary antibodies anti-OVA-IgG^{HRP}. For calibration of antigen and determination of the detection limit, solution of ovalbumin in range of concentrations between 1 and 60 µg was added to magnetic particles, containing 20 µg of primary antibodies. After 1 hour incubation necessary for immunocomplex formation, secondary antibodies anti-OVA^{HRP} were added. The optimal dilution of secondary antibodies was determined in previous experiments. In our case, commonly used ELISA method with spectrophotometric detection found the dilution 1:20000 as an optimum.

The main detection of formed immunocomplex was based on electrochemical in-time monitoring of the decrease of current response due the consumption of hydrogen peroxide (800 µl solution, 1 mg l^{-1}) by horseradish peroxidase (Fig. 4). Measurement of response was performed every 5th min after 4 min of incubation with substrate solution and 1 min separation of particles on the magnetic separator. Total analysis time was 15 min. The same procedure was also used for the blank (pure particles only).

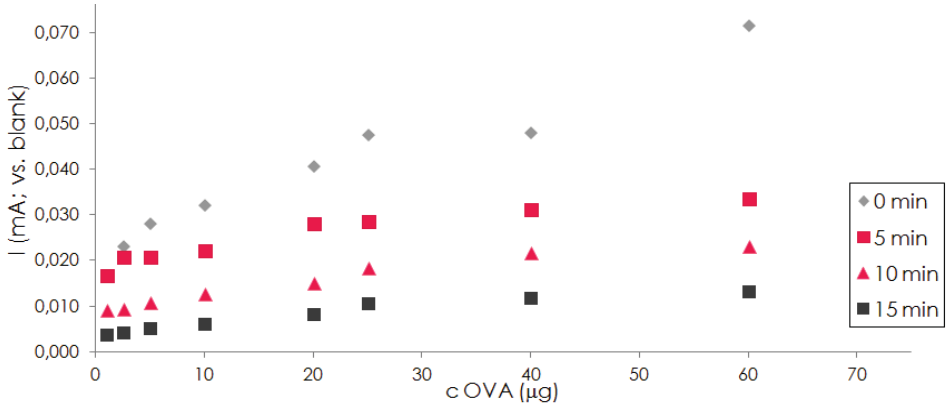

Fig. 4: *Decrease of H_2O_2 current response in presence of immunocomplex labelled with HRP.* 1-60 µg of ovalbumin; dilution of conjugate 1:20 000; 1 mg l^{-1} H_2O_2 in 0.1 M phosphate buffer pH 7.3 with 0.15 M NaCl; BST sensor Pt-Ag/AgCl-Pt; LSV, potential range 0 – 0.8 V.

It is evident from the results that the current decrease not only in time, but the rate of the decrease is also depending on the antigen concentration. This suggests that the assembled sandwich system of anti-ovalbumin - ovalbumin - anti-ovalbumin^{HRP} is fully functional and it is possible to detect as low as 2 µg of antigen using proposed method (Fig. 5).

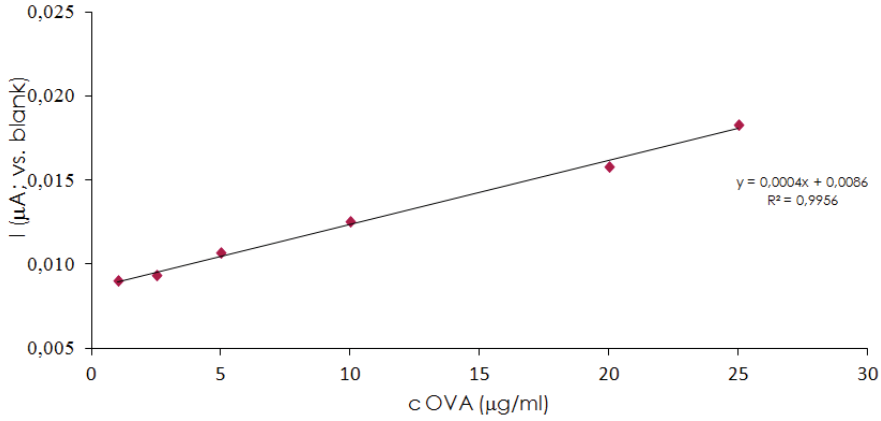

Fig. 5: *Calibration of ovalbumin with designed electrochemical immunosensor*. 1-25 µg ovalbumin; LSV, current reading after 10 min of incubation; other experimental conditions same as in Fig. 4.

Conclusions

The aim of this work was preparation of an immunomagnetic biosensor used for protein detection, which is based on specific capture of target antigen by immunocomplex formation on magnetic particles. The use of magnetic microspheres is suitable for easy separation from the sample using magnetic field and it prevents disadvantages of direct antibody sorption to the electrode surface.

Moreover, the use of screen-printed three-electrode sensors with platinum working electrode enables the analysis in one tube and in volume less than 1 ml. Using the elaborated method, up to 2 µg of antigen could be successfully detected. Finally, the anti-ovalbumin – ovalbumin – anti-ovalbumin labelled by HRP system served as a model example with a high potential to utilize it in the detection of any other protein after slight modification to the method and use of proper antibodies, labeled with suitable enzyme.

Acknowledgements

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