VILNIUS UNIVERSITY
CPST INSTITUTE OF CHEMISTRY

JUSTINA RUKŠNAITĖ

SURFACE PLASMON RESONANCE AND ELECTROCHEMICAL IMUNOSENSORS FOR HUMAN GROWTH HORMONE AND ANTIBODIES AGAINST HUMAN GROWTH HORMONE DETECTION

Summary of Doctoral Dissertation
Physical Sciences, Chemistry (03P)

VILNIUS, 2014

The research was carried out in Department of Analytical and Environmental Chemistry, Faculty of Chemistry, Vilnius University, in the period of 2009 – 2013.

Scientific supervisor:
Doc. Dr. **Asta Kaušaitė-Minkštimienė** (Vilnius University, Physical Sciences, Chemistry – 03P).

The defence council:

Chairman:

Prof. Dr. **Stasys Tautkus** (Vilnius University, Physical Sciences, Chemistry – 03P)

Members:

Prof. Habil. Dr. **Audrius Padarauskas** (Vilnius University, Physical Sciences, Chemistry – 03P)

Prof. Habil. Dr. **Saulius Balevičius** (State Scientific Research Institute Center of Physical Sciences and Technology, Physical Sciences, Physics – 02P)

Dr. **Daumantas Matulis** (Institute of Biotechnology, Physical Sciences, Chemistry – 03P)

Dr. **Sigita Jurkonienė** (Nature Research Centre, Institute of Botany, Biomedical Sciences, Biology – 01B)

Official opponents:

Prof. Dr. **Genė Biziulevičienė** (State Research Institute Center for Innovative Medicine, Biomedical Sciences, Biology – 01B)

Prof. Dr. **Vida Vičkačkaitė** (Vilnius University, Physical Sciences, Chemistry – 03P)

The defence of the dissertation will be held at 14 p.m. on 26th of September 2014 at the public meeting of the council at the Auditorium of Inorganic Chemistry at the Faculty of Chemistry of Vilnius University. Address: Naugarduko 24, LT-03225, Vilnius, Lithuania.

The summary of the dissertation was mailed on the 26th of August, 2014.
The dissertation is available at the Library of Vilnius University and at the Library of Institute of Chemistry.

VILNIAUS UNIVERSITETAS
FTMC CHEMIJOS INSTITUTAS

JUSTINA RUKŠNAITĖ

PAVIRŠIAUS PLAZMONŲ REZONANSO IR ELEKTROCHEMINIAI IMUNINIAI JUTIKLIAI ŽMOGAUS AUGIMO HORMONO IR ANTIKŪNŲ PRIEŠ ŽMOGAUS AUGIMO HORMONĄ NUSTATYMUI

Daktaro disertacijos santrauka
Fiziniai mokslai, Chemija (03P)

VILNIUS, 2014

Disertacija buvo ruošiama 2009 – 2013 metais Vilniaus universitete, Chemijos fakultete, Analizinės ir aplinkos chemijos katedroje.

Mokslinis vadovas:
Doc. dr. **Asta Kaušaitė-Minkštimienė** (Vilniaus universitetas, fiziniai mokslai, chemija – 03P)

Disertacija ginama Vilniaus universiteto Chemijos mokslo krypties taryboje:

Pirmininkas:

Prof.dr. **Stasys Tautkus** (Vilnius universitetas, fiziniai mokslai, chemija − 03P)

Nariai:

Prof. habil. dr. **Audrius Padarauskas** (Vilniaus universitetas, fiziniai mokslai, chemija – 03P)

Prof. habil. dr. **Saulius Balevičius** (Fizinių ir technologijos mokslų centro Puslaidininkių fizikos institutas, fiziniai mokslai, fizika – 02P)

Dr. **Daumantas Matulis** (Vilniaus universiteto Biotechnologijos institutas, fiziniai mokslai, chemija – 03P)

Dr. **Sigita Jurkonienė** (Gamtos tyrimų centro Botanikos institutas, biomedicinos mokslai, biologija – 01B)

Oponentai:

Prof. dr. **Genė Biziulevičienė** (Valstybinis mokslinių tyrimų institutas Inovatyvios medicinos centras, biomedicinos mokslai, biologija – 01B).

Prof. dr. **Vida Vičkačkaitė** (Vilniaus universitetas, fiziniai mokslai, chemija – 03 P)

Disertacija bus ginama viešame Chemijos mokslo krypties tarybos posėdyje 2014 m. rugsėjo mėn. 26 d. 14 val. Vilniaus universiteto Chemijos fakulteto Neorganinės chemijos auditorijoje. Adresas: Naugarduko g. 24, LT-03225 Vilnius, Lietuva.

Disertacijos santrauka išsiuntinėta 2014 m. rugpjūčio mėn. 26 d.
Disertaciją galima peržiūrėti Vilniaus universiteto ir FTMC Chemijos instituto bibliotekose

Introduction

Immunosensors have been used for over thirty years and are still among the most important diagnostic tools, which are widely applied in medical diagnostics (Saerens et al., 2008; Lin et al., 2004; Kassanos et al., 2008) food quality (Mitchell, J., 2010), environmental analysis (Schofield et al., 2007; Goryacheva et al., 2007) and even in explosive compound analysis (Smith et al., 2008). Immunosensors based on many kinds of detection methods, such as electrochemical (EL) (Rosales-Rivera et al., 2011), chemiluminescence (Grosso et al., 2011), ellipsometry (Balevicius et al., 2011), piezoelectric (Xu et al., 2011), surface plazmon resonance (SPR) (Baniukevic et al., 2013) and others, have been developed. These methods differ in analytical characteristics, such as specificity, sensitivity, accuracy, and precision. The use of different signal transducers enables various real-time monitoring and miniaturization possibilities, simplifies sample pretreatment procedures, and reduces analysis cost. In order to achieve higher immunosensor sensitivity the use of a combination of different detection methods is very promising.

SPR-immunosensors offer several significant advantages. First, there is no need for labeling and for this reason real-time detection is possible. Moreover, non-specific binding is reduced. In addition, it is possible to use small sample volumes and detect very low analyte concentrations. Furthermore, in case of the most successful immunosensors analysis of antigen-antibody or other biomolecular interactions can be completed within a few minutes and very often it is possible to reuse the sensor chip many times after proper regeneration of the sensing surface (Kausaite et al., 2007; Hu et al., 2008; Kausaite-Minkstimiene et al., 2009). Most important factors influencing the sensitivity and selectivity of immunosensors are the type of recognition molecule immobilization, concentration and orientation on the surface. It allows for detecting very low analyte concentrations in a relatively small volume of a sample (Brogan et al., 2003).

The aim of this work:

To develop surface plasmon resonance (SPR) and electrochemical (EL) immunosensors for detection of human growth hormone (HGH) and antibodies against human growth hormone (anti-HGH) and to adapt these sensors for real sample analysis.

The following main tasks were set to achieve the aim:

1. To compare four different techniques of anti-HGH immobilization on the SPR sensor chip and to evaluate the interaction between immobilized anti-HGH and HGH.
2. To determine the most efficient anti-HGH immobilization technique and to establish the optimal immobilization conditions.
3. To examine and evaluate linear detection ranges, limits of detection, selectivity, repeatability and stability of SPR immunosensors for HGH and anti-HGH detection.
4. To determine the optimal conditions of anti-HGH detection by pulsed amperometry (PA) and cyclic voltammetry (CV) techniques.
5. To examine and evaluate the limits of detection and repeatability of PA- and CV-based immunosensors for anti-HGH detection.
6. To investigate anti-HGH and HGH detection in real samples using developed SPRand EL immunosensors.

Statements for defense:

1. Oriented immobilization of antibodies increases SPR immunosensor sensitivity to HGH.
2. The indirect detection format increases SPR immunosensor sensitivity to anti-HGH.
3. The developed immunosensors demonstrate good selectivity, repeatability and stability.
4. The developed immunosensors are suitable for anti-HGH and HGH detection in real samples.

Experimental:

SPR measurements. A double channel SPR-analyzer 'Autolab ESPRIT' (ECO Chemie, Utrecht, Netherlands) was applied to SPR-measurements. One channel was used to perform assay. The second was used to run reference measurements. A laser-diode was used as the light source to produce monochromatic light with a wavelength of 670 nm. SPR-angle scan was performed around the manually fixed SPR-resonance position. The syringe and peristaltic pumps performed all liquid handling procedures. The syringe pump was used for sample mixing in the cuvette and for sample dispensing. The peristaltic pump was used to drain the cuvette, with the waste going into a waste flask. The SPR-angle was measured at a non-flow liquid condition, that is, with the circulating pump paused. All samples were monitored at a constant temperature of 24 ˚C using a thermostatically controlled cell.

Imaging by atomic force microscopy (AFM). Surface images of the bare SPR-chip, frag-anti-HGH modified SPR-chip layer and SPR-chip afer the immune complex formation between immobilised frag-anti-HGH and HGH were obtained with an 'AFM BioScope II' (Veeco Instruments Ltd., Santa Barbara, USA). The AFM imaging was performed in tapping mode in air at room temperature. Images were acquired with a silicon cantilever at 10 mm s^{-1} scan rate. For all AFM images a resolution of 200x200 pixels was applied.

EL measurements. The electrochemical cell of the SPR analyzer 'Autolab ESPRIT' was connected to the computerized potentiostat. EL measurements for the evaluation of optimal conditions for electrochemical immunosensor were performed using PA (E_{app} = +200 mV, t_p = 1 s) and CV (E = -75 - (+650) mV), v = 50 mV s^{-1}). A conventional three-electrode system comprising a prepared modified SPR chip as a working electrode, a platinum wire as an auxiliary electrode and an Ag/AgCl as a reference electrode was employed for electrochemical experiments. All experiments were performed in 0.05 mol/L PBS, pH 7.4, with 0.1 mol/L KCl. EL detection of analytical signal was performed at different concentrations of anti-HGH after the formed HGH/anti-HGH immune complex interaction with secondary anti-HGH-HRP antibodies in the presence of 0.25 mmol/L of TMB and 0.1 mmol/L of H_2O_2.

Results and diccussions:

Antibody immobilization is an essential process for the development of immunosensors because the choice of the immobilization technique greatly affects antibody-antigen interactions. The surface concentration and orientation of antibodies on sensor chip surface are important factors related to the sensitivity of SPR-immunosensor. To find the optimal immobilization protocol in this work four antibody-immobilization techniques were compared. Three of them are typical and are based on the binding of intact-anti-HGH antibodies; the fourth technique is very innovative and based on application of frag-anti-HGH.

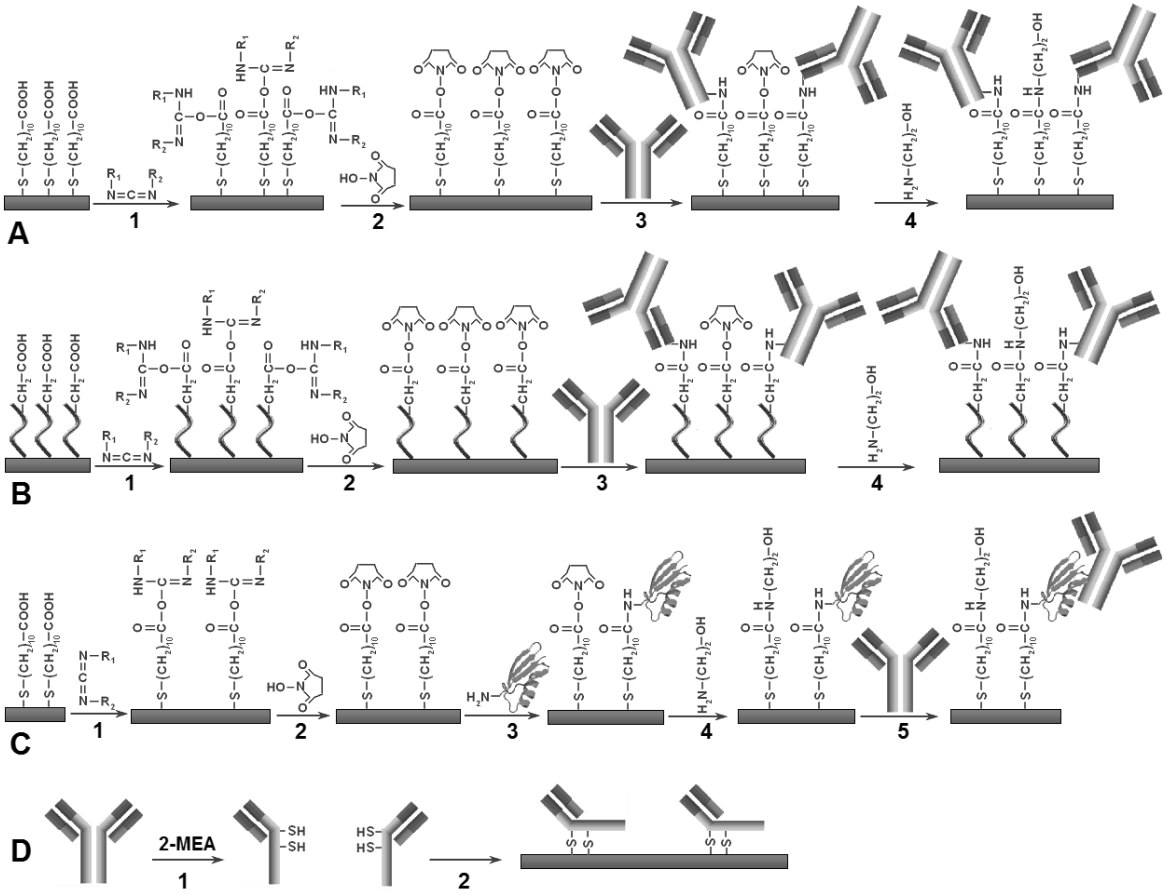

Figure 1. Principal immobilization schemes of (A-C) intact-anti-HGH and (B) frag-anti-HGH antibodies: (A) via self-assembled monolayer of MUA, (B) on carboxymethyldextran hydrogel, (C) on self-assembled MUA monolayer coupled protein-G, (D) directly on gold surface via native thiol functional groups present in frag-anti-HGH.

These techniques were (i) random intact-anti-HGH immobilization over the MUA self-assembled monolayer (Figure 1 A); (ii) random immobilization of intact-anti-HGH on CMD-hydrogel modified SPR-immunosensor chip by direct covalent amine coupling

(Figure 1 B); (iii) oriented intact-anti-HGH immobilization via MUA self-assembled monolayer coupled protein-G (MUA/pG) by indirect covalent coupling method due to affinity binding with an Fc-fragment of intact-anti- HGH (Figure 1 C); and (iv) oriented frag-anti-HGH immobilization on the gold by simple chemo-sorption of thiol-groups formed during the splitting of anti-HGH (Figure 1 D). Due to its advantages immobilization of biomolecules on SAMs is widely used in immunosensor design. One of the common strategies for immobilization of antibody on the electrode surface is covalent linking of the antibody amine groups to the terminal carboxylic acid groups of a SAM formed on gold. Covalent linking of antibodies to dextran hydrogels is presently the most popular due to low nonspecific binding and versatility of derivation methods suitable for modification of dextran matrices. Furthermore, the hydrophilic dextran hydrogels offer a large surface area, which enables considerably higher biomolecule immobilization in comparison with SAMs. Figure 2 represents real SPR-data for intact-anti-HGH immobilization over the MUA monolayer (B) and CMD-hydrogel (A). The immobilization was supported by the electrostatic interaction between the negatively charged carboxyl functional groups present in the CMD-hydrogel matrix and MUA monolayer with the positively charged amino acid residues in the intact-anti-HGH. Hence, in this case the immobilization was achieved by covalent coupling. Prior to immobilization, the modified SPR-chip surface was treated with coupling buffer (10 mmol/L Na-acetate buffer, pH 4.5), until a stable resonance angle was maintained (Figure 2, step 1). Then terminal carboxyl groups were activated by incubation with a mixture of EDC and NHS in deionized distilled water to make an active intermediate of N-hydroxysuccinimide ester (Figure 1A and B, steps 1-2). The activation step was carried out for 5 min (Figure 2, line 2). This activation was essential for effective immobilization because the formation of a reactive group allowed for a stable coupling of an intact-anti-HGH (Figure 1 A and B, step 3). After the activation step the surface was washed with coupling buffer (Figure 2, step 3) and solution of 1.64 μmol/L of intact-anti-HGH in coupling buffer was injected into the SPR-cell. At the same time registered sensorgrams showed a gradual increase in SPR-angle indicating binding of the intact-anti-HGH on the activated surface (Figure 2, step 4). The SPR-angle increased with time and reached steady-state condition within 25 min. Loosely bound intact-anti-HGH was washed out from the surface by passing coupling buffer solution over the

modified SPR-chip surface (Figure 2, step 5). However, after immobilization of the intact-anti-HGH some activated N-hydroxysuccinimide ester groups were left unreacted. To avoid the binding of other proteins to these groups they were transformed into amides via reaction with ethanolamine. After the deactivation step (Figure 2, l step 6), extent of ethanolamine was washed out by passing 10 mmol/L Na-acetate buffer solution over the intact-anti-HGH modified surface (Figure 2, step 7). Along with that, the SPR-angle was registered during the flow of coupling buffer (Figure 2, step 7) ant it was slightly higher compared to that registered after the immobilization step (Figure 2, step 5). The results illustrate that remaining unreacted N-hydroxysuccinimide ester groups are successfully blocked by ethanolamine (Figure 1 A and B, step 4). After the washing step intact-anti-HGH modified SPR-chip with coupling buffer (Figure 2, step 7) the SPR-chip was subsequently treated by regeneration solution (Figure 2, step 8) and later by PBS buffer, pH 7.4 (Figure 2, step 9). Binding of the antibodies via protein-A or protein-G on the sensor chip surface is a promising option for oriented immobilization of antibodies. Both proteins specifically interact with Fc domain of antibodies and hence the paratope of IgG can face the opposite side of the immobilized proteins leading to site-selective immobilization. However, the direct immobilization of these proteins on solid supports is less stable, for this reason they are generally immobilized through a spacer layer. Figure 2 C shows real SPR-data for protein-G coupling. In our study the protein-G was coupled on the SPR-chip surface by chemical binding to a formed SAM consisting of MUA (Figure 1 C, steps 1 - 4) in the same manner as described above. The shift of SPRangle obtained for the binding of protein-G to MUA was 418.43 ± 5 m°. From the shift of SPR-angle the surface concentration of protein-G was calculated to be 3.49 ± 0.04 ng/mm^2.

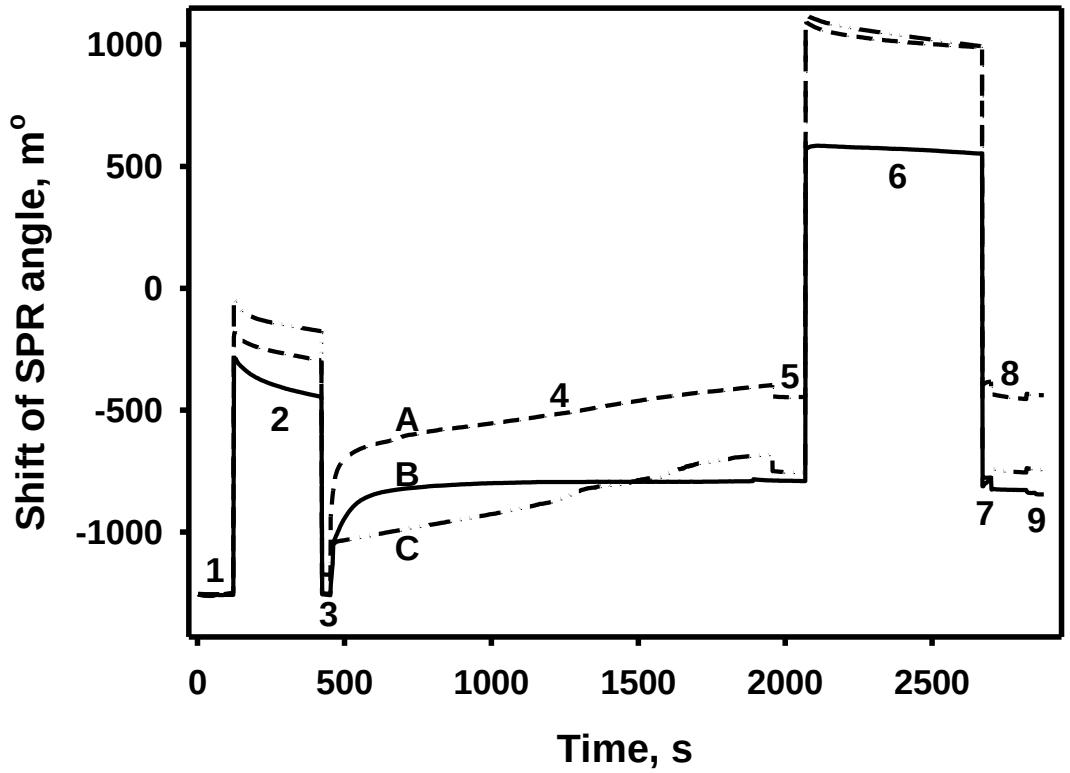

Figure 2. SPR sensograms of the intact-anti-HGH immobilization via: (A) CMD-hydrogel; (B) self-assembled monolayer of MUA; and (C) the protein-G coupling to self-assembled monolayer of MUA. SPR chip modification/treatment steps represented in sensogram: 1, baseline in 10 mmol/L Na-acetate buffer, pH 4.5 (from 1 to 120 s); 2, activation of the MUA or CMD-hydrogel with a mixture of EDC/NHS (from 120 to 420 s); 3, injection of Na-acetate buffer, pH 4.5 (from 420 to 450 s); 4, immobilization of intact-anti-HGH or coupling of protein-G (from 450 to 1960 s); 5, injection of Na-acetate buffer, pH 4.5 (from 1960 to 2070 s); 6, deactivation of remaining reactive NHS-esters with ethanolamine (from 2070 to 2670 s); 7, injection of Na-acetate buffer, pH 4.5 (from 2670 to 2700 s); 8, injection of regeneration solution consisting of 50 mmol/L NaOH and 0.5 % SDS (from 2700 to 2820 s); 9, injection of 10 mmol/L PBS buffer, pH 7.4 (this step was continued until stabile baseline was obtained).

The first step of intact-anti-HGH immobilization via MUA/pG was based on treatment with a 10 mmol/L PBS buffer, pH 6.0, until a stable resonance angle was maintained. After injection into the SPR-cell of PBS buffer, pH 6.0, with 1.64 μmol/L intact-anti-HGH a gradual increase of SPR-angle indicating binding of intact-anti-HGH on the MUA/pG modified sensor surface was detected in the sensorgram (Figure 1 C, step 4). The SPR-angle increased with time and reached steady-state conditions within 25 min. Any loosely bound intact-anti-HGH antibodies were removed from the surface by additional injection of PBS buffer, pH 4.5. The immobilization of biomolecules on gold supports is carried out by using bifunctional reagents containing thiol groups, which

bind strongly to the gold surface. On the other hand the biomolecule itself can contain sulphide and/or disulfide groups that enable direct immobilization on gold. The anti-HGH antibodies belong to a special class of glycoproteins named imunoglobulin-G (IgG). The IgG molecule contains two couples of polypeptide chains with a molecular weight of ca. 150 kDa and has Y-shape. Each couple contains the heavy chain and the light chain. The two heavy chains are linked via disulfide bonds. The structure of IgG comprises two Fab-fragments used for binding specific antigens and an Fc-fragment. It is possible to split antibody by reducing agents and to obtain two fragments of antibody. It is expected that such procedure does not affect the binding site of the antibody, which is formed by the coupling of heavy and light chains. To obtain frag-anti-HGH's the intact-anti-HGH antibodies were chemically reduced with 2-MEA. Three different concentrations of 2-MEA (15.0, 35.0, and 70.0 mmol/L) were used and prepared frag-anti-HGH were analyzed using gel electrophoresis. Lanes 2, 3, and 4 of Figure 3 show three sharp bands with a molecular weight around of 25, 50, and 80 kDa. The band representing 80 kDa is equal to half of molecular weight of intact-anti-HGH and can be assigned to the half-cleft anti-HGH (Figure 1 D, step 1). 25 and 50 kDa bands indicate that during the reduction some disulfide bridges between the heavy and light chains of some anti-HGH molecules were dissociated and separated light and heavy chains at some extent were also present in the sample. Since the SDS-PAGE analysis did not show any significant differences in anti-HGH fragments obtained using various 2-MEA concentrations the surface concentration of immobilized frag-anti-HGH and antigen binding capacity were investigated and compared. The immobilization of frag-anti-HGH was carried out using thiol-groups of the hinge part of anti-HGH (Figure 1 D, step 2). Prior to immobilization, the bare SPR-chip surface was treated with 10 mmol/L Na-acetate buffer, pH 4.5, until a stable resonance angle was maintained. Then Na-acetate buffer with frag-anti-HGH was injected into the SPR-cell. The SPR-angle indicating the binding of frag-anti-HGH on the SPR-chip increased with time and reached steady-state conditions within 25 min. Loosely bound frag-anti-HGH were washed out from the surface by passing Na-acetate buffer solution over the modified SPR-chip surface.

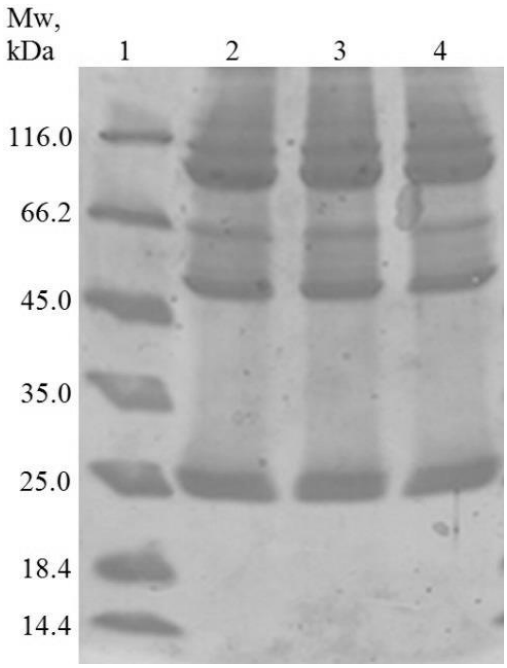

Figure 3. SDS-PAGE analysis of frag-anti-HGH fraction obtained by reduction of intact-anti-HGH antibodies with 15.0 (lane 2), 35.0 (lane 3), and 70.0 mol/L (lane 4) of 2-MEA. Molecular weight marker is on lane 1.

SPR-angle shifts for the binding of frag-anti-HGH obtained by reduction with 15.0, 35.0, and 70.0 mmol/L of 2-MEA were 583.95 ± 9, 625.41 ± 10, and 636.08 ± 10 m° respectively. The surface concentration of the frag-anti-HGH calculated from these angle shifts was 4.87 ± 0.08, 5.21 ± 0.08, and 5.30 ± 0.08 ng/mm^2. As shown in Figure 3, reduction of intact-anti-HGH with 2-MEA affected formation of frag-anti-HGH. In this study, both the cleavage of disulfide bridges between the two heavy chains of intact-anti-HGH and maintenance of the antibody binding site are important for the formation of biorecognition surface. The breakage of intact-anti-HGH disulfide bridges increases probability for proper orientation of immobilized frag-anti-HGH and decreases the loss of sensor selectivity, which is mainly caused by random orientation of biological recognition elements. Figure 4 shows the shift of SPR-angle caused by binding of frag-anti-HGH on the SPR-chip surface (black bar) and by formation of an immune-complex between immobilized frag-anti-HGH and HGH present in the sample (grey bar) with respect to the concentration of 2-MEA. When frag-anti-HGH antibodies were produced by reduction in 15 mM of 2-MEA the lowest shift of SPR-angle (583.95 m°) was registered. As the concentration of 2-MEA was increased, the surface concentration of immobilized frag-anti-HGH increased and a more significant shift of SPR-angle (625.41 m° and 636.08 m° for 35.0 mM and 70.0 mmol/L

of 2-MEA, respectively) was registered. But opposite effect was obtained for SPR-angle shifts, which were induced by HGH binding to immobilized frag-anti-HGH. When concentration of 2-MEA was increased, the SPR-angle shift induced by HGH binding was significantly decreased. Therefore, 15 mmol/L of 2-MEA was found to be the most optimal concentration for preparation of frag-anti-HGH.

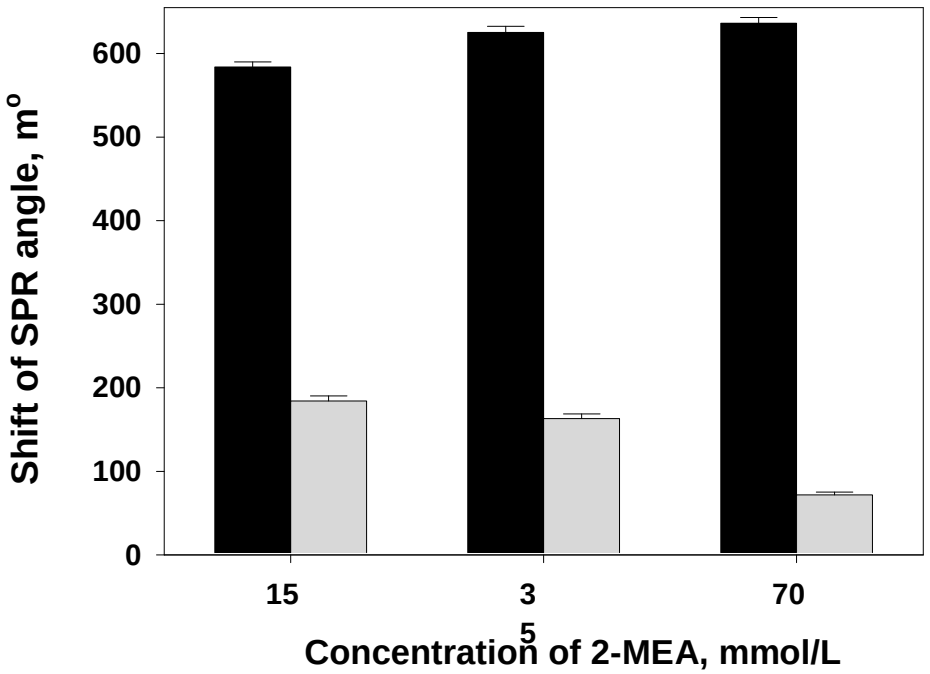

Figure 4. Variation of SPR-angle shift with respect to concentration of 2-MEA (black bars) and the exposure of 1.59 μmol/L of HGH (grey bars).

This concentration allowed us to reduce disulfide bridges between the two heavy chains of intact-anti-HGH and to maintain a high HGH binding capacity. Optimal condition for frag-anti-HGH immobilization was subsequently examined and 0.44 μmol/L of frag-anti-HGH was considered to be the most optimal concentration for modification of SPR-chip surface and it was used throughout the study. The influence of different immobilization techniques used in this study on the surface concentration of immobilized antibodies. SPR-angle shifts for the binding of antibodies on SPR-chip surface were calculated as the difference between the SPR-signal prior to binding and after treatment with coupling buffer. We found that absolute SPR-angle shifts caused by the immobilized molecules decreased by 807.53 ± 10 m° for the SPR-chip based on

intact-anti-HGH immobilized within CMD-hydrogel (CMD/intact-anti-HGH); 754.88 ± 9 m° for the SPR-chip based on intact-anti-HGH immobilized via MUA/pG (MUA/pG/intact-anti-HGH); 583.95 ± 6 m° for the SPR-chip based on frag-anti-HGH immobilized using native thiol groups of intact anti-HGH (Au/frag-anti-HGH); and 443.01 ± 5 m° for the SPR chip based on an intact-anti-HGH immobilized via MUA monolayer (MUA/intact-anti-HGH). From the shift of SPR-angle, the surface concentration of the antibodies was calculated to be 6.73 ± 0.08, 6.29 ± 0.08, 4.87 ± 0.05 and 3.69 ± 0.04 ng/mm^2 for CMD, MUA/pG, Au and MUA modified SPR-chips respectively; whereas the shift of SPR-angle by 120 m° is equivalent to the change of surface concentration of protein by 1 ng/mm^2. The highest antibody immobilization level was found in the case of random immobilization of intact-anti-HGH within the CMD-hydrogel, whereas the lowest level in the case of random immobilization of intact anti-HGH via MUA-based SAM. These results confirmed that dextran hydrogels offer a large surface area, which enables a considerably higher surface density of immobilized antibodies in comparison with SAM-based technique. However despite the fact that most of intact-anti-HGH antibodies were bound onto the CMD-hydrogel surface, the highest SPR-signal (184.75 m°) was observed by binding of HGH to the SPR-chip based on MUA/pG/intact-anti-HGH (Figure 5). The SPR-signals induced by HGH binding to the SPR-chips based on CMD/intact-anti-HGH (20.73 m°) and MUA/intact-anti-HGH (16.51 m°) were, respectively, 8.9 and 11.2 times lower if compared to the signal obtained for MUA/pG/intact-anti-HGH. These results indicate that majority of antigen binding sites on the randomly immobilized intact-anti-HGH were blocked due to incorrect antibody binding position, whereas in the oriented immobilization mode, the antigen-binding sites were more accessible from the solution front. It is expected that less than 10% of antibodies remain active when immobilized in a random orientation. Hence it can be concluded that CMD hydrogel is adsorbing a relatively high number of antibodies, but these antibodies might be (i) randomly oriented; many adsorbed antibodies are relatively far from sensing the gold surface and some of them are completely out of range, which has influence on the SPR-signal; (ii) dextran especially if it is modified with adsorbed antibodies forms a relatively thick diffusion layer and it increases the response time and to some extent it reduces sensitivity of the SPR-sensor. Meanwhile the attached protein-G layer favored oriented immobilization of the

intact-anti-HGH antibodies for sensitive detection of HGH. Since each protein-G molecule has more than one binding site for binding of antibody via Fc-domain, the binding of intact-anti-HGH antibodies to protein-G independent of orientation of the protein-G was possible. Because the binding of intact-anti-HGH antibodies occurred via Fc-domain, the antibody binding-site was not distorted. Thus, immobilization of the intact-anti-HGH antibodies via MUA/pG results in uniform, stable and sterically accessible biological recognition layer. However, the limitation of intact-anti-HGH immobilization via MUA/pG is the necessity to immobilize the protein-G layer. Furthermore, we could not find such regeneration solution, which will just dissociate antigen-antibody complex and will not dissociate intact-anti-HGH complex with immobilized protein-G. The next most intensive SPR-signal (142.68 m°) was observed by application of an SPR-chip based on Au/frag-anti-HGH (Figure 5 d). It was only 1.3 times lower than the signal obtained for the SPRchip based on MUA/pG/intact-anti-HGH (Figure 5 c). This is in agreement with reports, which claimed that immunosensing surfaces based on half-cleft IgG result in enhanced antigen-binding activity when compared to surfaces based on intact IgG. The binding-activity of frag-anti-HGH compared with that of intact-anti-HGH immobilized via MUA/pG may be influenced by partial inactivation of the antibody binding-site during the preparation of frag-anti-HGH and/or later by incubation of frag-anti-HGH modified SPR-chips in BSA solution with the aim of blocking empty nonspecific binding sites present on the SPR-chip. Nevertheless immobilization technique based on the interaction between the thiol-group of IgG fragment and gold surface is advantageous because the immobilized antibodies maintain both: high antigen binding constants and high stability. This immobilization technique provides proper orientation of IgG fragments; so that there is a similar distance between the binding site of the antibody and the surface of SPR-chip; it does not cause the distribution of apparent affinity constants. Due to sufficient antigen binding capacity, simplicity of accomplishment and low cost with respect to other evaluated techniques in this study, the immobilization based on application of frag-anti-HGH was found to be the most suitable for design of an SPR immunosensor devoted for HGH detection.

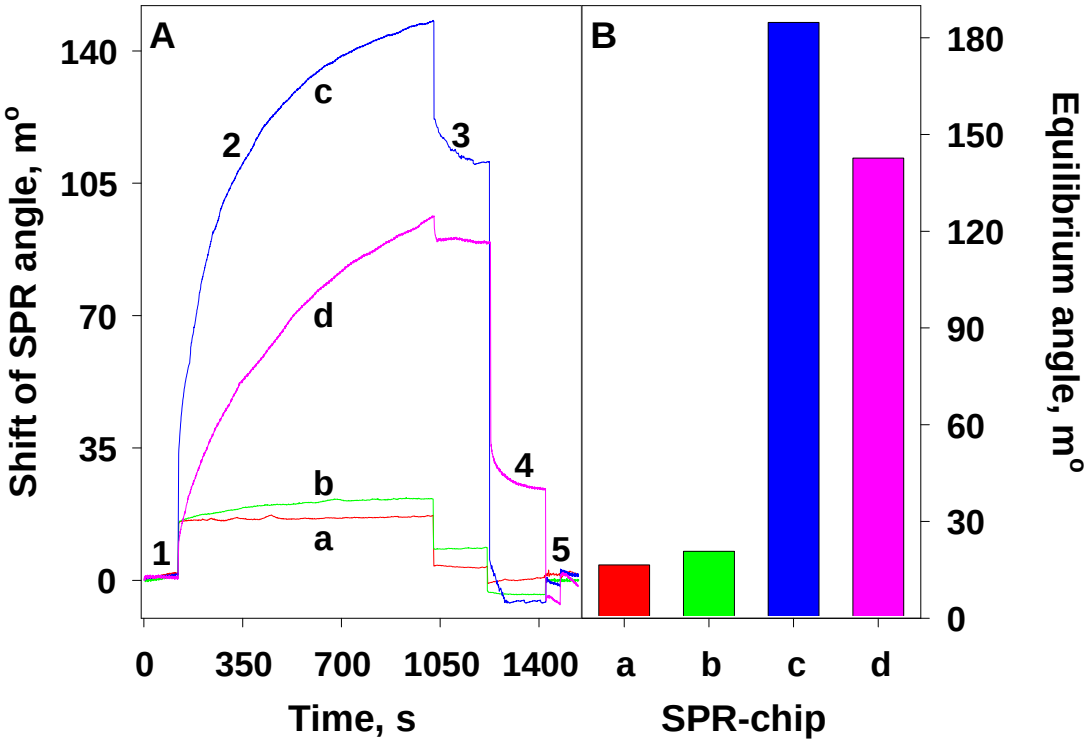

Figure 5. (A) The SPR-signals obtained by differently modified SPRchips. (B) Equilibrium angle dependence on antibody immobilization technique: a, MUA/intact-anti-HGH; b, CMD/intact-anti-HGH; c, MUA/ pG/intact-anti-HGH; d, Au/frag-anti-HGH. The HGH concentration in all tested samples was the same: 1.59 μmol/L.

Here the evaluated results correspond to results presented by other authors, and state that the method based on immobilization of antibody fragments eliminates the random orientation of antibody on the surface, consequently increasing the antibody antigen binding capacity, which increases the coupling of the antibody fragments, increases antigen binding capacity and decreases nonspecific binding. Comprehensive analysis of random immobilization of intact antibodies and oriented immobilization of fragmented antibodies via self-assembled monolayer was performed using the surface plasmon resonance technique. For a particular antibody/antigen system, the optimized fragmentation protocol in combination with an oriented immobilization of Fab' fragments on mixed SAMs leads to a >2-fold increase of the antigen binding signals when compared to signals registered with analytical systems based on randomly covalent immobilized intact antibodies.

The anti-HGH antibody belongs to a certain class of glycoproteins, namely immunoglobulin G (IgG). These molecules consist of two couples of polypeptide chains, all of which form a Y-shaped structure of ca. 150 kDa. Each couple contains a heavy chain and a light chain. Two heavy chains are linked via disulphide bonds to form the intact antibody. In the present study, native anti-HGH thiol groups were liberated after splitting the intact anti-HGH by the reducing agent 2-MEA into two fragments (frag-anti-HGH). This mild reduction reaction resulted in the splitting of S–S bridges between the two heavy chains, while retaining the activity of the antigen-binding sites (Figure 6, step 1). The immobilisation of frag-anti-HGH on the gold surface of the SPR-chip was carried out by incubation of the SPR-chip in frag-anti-HGH solution (Figure 6, step 2). Subsequently the remaining free immobilization sites on the Au-layer of the SPR-chip were blocked by BSA in order to avoid non-specific adsorption of HGH onto the surface of the SPR-chip. The described immobilisation technique provides: (i) site-oriented immobilisation of frag-anti-HGH, (ii) similar distance between the binding sites of immobilized frag-anti-HGH and the SPR-chip surface, which reduces the distribution of the apparent affinity constants and (iii) high antigen-binding capacity because the antigen-binding sites of the fragmented antibodies are not affected.

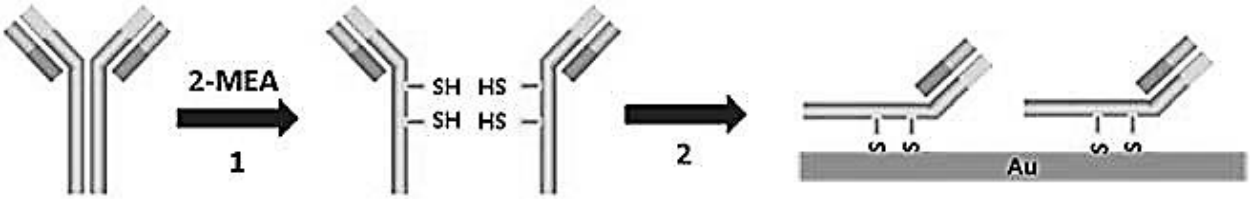

Figure 6. The immobilisation of frag-anti-HGH antibodies onto the gold surface using their native thiol groups.

The intact anti-HGH antibodies were chemically reduced with 15 mmol/L 2-MEA in order to obtain frag-anti-HGH. In our previous study, SDS-PAGE analysis of frag-anti-HGH confirmed the successful reduction of antibodies. The current concentration of 2-MEA was found optimal for frag-anti-HGH preparation, immobilisation and interaction with HGH.

The optimal concentration of anti-HGH used for the preparation of frag-anti-HGH (reduction with 15.0 mmol/L of 2-MEA) and modication on the SPR-chip surface was examined. Table 1 represents the variations in the SPR-angle shift: (i) with respect to the initial concentration of anti-HGH used for the preparation of frag-anti-HGH and (ii) after the formation of the immunecomplex between immobilised frag-anti-HGH and 5.0 mmol/L of HGH, which was present in the sample solution. According to the presented results, the highest SPR-angle shift (612.19 m°), which was caused by the immobilised frag-anti-HGH, was registered in the case of the maximal concentration of anti-HGH (1.65 mmol/L), whereas the lowest shift (583.95 m°) was detected in the case of the minimal investigated concentration (0.41 mmol/L). Thus, experimental results showed that by using increased concentrations of antibodies, the surface concentration of immobilised frag-anti-HGH increases as well and a more significant shift of the SPR-angle was registered. However, the highest SPR-signal (174.2 m°), which is caused by the formation of the frag-anti-HGH/HGH complex, was observed in the case of 0.41 mmol/L concentration of antibody. When the antibody concentrations were increased, the shifts of the SPR-angle after interaction with HGH decreased and were 155.6 and 147.4 m° for 0.83 and 1.65 mmol/L solutions of antibodies, respectively. Therefore, the antibody concentration of 0.41 mmol/L was considered to be the optimal concentration for the modification of SPR-chip surface and this concentration was used in all subsequent steps. The standard deviation (STDEV) of the SPR responses calculated for three similarly prepared SPR-chips is presented in Table 1.

Table 1. Variation of the SPR-angle shift with respect to the anti-HGH concentration and the reaction with 5.0 mmol/L of HGH.

Concentration of anti-HGH, mmol/L	**Shift of the SPR angle after frag-anti-HGH immobilisation, m°**	**Shift of SPR angle after frag-anti-HGH/ HGH immune complex formation, m°**
0.41	583.95 ± 6.10	174.20 ± 5.99
0.83	610.61 ± 7.20	155.57 ± 5.55
1.65	612.19 ± 7.32	147.37 ± 4.79

In our study AFM has been successfully applied for imaging of differently modified surfaces. Images of the frag-anti-HGH layer immobilised on the SPR-chip and the layer of the immune complex consisting of frag-anti-HGH/HGH were compared with the bare gold surface of the SPR-chip (Figure 7). In the present study, AFM images (Figure 7 A, C and E) and height distribution diagrams derived from these images (Figure 7 B, D and F) were compared. The obtained results clearly demonstrated changes in morphology of the gold surface after modification with frag-anti-HGH and after the formation of immune complexes with HGH.

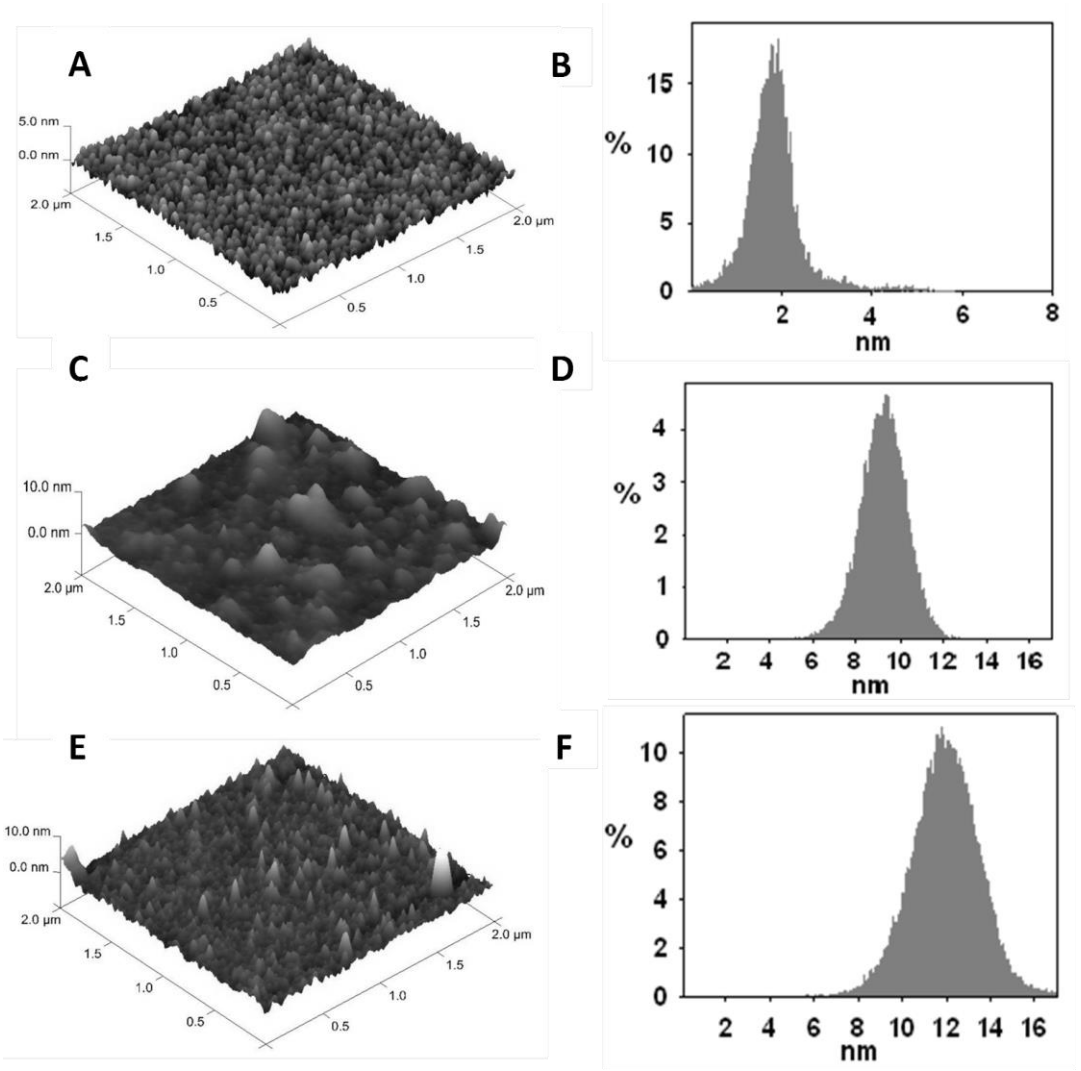

Figure 7. AFM images (A, C and E) and the height distribution diagrams (B, D and F) of surfaces of differently modified SPR-chips: bare gold surface of the SPR-chip (A and B), SPR-chip modified with frag-anti-HGH (C and D) and SPR-chip after the formation of frag-anti-HGH/HGH (E and F).

A topographic image of the gold coated SPR-chip was used as a control surface (Figure 7 A) and the periodic features of approximately 1.89 nm in height were observed

(Figure 7 B). The frag-anti-HGH molecules (80 kDa) were immobilised on the gold substrate via native thiol functional groups and yielded a high surface coverage. The evaluation of topography of the obtained protein monolayer showed a number of features of 9.25 nm in height (Figure 7 C and D). The obtained results coincide with the published results, where typical dimensions of IgG of 14.5 nm x 8.5 nm x 4.0 nm have been reported (Sarma et al., 1971; Saphire et al., 2002). Thus the frag-anti-HGH obtained after reduction of intact antibodies might be approximately of 7.25 nm. Taking into account the roughness of gold (1.89 nm in height), the height of immobilised frag-anti-HGH should be approximately 7.10 nm. The obtained value is slightly lower than the theoretical value of frag-Ab dimensions. After the specific interaction of frag-anti-HGH with HGH, the features of 11.90 nm in height (Figure 7 E and F) were registered. These distinct changes of surface morphology can be explained by the formation of complexes between the immobilised frag-anti-HGH and HGH molecules (22 kDa; 5.3 x 3.1 nm). The distance of frag-anti-HGH active sites from the surface might change after interaction with HGH, so the increase in height of features by 2.65 nm was slightly lower than the theoretical value for HGH. The observed differences among the three differently modified surfaces indicate that frag-anti-HGH molecules were adequately bound onto the gold substrate, and immune complexes were formed because of specific interactions between the antibody and the antigen.The sensitivity of developed SPR-immunosensor to HGH using anti-HGH with an initial concentration of 0.41 mmol/L was studied by the experiment described in this paragraph. The interaction of HGH with the immobilised frag-anti-HGH after blocking the sensing surface with BSA and the surface stabilization procedure was registered directly (without labelling) in real time. The SPR-chip modified by frag-anti-HGH was treated with 10 mmol/L PBS, pH 7.4, until a stable SPR-angle was established (Figure 8 A, “Baseline”). Then the aliquot consisting of HGH in PBS was injected into the SPR-cell. The formation of a frag-anti-HGH/HGH complex resulted in an increase in the SPR angle (Figure 8 A, “Association”). After the association phase, the modified SPR-chip surface was treated with PBS and this caused a partial dissociation of the frag-anti-HGH/HGH complex. This dissociation caused some decrease in the SPR-angle (Figure 8 A, “Dissociation”). In order to reuse the SPR-chip in multiple analyses, the HGH must be removed from the frag-anti-HGH surface after the dissociation, but the immobilised frag-anti-HGH must

stay intact and retain its binding capacity. Therefore, after the treatment with PBS the SPR-chip was treated with regeneration solution of 50 mmol/L NaOH and 17.3 mmol/L SDS. At this stage, the SPR-angle decreased sharply (Figure 8 A, "Regeneration"). Eventually, in the presence of PBS, the SPR-angle attained the original level, which was observed before the injection of HGH (Figure 8 A, "Returning to baseline"). The observed changes of SPR-angle indicated the removal of HGH from the active SPR-chip surface. Figure 8 A illustrates that the active SPR-chip surface was completely regenerated after treatment with the regeneration solution for 5 minutes and then with PBS for 3 minutes. The application of immunosensors in multiple analyses makes analytical procedures more cost-effective and in certain cases it reduces the analysis time.

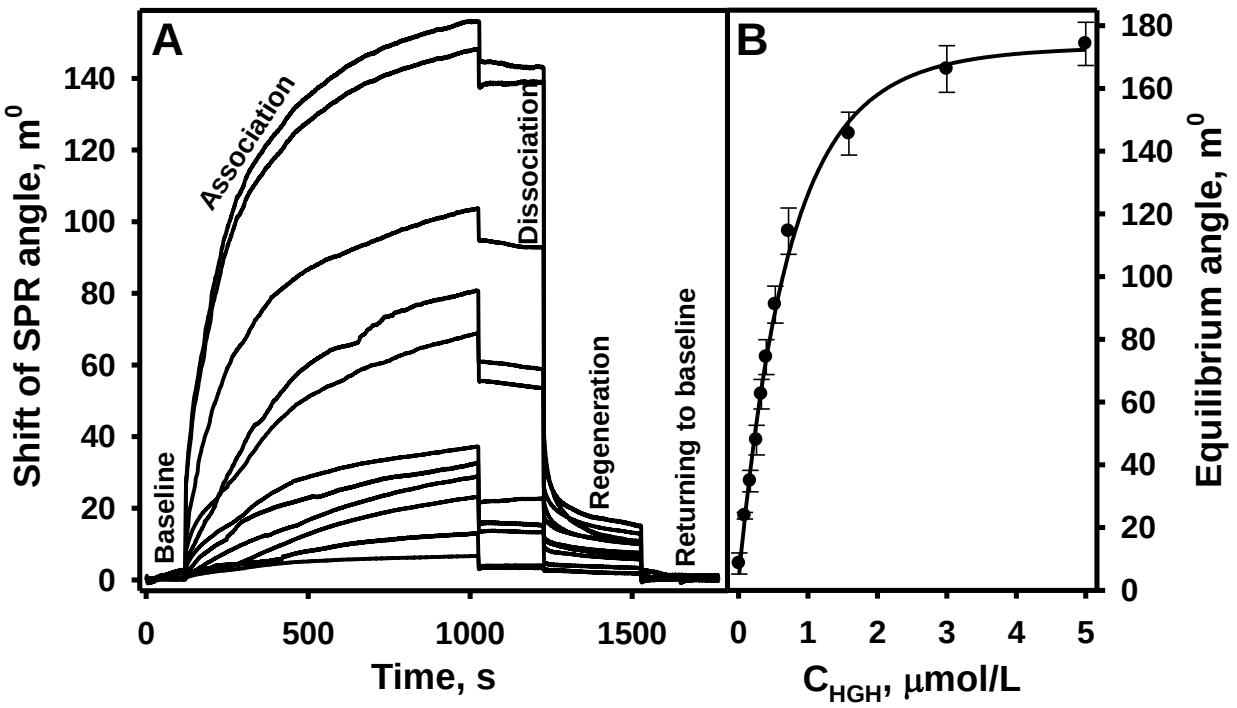

Figure 8. (A) The SPR study of the formation of a frag-anti-HGH/HGH complex at 0.01, 0.09, 0.17, 0.26, 0.33, 0.40, 0.53, 0.72, 1.59, 3.0 and 5 mmol/L concentrations of HGH. (B) Equilibrium angle vs. the concentration of HGH.

Therefore, the regeneration step is an important procedure in the SPR-based immunoassays. Figure 8 A shows the SPR sensograms of eleven different concentrations of HGH ranging from 0.01 to 5.0 mmol/L. At the end of each regeneration carried out on the same chip the SPR-angle reached a similar steady-state baseline, which was observed

before the injection of the HGH sample. The obtained results indicate a well-established regeneration procedure. The shift in the SPR-angle depended on concentrations of HGH in the sample. After the establishment of steady-state conditions (at "the equilibrium angle"), the SPR-signal was plotted against the concentrations of HGH (Figure 8 B). The error bars illustrate the STDEV for three replicates. At 0.01 mmol/L HGH concentration, the shift in the SPR-angle was approximately 8.6 m°. This SPR-angle shift was quite significant, as the signal to noise ratio was 86 (8.6 m°/0.1 m°). The increase in the HGH concentration influenced the proportional increase in the SPR angle in the range of 0.01 – 0.72 mmol/L. The SPR-angle shift was linearly dependent on the concentration of HGH. The SPR-angle shift reached saturation and did not change with increasing analyte concentration, when the concentrations of HGH were higher than 5.0 mmol/L. The analytical signal obtained at the lowest concentration of analytes plus three times its standard deviation was used to estimate the LOD. It has been demonstrated that the LOD is 0.0034 mmol/L for the developed SPR-immunosensor.

Recently, an SPR immunosensor based on a "binding inhibition" mode, which is suitable for the determination of HGH in human serum samples, has been reported. In the study, the LOD was 6 ng/mL (0.00027 μmol/L). The lower sensitivity of the proposed method might be explained by the principle used for HGH detection. In our study, the "direct detection" format of HGH was applied, e.g. the analytical signal increased with increased HGH concentration. Using the SPR immunosensor based on a HGH "binding inhibition" format, the analytical signal is inversely proportional to the HGH concentration, e.g. when small concentration of HGH is in the sample a higher signal is registered. Despite slightly lower sensitivity in the present study, the described HGH immunosensor is very promising for applications in the "direct detection" format. Selectivity and cross-reactivity investigations were performed by simultaneous injection of 5.0 mmol/L HGH and pGH in PBS into the different channels of the SPR cell. This study showed that the SPR-signal registered during the formation of the frag-anti-HGH/HGH complex (157.14 m°) was 11.57 times higher when compared to that obtained in case of the cross-reactivity of pGH to the frag-anti-HGH modified SPR-chip surface (13.58 m°).

The repeatability of the analytical signal is one of the most important parameters of any analytical system. For this reason, repeated detection–regeneration cycles were performed three times and consecutive injections of the same amount of HGH (0.41 mmol/L) showed extremely similar shifts in the SPR angle. These results indicated that the frag-anti-HGH modified SPR-chip surface retained its reactivity almost completely during the detection–regeneration cycle of the SPR-chip. The STDEV of the SPR responses, which were obtained on the same SPR-chip, was found to be 2.28 and the coefficient of variation (CV) was found to be 3.7 %, yielding a measurement repeatability of 96.3 %. This means that the destruction of the immobilised frag-anti-HGH based sensitive layer during the regeneration procedure was minimal. The STDEV of the SPR responses calculated for three similarly prepared SPR-chips was a little higher. It was found to be 2.45 and CV was found to be 6.2 %, yielding a measurement repeatability of 93.8 %. These minor differences between the different SPR-chips might be caused by slightly varying surface-concentrations of frag-anti-HGH immobilised on different SPR-chips. Operational stability of the SPR-immunosensor was investigated by the repeated injection of 1.59 mmol/L of HGH followed by regeneration. These experiments were performed using SPR chips modified by frag-anti-HGH after blocking the sensing surface with BSA (Figure 9, curve 1) and compared with the experiments performed without blocking (Figure 9, curve 2). Repeated detection–regeneration cycles were performed 22 times while the SPR-chips were stored in a controlled environment (PBS, pH 7.4, 20 °C) for 18 days. During the first day the differences between the 1^{st} and 5^{th} analytical signals were just 4 % (Figure 9, curve 1). If empty sites available for binding on the Au-layer of the SPR-chip were not blocked by BSA, the drop in the SPR-signal was more significant and exceeded 26 % (Figure 9, curve 2). After the first two days 6 % and 35 % decreases in the absolute SPR response were observed for the BSA-blocked and not-blocked SPR-chips respectively. Later the decrement of the absolute SPR-response became more significant and after the 7^{th}, 12^{th} and 18^{th} day the observed absolute SPR-responses for the BSA-blocked SPR-chip were 20, 30 and 38 % (Figure 9, curve 1) and for not-blocked SPR-chip – 66, 80 and 91 % lower, respectively (Figure 9, curve 2). Hence, BSA had blocked the empty sites available for binding, which were present on the Au-layer of the SPR-chip, and this blocking not only significantly reduced the non-specific adsorption of HGH on the

SPR-chip surface, but also increased the stability of the frag-anti-HGH layer and its capacity to interact with HGH for a longer period of time.

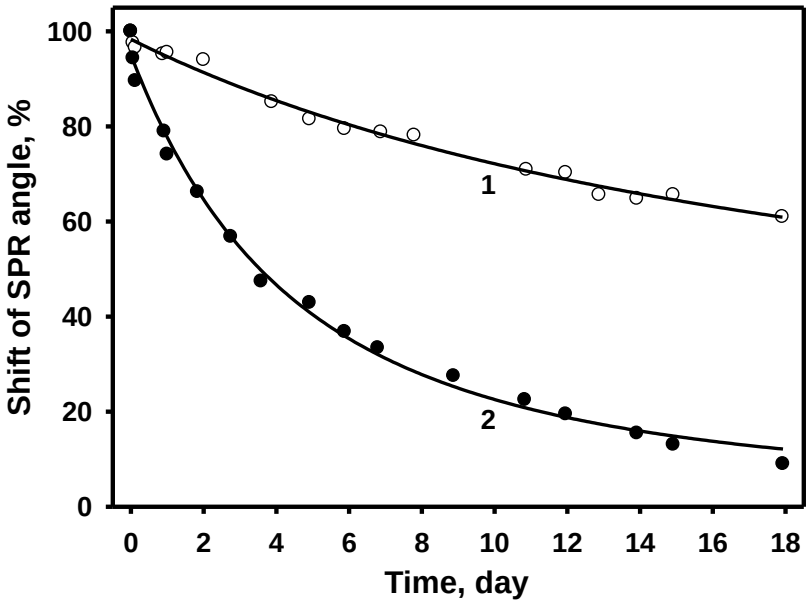

Figure 9. Operational stability of the SPR-chips modified by frag-anti-HGH after blocking of the sensing surface with BSA (1) and without blocking (2). The experiment was performed by injection of 1.59 mmol/L of HGH dissolved in PBS.

The feasibility of the SPR-immunosensor to detect HGH under optimized conditions was investigated in spiked human blood serum samples with 0.26 mmol/L of HGH. The recovery ratio of the proposed method was 109.6 % and the precision expressed as the relative standard deviation was 4.8 %.

An indirect immunoassay format with HGH immobilized on the self-assembled monolayer modified SPR-chip was chosen to detect specific anti-HGH antibodies using a combination of three different physical phenomena in the same channel of the SPR analyzer. For the enhancement of the analytical signal and sensitivity of immunosensor HRP labeled secondary antibodies specifically interacting with formed immune complexes were used. The serial use of different analytical instruments with the SPR analyzer is generally utilized to speed up and complement the bioanalysis procedure. The applicability and sensitivity of SPR and EL techniques detecting formed HGH/anti-HGH immune complexes on the same SPR-chip surface using secondary HRP labeled antibodies were investigated and compared.

SPR is one of the most advanced surface-sensitive optical techniques providing real-time and label-free monitoring of biorecognition events. Immobilization of HGH involves the formation of a mono molecular film of the biological recognition element in a controlled and stable manner on the gold surface modified with SAM. Covalent attachment of HGH to the SPR-chip surface was achieved via carbodiimide coupling between free amino groups of the protein and the carboxylend of SAM. SPR-chip modified by biological recognition element was used for the interaction with analyte of interest anti-HGH antibodies. The conjugation of HGH to the SAM modified gold surface and each step of sensor assembly (interaction with anti-HGH and secondary antibodies) was monitored using the SPR analyzer. The results obtained during HGH immobilization indicated the successful binding of HGH to MUA after SAM activation with a mixture of EDC and NHS. The SPR angle shift was ca. 370 ± 10 m° and showed quite good reproducibility up on repeated experiments, as it was reported in the previous study. From the SPR angle shift, the surface concentration of the HGH was calculated and was equalto 3.08 ± 0.08 ng/mm^2. The sensitivity of the fabricated immunosensor using indirect immunoassay format (HGH/anti-HGH/anti-HGH-HPR) (Figure 10) was studied during the subsequent recognition of anti-HGH antibodies of known concentration. In all tested samples the concentration of secondary antibodies was the same: 0.181 mmol/L. Figure 10 A shows SPR sensograms for nine different concentrations of anti-HGH antibodies ranging from 0.098 to 39.47 nmol/L. SPR angle shift could not reach the saturated steady statein the tested anti-HGH concentration interval. After the HGH/anti-HGH immune complex formation interaction with secondary anti-HGH-HRP antibodies resulted in an increase of the SPR signal (Figure 11).

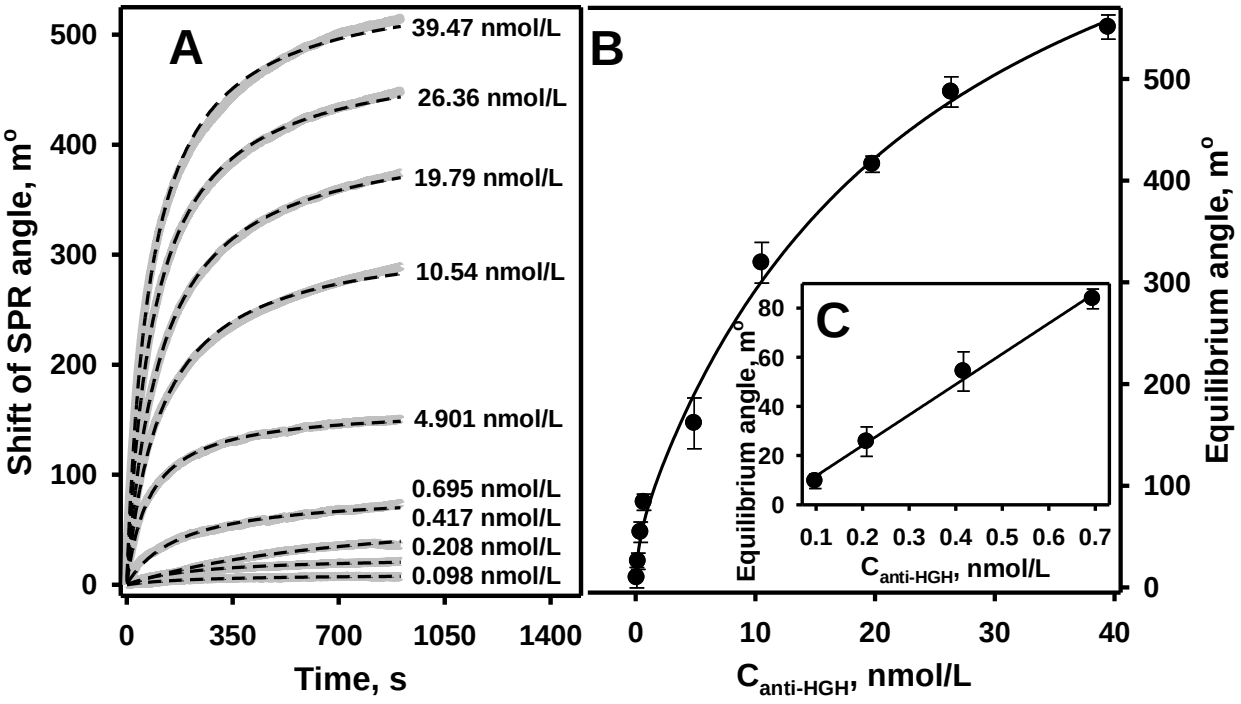

Figure 10. SPR study of secondary antibody binding to the immune complex formed between the immobilized HGH and different concentrations of anti-HGH. (A) Calculation of the equilibrium angle. Solid lines represent real-time kinetic measurement data obtained during the measurements of anti-HGH and secondary antibody interactions. Dashed lines were generated by Sigma Plot statistics soft-ware. Concentration of secondary antibodies in all tested samples was the same: 0.181 mmol/L. (B) and (C) Concentration of anti-HGH versus the equilibrium angle.

Indirect immunoassay format at 0.695 nmol/L of anti-HGH antibodies allows to register a 4.9 times higher SPR angle shift when compared to the direct format. Also this concentration was the minimal detectable concentration of anti-HGH indirect immunoassay format. In comparison, the minimal detectable concentration of anti-HGH in the control sample using secondary antibodies was 0.098 nmol/L; the SPR angle shift of ca. 9.60 m° was obtained. The SPR angle shift for 0.098 nmol/L of anti-HGH is quite significant, whereas the signal-to-base line noise ratio is 96.0 (9.6 m°/0.1 m°). SPR signal in the indirect immunoassay format was dependent on the concentration of anti-HGH antibodies and at steady-state conditions (equilibrium angle) was plotted against the concentrations of anti-HGH (Figure 10 B). In the range of 0.098 – 0.695 nmol/L the SPR angle shift was linearly dependent on anti-HGH concentration (Figure 10 C) with a correlation coefficient of 0.9927 (n=3). The relative standard deviation (RSD) of the SPR response, which was obtained by measuring

different SPR-chips, was found to be about 0.12. Å double-channel SPR analyzer allows for performing measurements in two channels simultaneously. One channel was used to perform the immunoassay; the second channel was used for the cross-reactivity study. Interactions of HGH with non-specific anti-pGH antibodies and with secondary antibodies specific to cattle antibodies were studied. SPR angle shift during the cross-reactivity study was evaluated and eliminated from the SRP angle shift registered during the specific interaction. SPR signal registered during the secondary antibody binding to the formed HGH/anti-HGH immune complex was over 12 times higher when compared to that obtained in the case of non-specific binding. It is worthwhile to mention that in our previous study the SPR signal registered using direct immunoassay format was about 10 times higher. The experimentally observed minimal detectable concentration of anti-HGH indirect, label-free immunoassay format using SPR analyzer was 2.47 nmol/L, while using the indirect immunoassay format we can detect 0.098 nmol/L of anti-HGH.

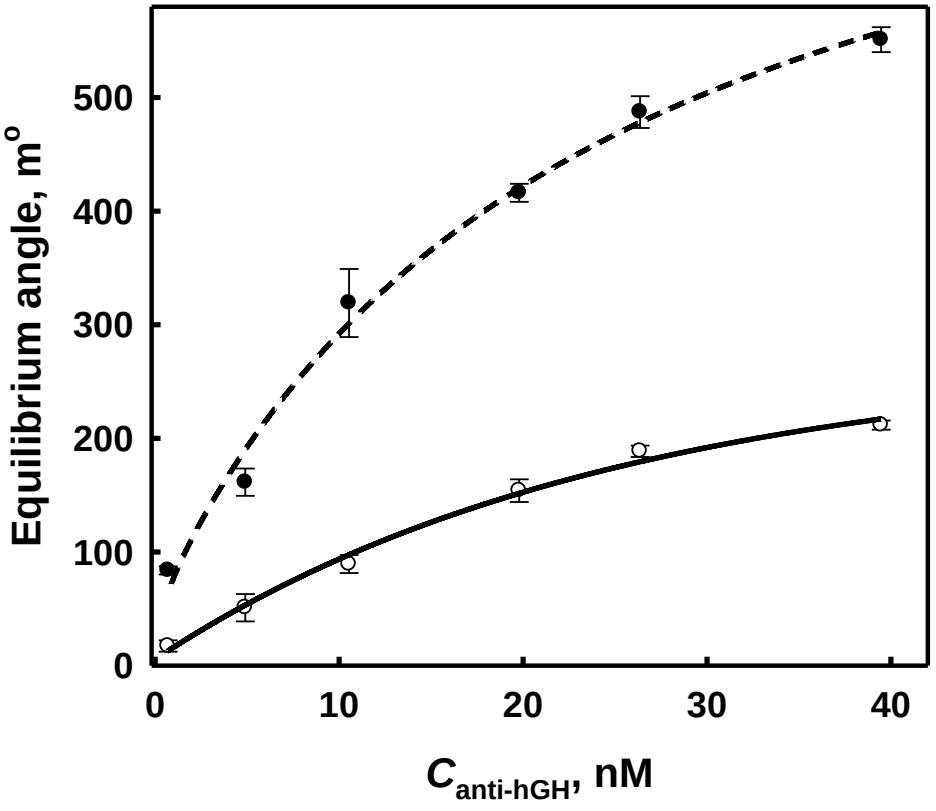

Figure 11. SPR signals registered during direct and indirect detection of anti-HGH. (1) HGH interaction with anti-HGH (HGH/anti-HGH); (2) secondary antibodies binding to the immune complex formed between the immobilized HGH and anti-HGH (HGH/anti-HGH/anti-HGH-HRP).

This minimal detectable concentration of the anti-HGH in direct immunoassay format can be reduced, because the SPR angle shift is quite significant (the signal-to-baseline noise ratio is 96.0). The repeated detection–regeneration cycles were performed only in the direct immunoassay format during the storage of SPR-chips for 21 days in a controlled environment (PBS buffer, pH 7.4; 20 °C). No changes in the original baseline level were detected during the 40 detection–regeneration cycles. This means that damage of the immobilized HGH-based sensitive layer during regeneration was minimal. It was demonstrated that the developed SPR-chip could be stored for at least 21 days before use without considerable loss of sensitivity towards anti-HGH. It might be predicted that the same HGH/anti-HGH system using a direct immunoassay format might be characterized by similar parameters. The combination of in direct immunoassay technology and EL methods provided the basis for a model system devoted to the detection of antibodies against HGH. The enzyme activity can be determined through the quantitative assessment of an electrochemically active product generated in the enzyme-catalyzed reaction (Garay et al., 2010). The redox mediator is very important for the amperometric registration of HRP-catalyzed reaction. In our previous study (Kurtinaitiene et al., 2008) TMB was found to be the most efficient redox mediator for design of amperometric immnosensor. Other authors have also reported TMB (Micheli et al., 2005; Tian et al., 2009) as efficient redox mediator for the registration of HRP-catalyzed reaction. Immunoassay was conducted on a gold film and it was analyzed using both SPR and EL approaches. After the HGH/anti-HGH/anti-HGH-HRP complex formation TMB is oxidized by HRP into a highly reactive cation radical indicated as TMBox‘, which is very quickly oxidized on the surface of an electrode into a more stable form indicated by TMBox‘‘. Optimal conditions for the EL immunosensor were initially evaluated. CV was used to determine the optimal voltage for the detection of anti-HGH and as another EL method. It was discovered that the optimal voltage for anti-HGH determination was +200 mV. To obtain the maximal EL intensity the effect of TMB and H_2O_2 concentration on the response of the EL immunosensor to analyte after the anti-HGH and secondary antibody interaction (Figure 12 A and B, respectively) was studied using PA and CV.

As presented in Figure 12 A the intensity of the analytical signal increased with increasing TMB concentration from 0.1 to 0.25 mmol/L and the decreased TMB

concentration of 0.25 mmol/L increased the sensitivity of developed analytical systems by about 1.70 times in both EL methods. EL signal intensity also depends on the concentration of H_2O_2. The change of the EL signal intensity with the concentration of H_2O_2 is shown in Figure 12 B. The 0.1 mmol/L concentration of H_2O_2 increased the analytical signals 2.37 and 1.77 times using PA and CV, respectively. Therefore, a 0.25 mmol/L concentration of TMB and 0.1 mmol/L concentration of H_2O_2 were selected for the subsequent experiments, because of the highest sensitivity of the analytical system. From the results of analytical signal obtained using different electrochemical methods and optimal concentrations of TMB and H_2O_2 it is visible, that detection using PA (Figure 12 B, 3 curve) is a more sensitive method – the analytical signal was 1.68 times higher compared to CV (Figure 12 B, 4 curve).

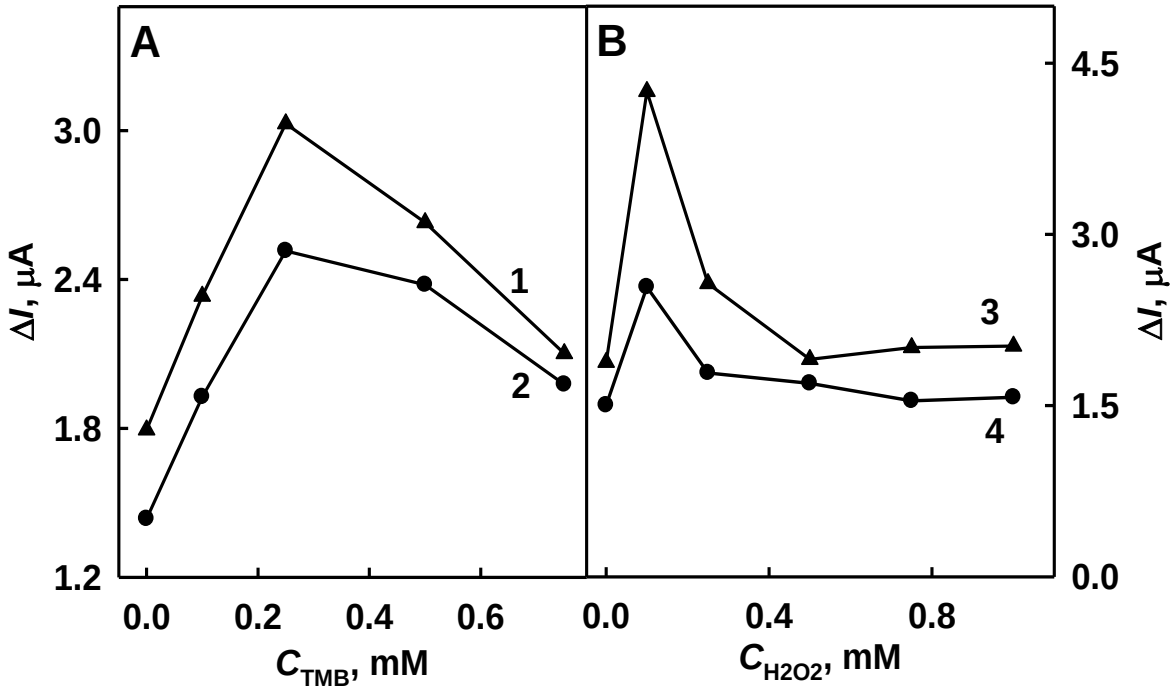

Figure 12. The effect of TMB (A) and H_2O_2 (B) concentration on the analytical signal after secondary antibody binding to the immune complex formed between the immobilized HGH and anti-HGH (HGH/anti-HGH/anti-HGH-HRP) (1, 3 – PA and 2, 4 – CV curves, respectively). Concentrations of anti-HGH and secondary antibodies in all tested samples were the same: 0.019 and 0.181 mmol/L, respectively; (B) concentration of TMB – 0.25 mmol/L.

Under optimized conditions it was found that when a mediator is used the registered currents depend on the anti-HGH concentration after the

HGH/anti-HGH/anti-HGH-HRP complex formation. EL signal during cross-reactivity with non-specific anti-pGH antibodies and secondary antibodies specific to cattle antibodies was evaluated and eliminated from the analytical signal registered during the specific interaction. EL signal registered during the secondary antibody binding to the formed HGH/anti-HGH immune complex for CV was about 23.0 times and for PA 32.9 times higher compared to that obtained in the case of non-specific binding. The results obtained using the PA method before and after binding of secondary antibodies (Figure 13 A) were compared and differences in peak currents interpreted as the analytical signal. In case of the CV method (Figure 13 B) differences between the highest and lowest current at potential values of +650 and +75 mV were interpreted as the analytical signal.

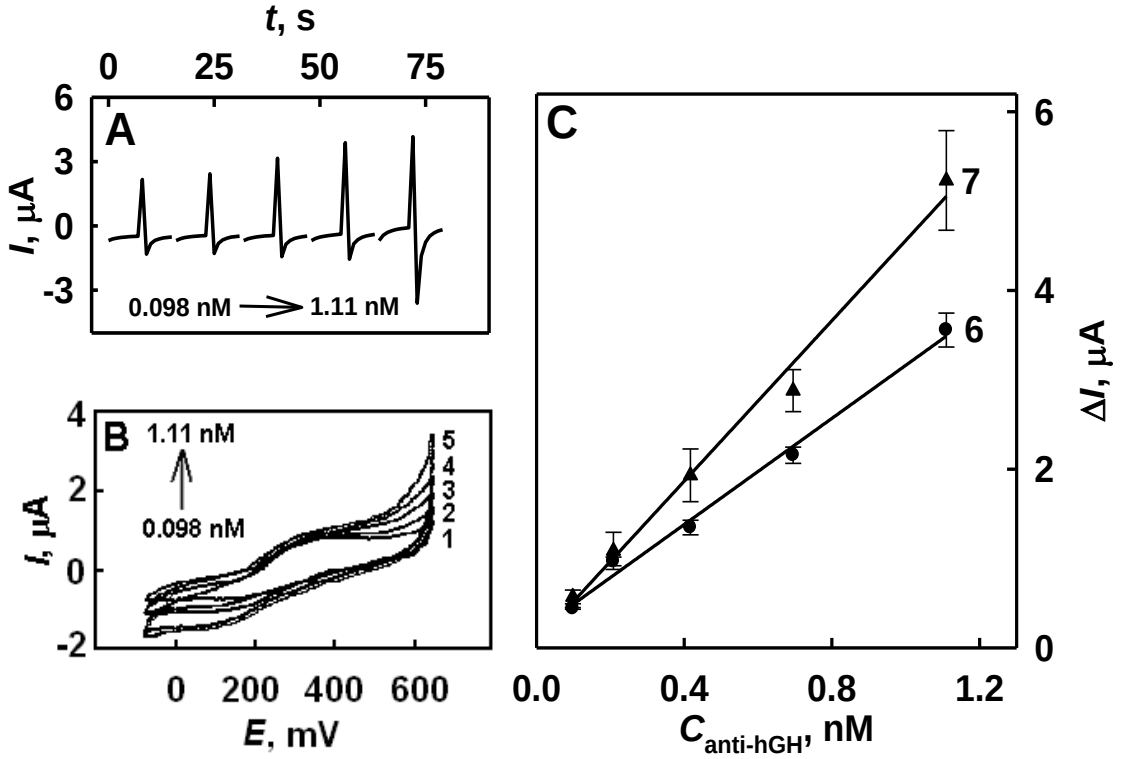

Figure 13. Electrochemical study of secondary antibody binding to the immune complex formed between the immobilized HGH and different concentrations of anti-HGH. (A) Pulsed amperomograms ($E_{app=}$+200 mV, t_p=1 s), (B) cyclic voltammograms (E=-75-(+650) mV), *v*=50 mVs^{-1}) (1 – 0.098, 2 – 0.208, 3 – 0.417, 4 – 0.695 and 5 – 1.11 nmol/L of anti-HGH antibody) and (C) calibration curves of analytical signal intensity versus the concentration anti-HGH antibodies (6 – CV and 7 – PA curves). Anti-HGH concentration ranges from 0.098 to 1.11 nmol/L, concentration of TMB – 0.25 mmol/L and H_2O_2 – 0.25 mmol/L, concentration of secondary antibodies in all tested samples was the same: 0.181 mmol/L.

This diapason of potentials was selected to reach the highest sensitivity of immunosensors, because it gave a much higher accuracy in comparison to traditional evaluation of oxidation and reduction peaks, which are attributed to TMB. While these oxidation–reduction peaks for TMB are not very well established especially at low concentrations of analyte the advantage of this kind of evaluation of analytical signal, which is based on a potentio dynamic method, is in agreement with the evaluation of other kinds potentio dynamic analytical signals, e.g. PA detection (Ramanaviciene et al., 2004; Ramanaviciene et al., 2006B), where differences between extreme values of currents in amperomograms were evaluated as the analytical signal. The dependences were measured in the anti-HGH concentration range of 0.098 – 1.11 nmol/L using the PA (Figure 13 C, 7 curve) and CV (Figure 13 C, 6 curve) methods. As seen in Figure 13 C the dependence of the EL signals at low analyte concentrations was close to linear without the intercepts on x- or y- axis and with correlation coefficients of 0.9886 and 0.9920 (n=3) for PA and CV, respectively. The analytical signals are reproducible – RSD for the PA and CV methods were 0.11 and 0.06 for the tested anti-HGH concentrations.

Many electrochemical, optical, piezoelectric and other type of immunosensors for specific recognition of immune complex formations between antigens and antibodies have been developed. Immunosensors are capable of direct and specific, as well as indirect, stable and reproducible measurements of very low concentrations of analytes of interest (Garay et al., 2010; Rosales-Rivera et al., 2011). The application of the HRP labeled secondary antibodies immobilized on gold nanoparticles enhanced amperometric signal by 2-fold and the limit of detection of neuronal protein asynuclein decreased from 3.2 ng/mL to 14.6 pg/mL (An et al., 2012). In our indirect immunoassay format SPR and EL techniques were used for the detection of the specific anti-HGH antibodies. The sensitivity, linear concentrations range of analyte and other parameters of developed immunosensors are summarized and compared in Table 2. Analytical signal at the zero concentration value of analyte plus three times its standard deviation could be used to estimate the detection limit of analyte. This result was very similar to that obtained by SPR, which was equal to 0.051 nmol/L. In the case of anti-HGH detection using PA the limit of detection was the lowest – 0.027 nmol/L. Lower reproducibility of the analytical signal and higher limit of detection was observed using CV and it was 0.056 nmol/L.

The PA detection shows 1.89 and 2.07 times higher sensitivity compared to SPR and CV, respectively. The advantages of the EL immunoassay in comparison with SPR were observed by other authors too (Tian et al., 2009; Rosales-Rivera et al., 2012). EL methods are less sensitive to the nonspecific adsorption related interferences and have a better detection limit than the SPR method. Looking at the sensitivity of our immunosensors with the LOD for SPR being 32.7 times higher, and 61.9 and 29.8 times higher for PA and CV compared to the results obtained by other authors using high-performance protein G affinity chromatography with fluorescence (Rosales-Rivera et al., 2012). Although indirect immunoassay format was characterized by high sensitivity and the possibility of detecting analyte at concentrations not detectable using direct immunoassay format, linearity of the concentration range is relatively narrow. Using the direct immunoassay format the SPR angle shift was almost linearly dependent on the anti-HGH concentration in the wider range of concentrations (2.47 – 250 nmol/L). Using indirect immunoassay format a wider linearity range (0.098 – 1.11 nmol/L) was observed using the PA and CV methods. In the case of SPR and ECL the linearity is relatively narrow, only 0.098 to 0.695 nmol/L of anti-HGH antibodies.

Table 2. Performance characteristics and comparative analysis.

	SPR		**EL**	
	Direct*	**Indirect**	**PA**	**CV**
Lineary range (nmol/L)	2.47 – 250	0.098 – 0.695	0.098 – 1.11	
LOD (nmol/L)	–	0.051	0.027	0.056
Assay time (min)	15	25	1	5
Surface regeneration	Yes	Yes	No	No

*Kausaite-Minkstimiene et al. (2009).

Feasibility of the immunosensor in detecting an anti-HGH using PA detection was investigated in spiked human serum samples obtained from the Rheumatological Department of Vilnius University Hospital ‘Santariskiu Klinikos’. Serum was diluted 10 times with 10 mmol/L PBS buffer, pH 7.4, and various concentrations of anti-HGH were

added (Table 3). In the previous section optimized immunoassay protocol for PA was applied. It was found that the recoveries were in the range of 93 – 98 %. Therefore, the developed EL immunosensor using PA detection is applicable for the determination of anti-HGH in real samples.

Table 3. Recovery of anti-HGH in human serum sample using PA detection.

Background content, nmol/L	**Added concentration, nmol/L**	**Detected concentration, nmol/L**	**RSD, %**	**Recovery ratio, %**
0.8	0	0.74	8.57	93
0.8	2.5	3.2	8.06	97
0.8	4.8	5.5	7.29	98

Conditions: potential +200 mV, 0.25 mmol/L of TMB, 0.1 mmol/L of H_2O_2, 0.181 mmol/L of secondary antibodies.

Conclusions:

1. Frag-anti-HGH immobilization *via* their native thiol-groups directly coupled to the SPR chip is the most suitable technique for the development of an SPR immunosensor for HGH detection. This anti-HGH immobilization method is of good antigen binding capacity, simple, and low cost in comparison to other techniques.
2. Optimal reagent concentrations to obtain frag-anti-HGH are 0.41 μmol/L anti-HGH and 15 mmol/L 2-MEA. These antibody reduction conditions enable reducing most of the anti-HGH molecules without damaging their antigen binding sites and result in the highest SPR angle shift following antibody/antigen interaction.
3. After frag-anti-HGH immobilization BSA blocks the remaining active sites on the SPR chip. BSA blocking reduces non-specific binding, improves frag-anti-HGH layer stability and enables frag-anti-HGH to sustain complexes with HGH for longer periods of time.
4. SPR HGH immunosensor is suitable for HGH detection with a LOD of 0.0034 μmol/L, good repeatability (96.3 % and 93.8 %, intra-assay and inter-assay repeatability respectively), and selectivity over porcine growth hormone. Active SPR-chip surface is fully regenerated after using a solution consisting of 50 mmol/L NaOH and 17.3 mmol/L SDS for 5 minutes and rinsing with 10 mmol/L PBS buffer, pH 7.4 for 3 minutes.
5. Optimal concentrations of TMB and H_2O_2 for electrochemical immunoanalysis are 0.25 and 0.1 mmol/L respectively. TMB concentration of 0.25 mmol/L increases the sensitivity of developed analytical systems about 1.70 times in both EL methods. The 0.1 mmol/L concentration of H_2O_2 increases the analytical signals 2.37 and 1.77 times using PA and CV respectively.
6. The LOD values of anti-HGH immunosensors are low (0.056, 0.051 and 0.027 nmol/L for CV-, SPR- and PA-based immunosensors, respectively). The analytical signals are reproducible (RSD values of PA, CV and SPR-based methods are 0.11, 0.06 and 0.12 for CV-, PA- and SPR-based immunosensors) and exhibit stability, high selectivity and short analysis times (1, 5 and 25 min for the PA-, CV- and SPR-based immunosensors). Electrochemical immunosensors are single-use only.
7. The developed PA and SPR immunosensors are suitable for anti-HGH and HGH detection in real samples.

References:

1. An, Y., Jiang, X., Bi, W., Chen, H., Jin, L., Zhang, S., Wang, C., Zhang, W. Biosensors and Bioelectronics, 2012, *32*, 224–230.
2. Balevicius, Z., Ramanaviciene, A., Baleviciute, I., Makaraviciute, A., Mikoliunaite, L., Ramanavicius, A. Sensors and Actuators B: Chemical. 2011, *160*, 555-562.
3. Baniukevic, J., Hakki Boyaci, I., Goktug Bozkurt, A., Tamer, U., Ramanavicius, A., Biosensors and Bioelectronics. 2013, *43*, 281-288.

4. Brogan, K.L., Wolfe, W.N., Jones, P.A., Schoenfish, M.H. Analytica Chimica Acta. 2003, *496*, 73-80.
5. Garay, F., Kisiel, G., Fang, A., Lindner, E. Analitical and Bioanalytical Chemistry. 2010, *397(5)*, 1873-1881.
6. Goryacheva, I., DeSaeger, S., Eremin, S., VanPeteghem, C. Food Additives and Contaminants. 2007, *24(10)*, 1169-1183.
7. Grosso, P., Carrara, S., Stagnia, C., Benini, L. Sensors and Actuators B: Chemical. 2010, *147(2)*, 475-480.
8. Hu, W., Li, C.M., Dong, H. Analytica Chimica Acta. 2008, *630(1)*, 67-74.
9. Kassanos, P., Iles, R.K., Bayford, R.H., Demosthenous, A. Physiological Measurments. 2008, *29(6)*, 241-254.
10. Kausaite, A., van Dijk, M., Castrop, J., Ramanaviciene, A., Baltrus, J.P., Acaite, P., Ramanavicius, A. Biochemistry and Molecular Biology Education. 2007, *35(1)*, 57-63.
11. Kausaite-Minkstimiene, A., Ramanaviciene, A., Ramanavicius, A. The Analyst. 2009, *134*, 2051-2057.
12. Kurtinaitiene, B., Ambrozaite, D., Laurinavicius, V., Ramanaviciene, A., Ramanavicius, A. Biosensors and Bioelectronics. 2008, *23(10)*, 1547-1554.
13. Lin, F.Y., Sherman, P.M., Li, D. Biomedical Microdevices. 2004, *6(2)*, 125-130.
14. Micheli, L., Grecco, R., Badea, M., Moscone, D., Palleschi, G. Biosensors and Bioelectronics. 2005, *21*, 588–598.
15. Mitchell, J. Sensors (Basel). 2010, *10(8)*, 7323-7346.
16. Ramanaviciene, A., Ramanavicius, A. Analytical and Bioanalytical Chemistry. 2004, *379*, 287–293.
17. Ramanaviciene, A., Snitka, V., Mieliauskiene, R., Kazlauskas, R., Ramanavicius, A. Central European Journal of Chemistry. 2006B, *4*, 194–206.
18. Rosales-Rivera, L.C., Acero-Sánchez, J.L., Lozano-Sánchez, P., Katakis, I., O'Sullivan, C.K. 2011, *26(11)*, 4471-4476.
19. Saerens, D., Ghassabeh, G.H, Muyldermans, S. Current Opinion in Pharmacology. 2008, *8(5)*, 600-608.
20. Schofield, D.A., Westwater, C., Barth, J.L., DiNovo, A.A. Applied Microbiology and Biotechnology. 2007, *76(6)*, 1383-1394.
21. Smith, R.G., D'Souza, N., Nicklin, S. Analyst. 2008, *133(5)*, 571-584.
22. Tian, D., Duan, C., Wang, W., Li, N., Zhang, H., Cui, H., Lu, Y. Talanta. 2009, *78*, 399–404.
23. Xu, T., Miao, J.M., Wang, Z.H., Yu, L., Li, C.M. Sensors and Actuators B: Chemical. 2011, *151(2)*, 370-376.

List of original publications by author:

Articles in journals:

1. A. Kausaite-Minkstimiene, A. Ramanaviciene, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius. A Comparative Study of Random and Oriented Antibody Immobilization Techniques on the Binding Capacity of Immunosensor. Analytical Chemistry, 2010, 82(15), 6401-6408.
2. A. Ramanaviciene, N. German, A. Kausaite-Minkstimiene, J. Voronovic, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius. Comparative Study of Surface Plasmon Resonance, Electrochemical and Electroassisted Chemiluminescence Methods Based Immunosensor for the Determination of Antibodies Against Human Growth Hormone. Biosensensors and Bioelectronics, 2012, 36(1), 48-55.
3. A. Kausaite-Minkstimiene, A. Ramanavicius, **J. Ruksnaite**, A. Ramanaviciene. A surface plasmon resonance immunosensor for human growth hormone based on fragmented antibodies. Analytical Methods, 2013, 5(18), 4757-4763.

Published contributions to academic conferences:

1. **J. Kirlytė (Rukšnaitė)**, A. Rapkauskaitė, A. Kaušaitė-Minkštimienė**.** Sąveikos tarp skirtingai imobilizuotų anti-HGH antikūnų ir HGH tyrimas PPR metodu. Neorganinių junginių chemija ir technologija. 2010, April 21– 22 d, Kaunas, Lithuania, p. 63–66.
2. **J. Kirlyte (Rukšnaitė)**, A. Kausaite-Minkstimiene, A. Ramanaviciene, A. Ramanavicius. The Impact of Antibody Immobilization Technique on the Binding Capacity of SPR Immunosensor. 12th conference Advanced Materials and Technologies 2010, August 27 – 31, Palanga, Lithuania, p. 39.
3. A. Ramanaviciene, A. Kausaite, **J. Kirlyte (Rukšnaitė)**, Z. Balevicius, I. Baleviciute, A. Makareviciute, Y. Oztekin, A. Ramanavicius. Evaluation of Immobilization Techniques on the Efficiency of Immunosensors. PITTCON 2011. CD-ROM abstract, 2011, March 13-18, Atlanta, USA, p. 2200-2212.
4. A. Ramanaviciene, A. Kausaite-Minkstimiene, A. Makareviciute, **J. Kirlyte (Rukšnaitė)**, I. Baleviciute, Y. Oztekin, A. Ramanavicius, J. Voronovic, N. German. Immunosensors Based on Surface Plasmon Resonance and Electrochemical Techniques. PITTCON 2012. CD-ROM abstract, 2012, March 11-15, Orlando, USA, p. 550-560.
5. N. German, A. Kausaite-Minkstimiene, J. Voronovic, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius, A. Ramanaviciene. The Creation of Immunosensors Based on Antigen-Antibodies Agaist Human Growth Hormone Interaction and the Application for the Analysis in Real Samples. 3-iasis Europos imunologų kongresas 2012. CD-ROM abstract, 2012 September 5–8, Glasgow, Scotland, p. 652–653.
6. J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius, A. Ramanaviciene. Comparison of Oriented and Random Antibody Immobilization Technique on the Efficiency of Immunosensor. EUROSENSORS 2012. CD-ROM abstract, 2012, September 9–12, Krakow, Poland, PM2 – 21.

Other publications:

1. A. Ramanavicius, N. Ryskevic, Y. Oztekin, A. Kausaite-Minkstimiene, S. Jursenas, J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, U. Bubniene, A. Ramanaviciene. Immunosensor based on fluorescence quenching matrix of the conducting polymer polypyrrole. Analytical and Bioanalytical Chemistry, 2010, 398(7-8), 3105-3113.
2. N. German, A. Kausaite-Minkstimiene, **J. Kirlyte (Rukšnaitė)**, A. Makaraviciute, A. Ramanavicius, L. Mikoliunaite, A. Ramanaviciene. Determination of antibodies against human growth hormone using a direct immunoassay format and different electrochemical methods. Analyst, 2013, 138(5), 1427-1433.
3. J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius, A. Ramanaviciene. Comparison of Oriented and Random Antibody Immobilization Techniques on the Efficiency of Immunosensor. Procedia Engineering, 26th European Conference on Solid-State Transducers, EUROSENSOR 2012, 2013, 47, 837-840.
4. J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius, A. Ramanaviciene. Application of oriented and random antibody immobilization methods in immunosensor design.Sensors and Actuators B: Chemical, 2013, 189, 217-223.

Other academic conferences:

1. A. Ramanaviciene, J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, A. Kausaite-Minkstimiene**,** Z. Balevicius, A. Makaraviciute, A. Ramanavicius, T. Romaskevic. SPR and QCM Based Sensors for Detection of Bovine Leukemia Virus Antigen. PITTCON 2010. CD-ROM abstract, 2010, February 28 – March 5, Orlando, USA, p. 670–18.
2. **J. Kirlyte (Rukšnaitė)**, T. Mairal, P. Nadal, V. Beni, A. Kausaite-Minkstimiene and C. K. O'Sullivan. SPR and fluorescence aptasensors for Allergen Lupine detection. IMA 2011. CD-ROM abstract, 2011, September 18–22, Chania, Crete, Greece, P134.
3. N. Ryskevic, U. Bubniene, S. Jursenas, J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, A. Ramanaviciene, A. Ramanavicius. Conducting Polymer Based Fluorescence Quenching Matrix for Immunosensor Design. 9th international electrochemistry meeting in Turkey. CD-ROM abstract, 2011, September 25–29, Izmiras, Turkey.
4. **J. Kirlyte (Rukšnaitė)**, P. Nadal, T. Mairal, V. Beni, A. Kausaite-Minkstimiene, C.K. O'Sullivan. Aptasensors for Allergen Lupin Detection. 10thinternational chemistry conference– Chemija 2011. CD-ROM abstract, 2011, October 14–15, Vilnius, Lithuania, p. 70.
5. J. Baniukevic, **J. Kirlyte (Rukšnaitė)**, A. Ramanavicius, A. Kausaite-Minkstimiene, A. Ramanaviciene. Bovine Leukemia Virus Antigen-Antibody Interaction Affinity Kinetic Evaluation. 10th international chemistry conference–Chemija 2011. CD-ROM abstract, 2011, October 14–15, Vilnius, Lithuania, p. 28.

Acknowledgements

I would like to thank to my scientific supervisor doc. dr. Asta Kaušaitė-Minkštimienė for the supervision, valuable ideas and the opportunity to develop this interesting subject of reseach.

I wish to thank colleagues form the Faculty of Chemistry, Vilnius University: doc. dr. Almira Ramanavičienė and prof. dr. Arūnas Ramanavičius and from the State Research Institute Center for Innovative Medicine: dr. Natalija German for their consultations and encouragement.

I would like to thank Lithuanian State Studies Foundation and The Research Council of Lithuania for financial support.

Curriculum vitae

Name and surname:	**Justina Rukšnaitė**
Birth date and place:	**1984 – 09 – 28, Vilnius**
Education:	
2003 – 2007	Bachelor degree of Bioengineering, Vilnius Gediminas Technical University, Faculty of Fundamental Sciences
2007 – 2009	Master degree of Chemistry, Vilnius University, Faculty of Chemistry
2009 – 2013	Doctoral studies at Vilnius University, Faculty of Chemistry
Work experience:	
2012 – 2013	Engineer, National Food and Veterinary Risk Assessment Institute
From 2013	Quality manager, BIOK Laboratory
Email address:	justina.ruksnaite@gmail.com

PAVIRŠIAUS PLAZMONŲ REZONANSO IR ELEKTROCHEMINIAI IMUNINIAI JUTIKLIAI ŽMOGAUS AUGIMO HORMONO IR ANTIKŪNŲ PRIEŠ ŽMOGAUS AUGIMO HORMONĄ NUSTATYMUI

SANTRAUKA

Šioje daktaro disertacijoje apibendrintų mokslinių tyrimų tikslas - sukurti paviršiaus plazmonų rezonanso (PPR) ir elektrocheminius imuninius jutiklius žmogaus augimo hormono (ŽAH) ir antikūnų prieš žmogaus augimo hormoną (anti-ŽAH) nustatymui bei pritaikyti juos realių mėginių analizei.

Darbe ištirti skirtingi anti-ŽAH imobilizavimo būdai PPR jutiklio lusto paviršiuje ir įvertintas imobilizuotų antikūnų gebėjimas sąveikauti su ŽAH ir parinktos optimalios imobilizavimo sąlygos. Taip pat ištirtas ir įvertintas PPR imuninių jutiklių ŽAH ir anti-ŽAH koncentracijos nustatymo intervalas, aptikimo ribos, atrankumas, analizinio signalo atsikartojamumas ir stabilumas. Nustatytos optimalios anti-ŽAH nustatymo sąlygos impulsinės amperometrijos (PA) ir ciklinės voltamperometrijos (CV) analiziniais metodais, koncentracijos nustatymo intervalas, aptikimo ribos ir analizinio signalo atsikartojamumas. Taip pat patikrinti sukurtų imuninių jutiklių veikimas realiuosuose žmogaus serumo mėginiuose.

Buvo nustatyta, kad tinkamiausias iš tirtų anti-ŽAH imobilizavimo PPR jutiklio lusto paviršiuje būdų yra anti-ŽAH fragmentų gautų redukuojant anti-ŽAH 2-MEA imobilizavimas. Lyginant su kitais, šis mobilizavimo būdas pasižymi pakankamai didele imobilizavimo talpa, atlikimo paprastumu bei maža kaina, o antikūnai imobilizuojamai kryptingai. Optimalios sąlygos anti-ŽAH fragmentų gavimui yra 15 mmol/L 2-MEA ir 0,41 μmol/L anti-ŽAH koncentracija, nes esant šioms sąlygoms didžiausia anti-ŽAH molekulių dalis redukuojama nepažeidžiant jų aktyviojo centro ir gaunamas didžiausias PPR kampo pokytis vykstant imobilizuotų frag-anti-ŽAH ir ŽAH sąveikai. JSA užblokuojapo anti-ŽAH fragmentų imobilizavimo likusius aktyvius centrus PPR jutiklio lusto aukso paviršiuje, tai sumažina nespecifinę sąveiką, padidina frag-anti-ŽAH sluoksnio stabilumą bei jo gebėjimą sąveikauti su ŽAH ilgesnį laiką. PPR ŽAH imuninis jutiklis pasižymi maža aptikimo riba (3,4 nmol/L), pakankamu analizinio signalo atsikartojamumu (96,3 % ir 93,8 %, atitinkamai tam pačiam ir skirtingiems jutikliams) ir stabilumu, plačiu analizuojamų koncentracijų intervalu (0,01 – 0,72 μmol/L) bei dideliu atrankumu. Aktyvus PPR jutiklio lusto paviršius visiškai regeneruojamas jį 5 minutes veikiant 0,5 % SDS ir 50 mmol/L NaOH tirpalu, o po to 3 minutes 10 mmol/L PBS buferiniu tirpalu, pH 7,4.

Optimali elektrocheminei imumoanalizei TMB ir H_2O_2 koncentracija yra 0,25 ir 0,1 mmol/L atitinkamai. Esant 0,25 mmol/L TMB koncentracijai PA ir CV analizės metodais registruojamas analizinis signalas padidėja 1,7 karto. 0,1 mmol/L H_2O_2 koncentracija padidina analizinį signalą 2,37 ir 1,77 karto, atitinkamai PA ir CV atveju. Anti-ŽAH imuniniai jutikliai pasižymi maža aptikimo riba (0,056, 0,051 ir 0,027 nmol/L, atitinkamai CV, PPR ir PA), pakankamu analizinio signalo atsikartojamumu (RSD = 0,06, 0,11 ir 0,12, atititinkamai PA, CV ir PPR) ir stabilumu, dideliu atrankumu ir trumpa analizės trukme (1, 5 ir 25 min, atitinkamai PA, CV ir PPR). Elektrocheminių imuninių jutiklių regeneracija negalima.

PA ir PPR imuniniai jutikliai gali būti taikomi atitinkamai anti-ŽAH ir ŽAH koncentracijos nustatymui realiuose mėginiuose.