Highly sensitive immunosenosors for determination of organochlorinated herbicides and PCBs based on the piezoelectric and surface plasmon resonance transducers

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Introduction

Immunochemical detection of toxic pollutants has become an important part of analytical chemistry. Antibody-based assays provide several advantages, including excellent sensitivity combined with high selectivity, theoretical and experimental simplicity, low costs and minimal sample pretreatment. Biosensors bring to the area of immunoanalytical techniques new potential, especially consisting of the possibility of full automatization, higher detection limits and lower material consumption. The Quartz Crystal Microbalance[1] (QCM) and Surface Plasmon Resonance[2] (SPR) biosensors allow real-time monitoring of immunoaffinity interactions without any additional labels, the QCM biosensors in many cases provides significant decrease of assay costs, SPR biosensors offer high sensitivity and time saving due the multichannel arrangement.

Two basic principles would be employed in construction of immunosensors, either the direct or indirect form of assay. The former one allows simple performance, when only the binding of analyte to the surface-bound antibody molecules is monitored. As the sensitivity of the QCM and SPR-based biosensors depends on the change of surface mass, the indirect (competitive) form of assay is usually preferred.

Numerous of methods for immunochemical detection of atrazine and 2,4-dichlorphenoxyacetic acid has been developed in recent years. Wide range of biosensor transducers was tested for this purpose; e.g. amperometric[3], optical[4] or piezoelectric[5]. Contrariwise, publications about development of biosensors for detection for PCBs, acetochlor and especially endosulfan have been published rarely[6,7].

In our studies, both formats of immunoanalysis were employed in the detection of herbicides and PCBs. 2,4-dichlorophenoxyacetic acid (2,4-D), atrazine, acetochlor, endosulfan, and three PCB congeners were chosen as the target compounds to be monitored by the immunobiosensors. In case of atrazine and PCBs, the direct assay using the QCM biosensor was successfully accomplished. Moreover, PCBs were analyzed using method analyzing competition between DCB-polystyrene conjugate and free PCB molecule. Simultaneous detection of 2,4-D, atrazine and acetochlor was performed in competitive format using multichannel, highly sensitive SPR instrument. Similarly, competitive assay for endosulfan analysis running on piezoelectric biosensor is described in this contribution.

Materials and Methods

Chemicals and materials

Ethyleneglycol-terminated mercaptoundecan ([11-mercaptoundecyl)oxy] methanol; C_{11}-PEG) was purchased from ProChimia (Sopot, Poland). Deionized water (Millipore apparatus) was used throughout and 50mM phosphate buffer pH 7.0 was used in all experiments as the working medium.

DCB-polystyrene conjugate was prepared as was published elsewhere [9].

Standards (solid) were purchased from Accustandard (New Haven, CT, USA).

All other chemicals were purchased either from Sigma (St. Louis, MO, USA) or Fluka (Buchs, Switzerland), and were used as supplied without further purification.

Measurements with QCM biosensor were performed using smooth, gold, 10 MHz, AT-cut quartz crystals (ICM, Oklahoma City, OK, USA). The SPR sensor chips[8] coated by an adhesion promoting chromium layer (thickness, 2 nm) and SPR-active gold layer (thickness, 55 nm) were prepared at the Institute of Radio Engineering and Electronics, Prague.

Immobilization procedures

Prior the biomolecules attachment, the gold surface was thoroughly cleaned with either chromium-sulphuric acid or with "piranha solution" (70% H_2SO_4–30% H_2O_2), finished by dipping into acetone.

For immobilization of antibodies, the previously[9] tested method, consisting of DTSP activation of gold (20 mg/mL in DMSO), deposition of protein A (1 mg/mL in water) and antibody (2 mg/mL in buffer) sublayers and covalent cross-linking with the dimethylpimelimidate (10 mg/mL in water), was employed.

Immunoconjugates were immobilized to the gold surface via various thiocompunds. Following compounds were used: cystamine (20 mg/mL in water), ATP, DTSP (20 mg/mL in DMSO), lipoic acid, 11-MUA, 11-MUOL and mixtures of 16-MHDA with C_{11}-PEG (1 mM, absolute ethanol). Amino-group layer was activated with glutaraldehyde (GA, 3% aquatic solution), carboxygroups were functionalized with TSTU solution (5 mg/ml, DMF). OVA-endosulfan conjugate (0.5 mg/mL) was deposited by its dropwising, the other conjugates (100 µg/mL) were deposited in flow. Layers containing Schiff bonds were stabilized by reduction with $NaBH_4$.

Equipment

MultiLab+ instrument (MultiLab, Brno, Czech Republic) combining oscillator and resonant frequency counter (resolution 1 mHz, sensitivity 0.686 ng/Hz) was used for experiments with piezoelectric biosensor. The eight channel instrument[8] (developed and made in Institute of Radio Engineering and Electronics, Prague) with wavelength division multiplexing was used for SPR experiments.

Measuring procedures

All measuring procedures have employed quite similar protocol: base line stabilization (working solution, 2-3 minutes) was followed by association (direct measurements – antigen; competitive experiments – preincubated mixture of antibody solution with antigen, 5-7 min.) and equilibration phase (working solution). In last step, the bound molecules were dissociated from surface using various solutions.

Pretreatment of soil and water samples

Soil samples for analysis of PCBs were extracted according to the modified procedures[10] and[11], mainly consisted of cold solvent extraction (by shaking) to toluene and filtration.

Water samples analysed for atrazine content using competitive assay on SPR instrument were collected from Prague water tap. The only preparation of thus samples for analysis consisted in their dilution with working buffer.

Results and Discussion

As a first step of all projects, optimization of studied assay was performed. For maximal sensitivity of biosensors, optimal density of binding places should be immobilized on the sensor surface. The antibody immobilization was not optimized, as this was done in previous studies[5,9]. On the contrary, attachment of protein-herbicide conjugates was deeply studied. As the sensor Gold-LA-OVA-END provided the highest response to the anti-endosulfan Ab, the maximal specifity and stability was obtained when the Gold-ATP-GA-OVA-END sensor was used. During optimization of the indirect experiments on SPR chip, a mixture of 16-MHDA with C_{11}-PEG (1:1 and 1:4, respectively) was additionally tested. An innovative immobilization procedure, employing equimolar mixture of thiocompounds with "binding" and protecting groups, was found to provide not only the highest response, but also extremely low inspecific

binding of biomolecules.

For direct (non-competitive) analysis of PCBs, previously optimized buffer constitution (neutral pH, 5% DMSO content) was employed. Perfect regeneration of sensing surface is one of the most important conditions for good reproducibility of experiments; 25% solution of acetonitrile (2 min.) disintegrates the PCB-Ab complex completely. Also composition of buffer for competitive analysis of herbicides on SPR chip was optimized and following values were found: neutral pH, low ionic strange (50mM buffer with no addition of salts) and flow rate 20 μl/min (gives the best response/noise ratio). For removing of bound antibodies, 3 minutes pulse of 25% solution of acetonitrile in 0.1M NaOH was employed. The addition of solvents was chosen according to solubility of analytes: atrazine, 2,4-D and acetochlor herbicides were analyzed in pure water (buffer), endosulfan was analyzed in buffer-solvent mixture and PCBs in either buffer-solvent mixture (non-competitive assay) or in pure organic solvents. Binding of heavy mass conjugate dissolved in toluene (most hydrophobic solvent) showed the highest response comparing to other used solvents (acetonitrile, DMSO, dichloromethane, trichloromethane and toluene - ordered according to increasing response). Mixture (1:1) of dichloromethane with toluene applied for 3 minutes to flow fully disintegrates the Ab-DCB-PS complex.

Subsequently, anti-atrazine and anti-2,4-D monoclonal antibody D6F3 and E2B5 were found to have the highest affinity to immobilized antigen, from range of eight other antibodies.

The constructed direct biosensor for atrazine (Gold-DTSP-pA-Ab(D6F3)) was calibrated (in duplicate) using atrazine solutions in range from 1 to 200 ng/mL. Calibration curve was linear among whole concentration range; minimum detectable concentration of atrazine was calculated to be 1.5 ng/mL (fivefold of signal noise). Described biosensor is able to perform up to 40 experiments without significant loosing of sensitivity. However, further optimization should be done, as the detection limit is close below the US EPA and WHO limits for atrazine content in drinking water (3 and 2 ppb), but about one order higher than the legal limits in EU (0.1 ppb). Even higher detection limits provided the direct assay of PCBs, due to the usage of polyclonal antibody with lower specifity (significant values of Ab cross-reactivity were found for following PCB congeners: 100% for 2,4,4'-TCB, 40% for 4,4'-TCB and 15% for 3,3',4,4'-TCB). Detection limits were determined to be 0.37 and 0.11 μg/mL for 4,4'-DCB and 2,4,4'-TCB, respectively. This biosensor is stable for about 30 experiments and would be used in analysis of extracts from highly contaminated samples.

An original, highly resistant flow-trough setup was constructed for performing of direct-competitive analysis of PCBs in pure solvents. Assay in toluene was calibrated, detection limit 6.0 ng/mL was calculated as I_{20} (sigmoidal fit).

The first immuno-biosensor for endosulfan detection (indirect arrangement) was developed, providing detection limit 0.1 ppb.

Experiments on SPR chip employed the indirect format, too. Solution of antibody was mixed with solution of antigen (10^{-4} to 10^{3} ng/mL) and used for analysis of binding to the biospecific surface. Response of SPR biosensor was determined as the slope of linear part of the association curve corrected to instability of base line. Detection limits were 0.01 ppb (atrazine and 2,4-D) and 10 ppb (acetochlor).

Assay for PCBs analysis (in direct-competitive arrangement) was tested for its ability to be used in soil extract analysis. Three different kinds of samples were used in experiments: samples from a clean environment (area near Pelhřimov, Czech Rep.), the same samples spiked with a high concentration of 4,4´-DCB, and samples from a highly-industrial area (Milovice, Czech Rep.). The differences between biosensor and HPLC results proceeded from 10 (higher concentrations) to 50% (low PCBs concentrations). This results, in combination with low inspecific response o extracts (below 1%), makes a good basement for further assay development. SPR -based competitive method was tested in tap water analysis (non -contaminated and atrazine -spiked samples). Error values up to 15% show an optimistic overview in further usage of developed assay.

Abbreviations

16-MHDA, 16 -mercaptohexadecanoic acid; 11 -MUA, 11 -mercaptoundecanoic acid; 11 -MUOL, 11 -mercaptoundecanol; ATP, 4 -aminothiophenol; C_{11}-PEG, ethyleneglycol -terminated mercaptoundecan; GA, glutaraldehyde; LA, lipoic acid; OVA-END, ovalbumine-endosulfan conjugate.

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