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Disposable Electrochemical Magnetic Beads-Based Immunosensors for Monitoring Polychlorinated Biphenyl (PCBs) Pollutants

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Screen-printed carbon electrodes (SPCEs) and magnetic beads were used in a combination to obtain immunosensors able to detect different groups of PCBs. The proposed devices were based on a direct competitive immunoassay scheme. Magnetic beads were employed as solid phase, whereas screen-printed electrodes as transducers to evaluate the progress of the immunochemical reaction. Alkaline phosphatase (AP) enzyme was used for labelling. Detection of the reaction product was performed applying differential pulse voltammetry (DPV). Different antibodies and tracers were combined in order to obtain immunosensors for different PCB mixtures and congeners. Combining IgG-against-PCB28 „antibody" and a PCB28-AP tracer, an immunosensor for Aroclor mixtures was developed. Using in turn an IgG-against-PCB77 and PCB77-AP tracer, detection of coplanar congeners (so called „dioxin like" molecules) was performed. Calibration studies of some mixtures and congeners were carried out with both developed systems. The dose-response curves of the competitive immunoassays exhibited typical sigmoidal shape and good detection limit. The developed immunosensors were applied for detection of PCBs in marine sediment extracts. The results were encouraging for future applications of the designed sensors for the analysis of food, soil, and other environmental samples.

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W pracy sitodrukowane elektrody węglowe (SPCEs) łączono z kulkami magnetycznymi, w celu otrzymania immunosensorów zdolnych do detekcji różnych grup polichlorowanych bisfenoli (PCBs). Prezentowane urządzenia są oparte na bezpośredniej kompetytywnej analizie przeciwciał. Urządzenia te są zbudowane z kulek magnetycznych, które pełnią rolę stałego podłoża, a sitodrukowane elektrody są przetwornikami służącymi do oceny postępu reakcji immunochemicznej. Fosfataza zasadowa (AP) została użyta jako znacznik enzymatyczny, a detekcję produktu reakcji prowadzono przy użyciu pulsowej woltamperometrii różnicowej (DPV). Różne przeciwciała i wskaźniki łączono w celu otrzymania immunosensorów dla różnych mieszanin i kongenerów polichlorowanych bisfenoli. Używając IgG przeciwko PCB28 jako przeciwciała i PCB28-AP jako wskaźnika, opracowano immunosensor zdolny do detekcji mieszanin Aroclor, podczas gdy użycie IgG przeciwko PCB77 i PCB77-AP umożliwiło detekcję koplanarnych kongenerów, nazywanych także cząsteczkami typu „dioxin-like”. Krzywe kalibracji dla niektórych mieszanin i kongenerów wyznaczono przy użyciu dwóch opracowanych systemów; krzywe odpowiedzi charakteryzowały się typowym sigmoidalnym kształtem kompetytywnej analizy przeciwciał i niską granicą detekcji. Opracowane immunosensory zostały użyte do oznaczenia PCBs w ekstraktach z osadów z dna morskiego i przewiduje się ich wykorzystanie do oznaczeń polichlorowanych bisfenoli w żywności, glebie i innych próbkach środowiskowych.

Polychlorinated biphenyls (PCBs) are toxic organic compounds. It is currently assumed that food is the major source of the PCB exposure. Since PCBs are lipophilic and accumulate in the food chain, food of animal origin is an important source of exposure. In the recent years several researchers have determined the concentration of PCBs in aquatic environmental samples. They have attributed the results, at least partially, to the food-chain biomagnification [1–4]. The level of PCBs in the environment depends on the matrix. In soil, for example, concentrations of PCBs can range between several ng g^{-1} up to mg g^{-1} (the upper limit corresponds to highly contaminated sites), whereas in sediments the upper limit is generally hundreds of μg g^{-1}. The limit of 4 ng g^{-1} for total PCB concentration in marine sediments has been fixed by the Italian Legislation [5].

There are 209 polychlorinated biphenyl congeners that persist worldwide in the environment and food chain. These congeners are divided into three classes based upon orientation of chlorine moieties, *i.e.*, coplanar, mono-ortho coplanar, and non--coplanar [6, 7]. Due to their high binding affinity to the aryl hydrocarbon (Ah) receptor, coplanar congeners are potentially the most toxic [8] and have been also shown to impact the immune system suppressing immunocompetence.

PCBs were produced and sold under many names; the most common are the „Aroclor series”. Aroclor refers to the mixture of individual chlorinated biphenyl compounds of a varying degree of chlorination. Aroclor mixtures usually have their own numbers, which provide additional information on the properties of the mixture. The most common mixtures in the Aroclor series are Aroclor 1016, Aroclor 1242, Aroclor 1248, Aroclor 1254, and Aroclor 1260. Bio-accumulation through the food

chain tends to concentrate congeners of high chlorine content, producing residues that are considerably different from the original Aroclors [9]. For this reason, there is always the need to build up the systems able to detect different classes of PCBs in food and environmental samples [10–11].

Conventional techniques used for the analysis of PCBs are generally based on gas chromatography coupled with mass spectrometry (GC–MS) [12]. Alternative techniques based for example on immunoassays, are inexpensive and rapid screening tools for sample monitoring in laboratory and field analysis. Moreover, immunoassays are simple, sensitive, reliable, and relatively selective for PCBs testing. Among several immunoassay techniques, the enzyme-linked immunosorbent assay (ELISA) combined with colorimetric end-point detection are the most popular [13, 14]. Another interesting approach is the use of immunosensor technology. In this case either antibody or antigen molecules are directly immobilised at the sensor surface (transducer) giving rise to a compact and miniaturised system. Examples are electrochemical immunosensors with voltammetric transducers, which have gained considerable attention in the recent years. Electrochemical immunosensors are usually based on the enzyme-labelled reagent that generates an electroactive product, which is in turn detected at the electrode surface [15, 16]. However, the use of a solid electrode surface as well as electrochemical transducer is not very convenient: shielding of the electrode surface by biospecifically bound antibody molecules may hinder electron transfer and make a voltammetric signal decrease. An interesting approach to improve the sensitivity involves the use of electrodes for the transduction step, whereas the affinity reaction is performed using another physical support. For such affinity--based biosensors [17–20], antibody-coated magnetic beads as a solid phase for immunochemical test in combination with screen-printed carbon-based electrodes (SPCEs) as electrochemical transducers have been proposed.

In this work, electrochemical immunosensors for detection of different classes of PCBs have been developed. These sensors are based on the combination of functionalised immunomagnetic beads and screen-printed electrodes. Different antibodies and the corresponding tracers were used to distinguish between different classes of PCBs (coplanar and non-coplanar molecules) and to characterise PCB pollution. The developed immunosensors were used for the detection of Aroclor mixtures and dioxin-like congeners. Preliminary analysis of marine sediment samples was also performed. The obtained results were encouraging for future applications of the designed immunosensors for PCB pollution screening.

EXPERIMENTAL

Reagents and chemicals

Magnetic beads coupled with protein G were purchased from Dynal Biotech (Milan, Italy). Sheep polyclonal antibodies against PCB28 (IgG anti-PCB28) congener, rabbit polyclonal IgG against PCB77 (IgG anti-PCB77), and tracer solutions containing PCB28-alkaline phosphatase conjugate (PCB28-AP) and PCB77-alkaline phosphatase conjugate (PCB77-AP) were provided by Prof. M. Fránek, Veterinary Research Institute, Brno, Czech Republic.

Standard solutions of PCB77 and 126 congeners, as well as Aroclor 1242, 1248, and 1016 mixtures were purchased from AccuStandard, Inc. (New Haven, USA).

α-Naphthyl phosphate, diethanolamine, and sodium chloride were purchased from Sigma-Aldrich Company (Milan, Italy).

Methanol, $Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, and $MgCl_2$ solution were purchased from Merck (Milan, Italy).

All solutions were prepared using a Milli–Q Water (Milli–Q Water Purification System (Millipore, UK).

The following buffers and solutions were used:

- 0.1 mol L^{-1} Na_3PO_4 solution, pH = 5, for washing and coating magnetic beads, according to the manufacturer's instructions;
- the mixture of 3×10^{-1} mol L^{-1} phosphate buffer, pH 7.2, 5×10^{-3} mol L^{-1} NaCl, and 1% methanol (v/v) as the working buffer for the competitive assay (PBS buffer)
- the mixture of 0.1 mol L^{-1} diethanolamine buffer (DEA buffer) solution, pH 9.6, 1×10^{-3} mol L^{-1} $MgCl_2$ and 0.1 mol L^{-1} KCl solution as a buffer for electrochemical detection studies

Sample mixer with a 12-tube mixing wheel and a magnet was purchased from Dynal Biotech (Milan, Italy).

Marine sediment extracts without PCBs were provided by the Fisheries Research Service of Marine Laboratory (FRS-ML), Aberdeen, Scotland (UK).

Instrumentation

Planar three-electrode strips comprising a carbon working electrode, a carbon counter electrode and a silver pseudo-reference electrode (Fig. 1) were used as electrochemical cells. The electrodes were screen--printed using a DEK 248 screen-printing machine (DEK, Weymouth, UK). Silver-based (Electrodag PF–410) and graphite-based (Electrodag 423 SS) polymeric inks were obtained from Acheson (Milan, Italy). A Vinylfast 36–100 insulating ink was purchased from Argon (Lodi, Italy). A polyester flexible film (Autostat CT5) from Autotype (Milan, Italy) was used as the printing substrate. Silver ink was printed to obtain conductive tracks and silver pseudo-reference electrode. Carbon was printed to obtain the working and the auxiliary electrodes. After each step, silver and carbon inks were treated at 120°C for 10 min. The insulating ink was also used to define the working electrode surface area (Ø = 3 mm). Then, the treating was performed at 70°C for 20 min.

Electrochemical measurements were performed using an Autolab PGSTAT 10 electrochemical analyser controlled *via* GPES 4.9 software (Metrohm, Rome, Italy). The Autolab analyser was combined with a multiplexer module with eight channels, even if only five channels were used for measurements. A connector for five screen-printed electrodes was connected with a bar with five magnets in order to perform serial measurements.

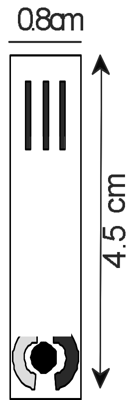

Figure 1. A scheme of a carbon screen-printed electrode

All measurements were carried out at room temperature. In differential pulse voltammetric (DPV) studies the following parameters were applied: range potential 0/+ 600 mV, step potential 7 mV, modulation amplitude 70 mV, standby potential 200 mV, interval time 0.1 s.

Assay scheme

A competitive assay was performed according to the scheme presented in Figure 2. All parameters of the competitive assay were optimised for both immunosensors and are reported in the next section.

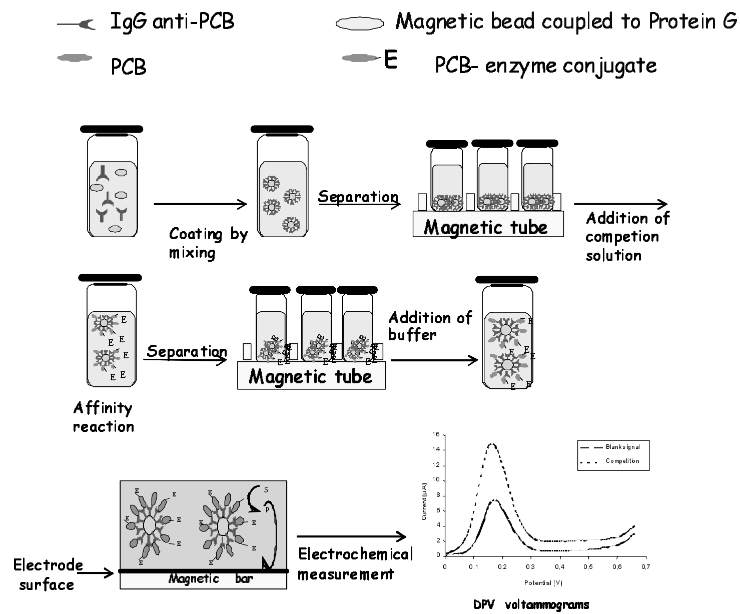

Figure 2. Schematic representation of immunochemical reaction, magnetic separation, electrochemical measurement, and DPV voltammograms

Beads preparation

All preparation steps were performed at room temperature. Washing, coating and competition steps were carried out under gentle stirring in the sample mixer.

Before use, magnetic beads coupled with protein G were washed with 0.1 mol L^{-1} Na_3PO_4 solution, pH = 5 to remove NaN_3 preservative, as advised by the manufacturer.

10 μL of bead suspension was introduced in a tube containing 500 μL of 100 mg L^{-1} antibody (IgG anti-PCB28 or IgG anti-PCB77) solution in 0.1 mol L^{-1} Na_3PO_4 solution, pH = 5. After 20 min incubation time, the tube was fixed on a magnet holding block to allow the beads to precipitate on the bottom of the test tube. The supernatant was then removed and the beads were washed twice with 500 μL of the washing solution (0.1 mol L^{-1} Na_3PO_4 solution, pH = 5). Each washing step consisted of re-suspension of the beads in the washing solution for 2 min, followed by the separation with the magnet holding block to remove the supernatant. In this way, the antibody-coated beads were obtained. They could be also prepared in advance and stored at +4°C for several weeks.

Immunochemical reaction and electrochemical measurement

50 μL of the suspension containing antibody-coated beads were mixed with 940 μL of the sample solution. 10 μL of the tracer solution (PCB28-AP or PCB77-AP) were added to this mixture. After incubation for 20 min, the beads were magnetically separated and the supernatant was removed.

After two washing steps, the beads were re-suspended in 100 μL of the working assay buffer and 10 μL of the suspension were transferred onto the surface of the working electrode. To better localise the beads on the electrode, the magnet holding block was placed on the bottom part of the electrode. Then, 60 μL of 1 g L^{-1} solution of the enzymatic substrate (α-naphtyl phosphate) in DEA buffer were deposited on the screen-printed strip to close the electric circuit. After 5 minutes, the enzymatic product was determined by DPV (Fig. 2).

Sample analysis

Marine sediment extracts. PCBs-free samples of marine sediments were collected, extracted with organic solvent using Soxhlet apparatus, and characterised by GC-ECD in FRS-ML laboratory.

For the analysis, 1 mL of the sample was left to evaporate in order to remove the organic solvent and then reconstituted in the same volume of PBS buffer. Subsequently, 10 μL of the standard solution (Aroclor mixture, or single PCB congener) were added to the non-contaminated solution in order to obtain the final concentration in the range: 2.5–1000 μg L^{-1}. Afterwards, the competition reaction was performed (see Section Immunochemical reaction and chemical measurement).

RESULTS AND DISCUSSION

Optimisation of immunoassay

In order to optimise the immobilization time of the antibodies onto magnetic beads, different coating times of beads suspension with the antibody solution (100 mg L^{-1} phosphate solution of IgG anti-PCB28) in the range 5–40 min were tested. Then, the beads were incubated for 30 min with the corresponding tracer solution (1000-fold

diluted with respect to the stock solution). Low current values were measured when incubation time was shorter than 10 min. The current started to increase when antibodies and beads were in contact for more than 10 min. For incubation times longer than 20 min, the current became constant and the reaction was terminated. Hence, a incubation time of 20 min was chosen as the minimum necessary for binding the antibodies to the magnetic beads.

Another optimised condition was the time necessary to complete the affinity reaction. The experiments were performed incubating the magnetic beads with IgG anti-PCB28 (100 mg L^{-1} in phosphate solution) for 40 min and then the antibody-coated beads were exposed for different times to the solution containing the corresponding tracer (1000-fold diluted with respect to the stock solution) and the competitor (1 mg L^{-1} Aroclor 1248). The obtained res-ponses were compared to those obtained in the same experiment but in the presence of the tracer only. In the absence of the competitor, the current signal increased until reaching a constant value after 20 min, whereas in the presence of the competitor the current was very low and constant for all times. Thus, 20 min was chosen as the minimum time necessary to complete the affinity reaction.

In a direct competitive assay it is also important to optimise the concentration of the tracer. The content of the tracer must be sufficient to saturate the antibodies immobilised on the solid phase. The concentrations of both PCB28-AP and PCB77-AP were optimised. Magnetic beads were incubated for 20 min in the antibody solution (100 mg L^{-1} in phosphate solution). Then, the antibody-coated beads were incubated for 20 min with the corresponding tracer solutions at different concentrations. The results obtained for both tracers are plotted in Figures 3 and 4, respectively.

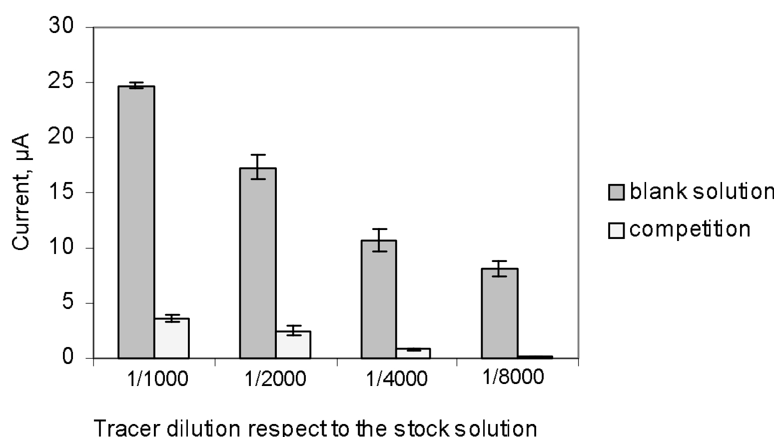

Figure 3. Optimisation of the PCB28-AP dilution with respect to the stock solution. IgG anti-PCB28 concentration: 100 mg L^{-1}

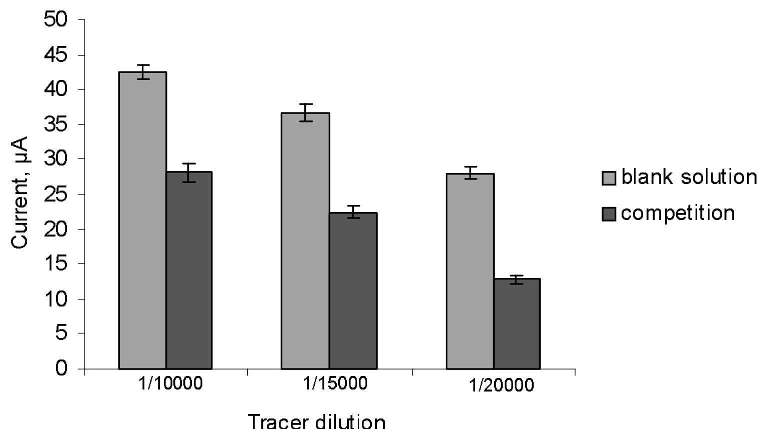

Figure 4. Optimisation of the PCB77-AP dilution with respect to the stock solution. IgG anti-PCB77 concentration: 100 mg L^{-1}

The measured response was compared to the signal recorded in the presence of the corresponding analyte (1 mg L^{-1} Aroclor 1248, or 1 μg L^{-1} PCB77 solution). The best dilutions for competition reaction were 1:1000 and 1:20000, respectively for PCB28-AP and PCB77-AP.

Application of immunosensors to the analysis of PCB standard solutions

Under optimised conditions, the developed immunosensors were applied to the detection of different PCBs solutions.

The immunosensor with IgG anti-PCB28 as antibody and PCB28-AP as tracer was applied to the detection of PCBs in some Aroclor mixtures. The corresponding calibration plots are shown in Figure 5. The measured signal is expressed in percentage (relative) units as Bx/Bo (*i.e.* measured signal-to-blank signal ratio) and plotted *vs* logarithm of the mixture's concentration. All plots exhibit a sigmoidal shape typical for competitive immunoassays. This result is consistent with our expectations because Aroclor 1242, 1248, and 1016 contain a significant amount of PCB28 and other structurally similar congeners. The shapes of the responses are, however, different because of different affinities of the antibodies towards particular congeners present in each Aroclor mixture. This conclusion has been confirmed by IC50 values measured for each mixture (IC50 is the concentration of target analyte that lowers the assay by 50%), as well as by different detection limits (DL). DL values were calculated as the average blank solution response (containing only the tracer) minus two times the standard deviation. IC50 values ranged between 8 and 94 μg L^{-1}, whereas DLs were between 0.3 and 0.8 μg L^{-1} (Tab. 1).

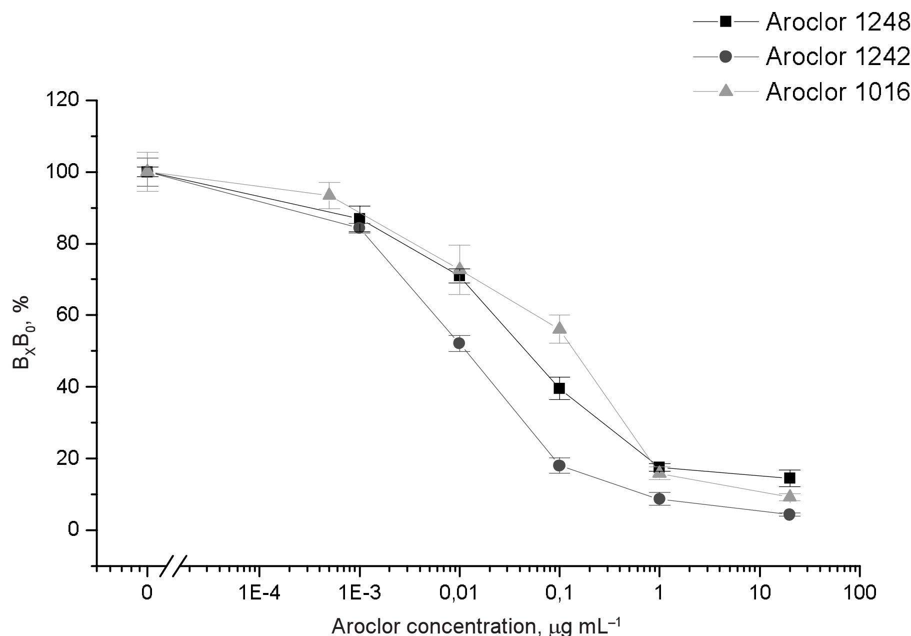

Figure 5. Calibration plot for selected Aroclor mixtures obtained using an immunosensor based on IgG anti-PCB28 as an antibody and PCB28-AP as a tracer. IgG anti-PCB28 concentration: 100 mg L^{-1}, tracer dilution 1:1000 with respect to the stock solution

Table 1. Results of the analysis of different Aroclor mixtures obtained with an electrochemical immunosensor

PCBs mixture	DL µg L^{-1}	[log IC_{50} (mg L^{-1})]	IC_{50} µg L^{-1}
Aroclor 1242	0.3	–2.1 ± 0.1	8
Aroclor 1248	0.4	–1.6 ± 0.1	24
Aroclor 1016	0.8	–1.0 ± 0.3	94

The immunosensor based on IgG anti-PCB77 and PCB77-AP was applied to the detection of PCB77 congener in the concentration range 0–0.250 mg L^{-1}. As shown in Figure 6, IC_{50} is 6.2 µg L^{-1} and the calculated DL is 5.1×10^{-1} µg L^{-1}. The same immunosensor was also applied to detect coplanar congener PCB126. The corresponding calibration line for the concentration range 0–1000 µg L^{-1} is shown in Figure 7. In this case IC_{50} equals 88.7 µg L^{-1}. This value is ca. ten times higher than that obtained for PCB77.

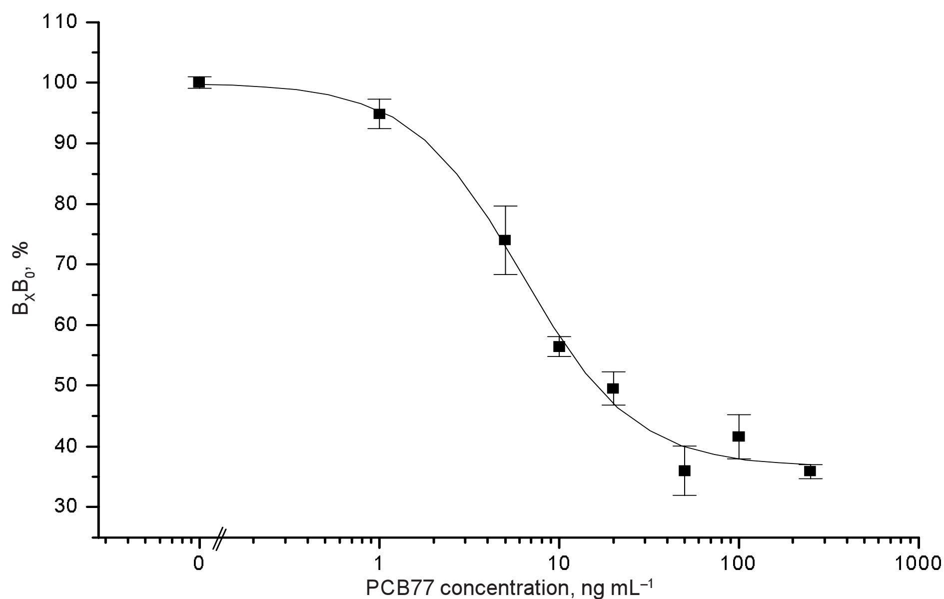

Figure 6. Calibration plot for PCB77 obtained using an immunosensor based on IgG anti-PCB77 as an antibody and PCB77-AP as a tracer. IgG anti-PCB77 concentration: 100 mg L^{-1}, tracer dilution 1:20000 with respect to the stock solution

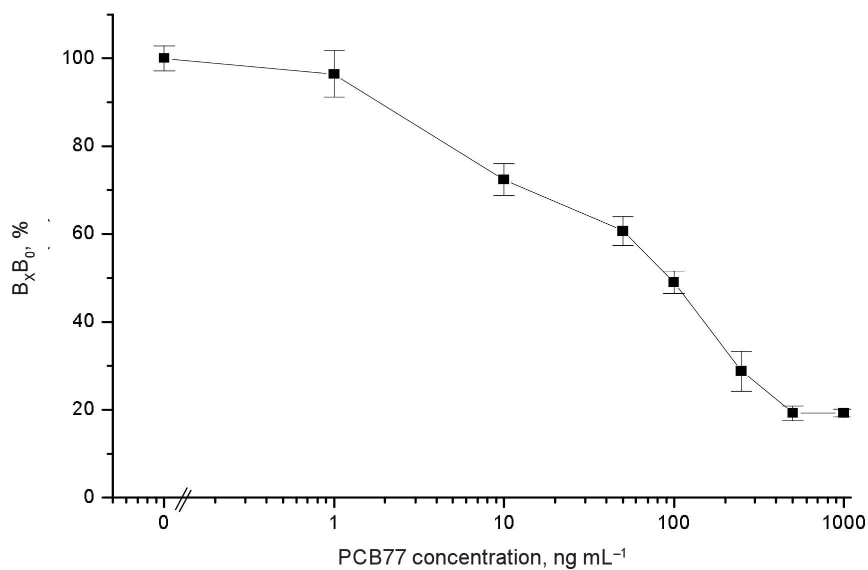

Figure 7. Calibration plot for PCB126 obtained using an immunosensor based on IgG anti-PCB77 as an antibody and PCB77-AP as a tracer. IgG anti-PCB77 concentration: 100 mg L^{-1}, tracer dilution 1:20000 with respect to the stock solution

Cross-reactivity of the immunosensor for PCB77 was also tested in some Aroclor mixtures. Figure 8 shows the calibration curves obtained for Aroclor 1260 and 1248. The immunosensor was occurred to be more reactive towards Aroclor 1260 (IC_{50} = 1.9×10^{-1} mg L^{-1}) than towards Aroclor 1248 (IC_{50} = 1.0 mg L^{-1}). The reason can be different compositions of both mixtures. Aroclor 1260 is characterised by a high content of high-chlorinated congeners, whereas Aroclor 1248 contains mainly low-chlorinated congeners and different coplanar PCBs. Therefore, PCB77 can be used as a precursor for detection of high-chlorinated mixtures, like, for example, Aroclor 1260.

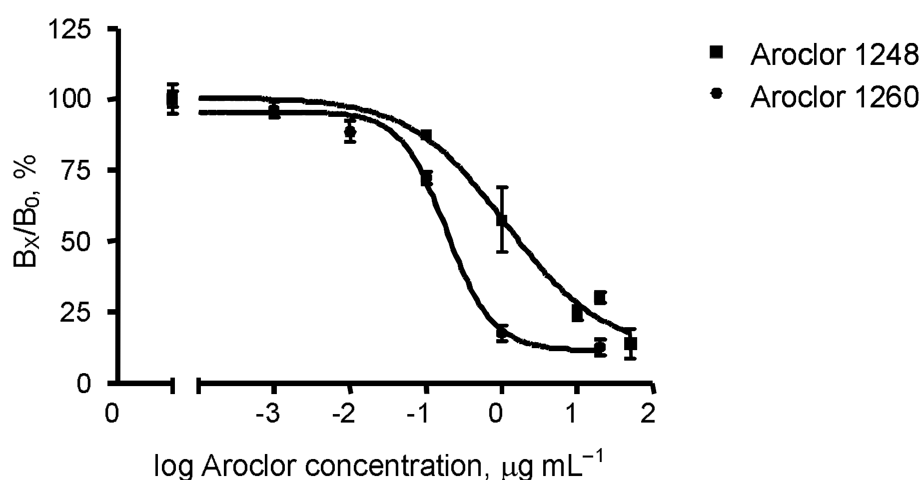

Figure 8. Calibration plots for Aroclor 1260 and 1248 obtained using an immunosensor based on IgG anti--PCB77 as an antibody and PCB77-AP as a tracer. IgG anti-PCB77 concentration: 100 mg L^{-1}, tracer dilution 1:20000 with respect to the stock solution

The above results have evidenced the possibility of coupling two immunosensors in order to have a complementary evaluation of PCB contamination in real samples.

Application to real samples analysis: marine sediment samples

Marine sediment extracts were spiked with various concentrations of different standard PCB solutions and analysed using the designed immunosensors. The obtained results are shown in Table 2. The measured signals are expressed with respect to the blank signal, which is assumed as 100%. Non-spiked samples were also analysed in order to evaluate potential matrix effect; this was, however, not detected (data not shown).

All samples spiked with a commercial formulation (A, B, C, and D) gave a decreased signal when analysed with both immunosensors.

Table 2. Results of the analysis of marine sediment samples obtained with the developed immunosensors. A comparison of signals obtained obtained for Aroclor mixtures and coplanar congeners is shown. The B_x/B_o ratios are accompanied by the corresponding SD values calculated for 3 repetitive determinations

Sample code	Spiked Aroclor mixture	Spiked concentration, µg L^{-1}	Immunosensor response in Aroclor mixtures B_x/B_o, %	Immunosensor response towards coplanar congeners B_x/B_o, %
A	Aroclor 1242	1000	22 ± 5	92 ± 4
B	Aroclor 1242	50	45 ± 1	99 ± 2
C	Aroclor 1248	20	59 ± 2	90 ± 2
D	Aroclor 1016	20	79 ± 4	98± 6
E	PCB77	2.5	93 ± 5	85± 2
F	PCB77	5	95 ± 3	81 ± 2
G	PCB77	500	99 ± 4	33 ± 2
H	PCB126	5	102 ± 6	80 ± 6

The signals recorded in samples E, F, G, and H were not decreased when measured with the immunosensor specific for PCB28. In all samples, however, the measured signal was decreased when they were analysed with immunosensor for coplanar congeners. In addition, the percentage signal measured in sample G was lower than the value obtained for samples F and H, containing the same congener (PCB77) at a lower concentration (2.5 and 5 µg L^{-1} *vs* 500 µg L^{-1}). Similar signals were measured for samples F and H, containing different congeners (PCB77 and PCB126) at equal concentrations.

In samples A, B, C, and D the presence of PCBs was confirmed using both immunosensors. In samples E, F, G, and H the presence of PCBs was evidenced only when the immunosensor specific for coplanar congeners was used.

The combination of the two immunosesors – one specific for PCB28 and another for PCB77 – allows to distinguish between different classes of PCB compounds present in real contaminated samples.

CONCLUSIONS

In this paper two different sensitive and reproducible immunosensors for detection of PCBs have been characterised. The proposed immunoassay scheme is based on the use of magnetic beads in combination with disposable and cheap screen-printed

sensors. Two different classes of PCBs (dioxin-like and non-coplanar congeners) can be detected in a short time.

Screening measurements allow one to either confirm or exclude sample contamination in a short time, as well as to identify the type of congeners. They are important in the analysis of a big number of samples. The proposed approach seems to be a useful analytical tool for fast screening analysis of many samples. Conventional methods can be then applied only to the positive samples in order to exactly quantify concentration of PCBs.

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