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Biacore biosensor immunoassay for 4-nonylphenols: assay optimization and applicability for shellfish analysis

Jeanne V. Samsonova [a,*], Natalya A. Uskova [a], Alexey N. Andresyuk [b], Milan Franek [c], Christopher T. Elliott [d]

[a] *Department of Chemical Enzymology, Chemistry Faculty, M.V. Lomonosov Moscow State University, 119992 Moscow, Russia*
[b] *"Chimmed", Kashirskoe shosse 9, Bld. 3, 115230 Moscow, Russia*
[c] *Department of Biotechnology, Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic*
[d] *Department of Veterinary Science, Queen's University of Belfast, Stoney Road, Stormont, BT4 3SD Belfast, UK*

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Abstract

A rapid Biacore biosensor immunoassay of 4-nonylphenols was developed. Two types of antibodies were used in the study: polyclonal antibodies with high cross-reactivity towards technical 4-nonylphenol and a monoclonal antibody very specific to 4-*n*-nonylphenol. 9-(*p*-Hydroxyphenyl)nonanoic acid was immobilized onto surface of a sensor chip. The best assay sensitivity was achieved using a flow rate of 50 μl min^{-1} and injection time of 2 min. For the assay incorporating monoclonal antibodies a limit of detection 2 ng ml^{-1} for 4-*n*-nonylphenol was achieved. With polyclonal antibodies one order lower sensitivity was observed for 4-nonylphenols. High background level of calibration curve for technical 4-nonylphenol was decreased by using IgG fraction of polyclonal antibodies in combination with lower amount of immobilised 9-(*p*-hydroxyphenyl)nonanoic acid. Sensitivity of the assay was improved by using a chip with a new derivative on a surface-*N*-aminobutyl [2-(4-hydroxyphenyl)ethylamine] (limit of detection—5 ng ml^{-1}). Applicability of the developed assays to ecological monitoring was checked in experiments using shellfish samples. 4-*n*-Nonylphenol from spiked samples was extracted into hexane followed by clean-up on NH2 SPE columns. Calibration curves generated for cockles, mussels and oyster samples were identical (limit of detection about 10 ng g^{-1}) whereas for scallop samples a slight decrease (about 5–10%) of absolute response was observed. In the assay using the monoclonal antibody specific to 4-*n*-nonylphenol 31 shellfish samples were found to be negative. Results obtained with polyclonal antibodies indicated that two scallop samples contained a quantity of 4-nonylphenols. The developed biosensor assay could be applied for shellfish analysis as a preliminary screening method.

Keywords: 4-nonylphenols; Endocrine disruptors; Biacore biosensor; Shellfish

1. Introduction

Non-ionic synthetic detergents—polyethoxylated alkylphenols—are widely used in different domestic and industrial products. Huge amounts of these detergents are directly released into the environment

* Corresponding author. Tel.: +7 095 9393407; fax: +7 095 9392742.
E-mail address: jvs@enz.chem.msu.ru (J.V. Samsonova).

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where they degrade to more toxic and persistent metabolites than the parent compounds (Ying et al., 2002). Alkylphenols such as nonylphenol (NP) and octylphenol (OP) are the most dangerous metabolites because of theirs enhanced resistance towards biodegradation, toxicity, strong estrogenic effects (Fig. 1). They can mimic natural hormones and interact with the estrogen receptor causing different adverse effects such as feminisation in fish (Schwaiger et al., 2002). There are a large number of investigations reported with regard to the toxic and estrogenic properties of 4-NP. Alkylphenols occurrence in the environment is quite common, e.g., 4-NP residues were discovered in water sources, sediments and aquatic organisms even far away from a cost (Tsuda et al., 2000b; Bester et al., 2001; Ferrara et al., 2001; Guenther et al., 2001; Petrovich et al., 2001). Recent studies showed that 4-NP residues are ubiquitous in food (Guenter et al., 2002). It was also reported about migration of 4-NP from polyvinyl chloride food packaging films into food (Inoue et al., 2001). These findings have raised increasing concern of 4-NP impact on the environment and human health.

For detection of nonylphenol ethoxylates and 4-NP in the environmental and food samples, the chromatographic methods such as gas, liquid or high pressure liquid chromatography are used (Ferrara et al., 2001; Guenther et al., 2001; Corsi and Focardi, 2002). However, simple and quick analytical methods are required to detect 4-NP presence in the environment. Chromatographic methods are usually time-consuming and demand sophisticated equipment. Immunoassays based on very specific antibody-antigen interaction are usually cheaper and more sensitive then chromatographic methods and are very suitable for use in routine monitoring programmes. Biosensor techniques combined

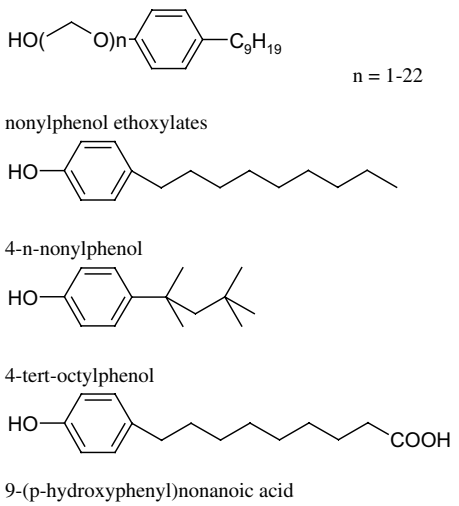


Fig. 1. Structure of nonylphenols ethoxylates, alkylphenols and 9-(*p*-hydroxyphenyl)nonanoic acid.

properties of biological recognising molecules such as antibodies, receptors, etc., with sensitive signal transformation allowed to develop faster and simpler methods which found application in different fields (Nakamura and Karube, 2003).

Recently polyclonal and monoclonal antibodies with a range of different specificities to alkylphenols were produced (Franek et al., 2001; Zeravik et al., 2004). The polyclonal antibodies were used for establishment of an indirect ELISA, polarisation fluoroimmunoassay and flow injection immunoassays for 4-NPs operating in the mg l^{-1} range (Franek et al., 2001). In the recently published study a more sensitive direct ELISA was developed (Zeravik et al., 2004). The polyclonal antibodies and new monoclonal antibody showed IC_{50} values around 40 μg l^{-1} for technical 4-NP and 11.5 μg l^{-1} for the linear isomer 4-*n*-NP. Cross-reactivity pattern of the antibodies was thoroughly investigated. Two polyclonal antibodies showed a strong tendency to recognise a total amount of 4-alkylphenols whereas other two polyclonal antibodies and monoclonal antibody allowed sensitive detection of linear forms of 4-alkylphenols (Zeravik et al., 2004). Very sensitive chemiluminescent ELISA and simple dipstick test for 4-*n*-NP were also recently developed (Samsonova et al., 2003).

The purpose of the present study was to develop a rapid and sensitive biosensor method for 4-NPs determination. For the assay development two 4-NP derivatives for immobilization on a surface of a sensor chip and two types of antibodies with different cross-reactivity pattern were used. Applicability of the developed assays for shellfish analysis was investigated.

2. Materials and methods

2.1. Materials

4-*n*-Nonylphenol (99.7% purity) was purchased from Supelco (Bellefonte, PA, USA); technical 4-nonylphenol, 4-(*tert*-octyl)phenol (97%), glycidyl 4-nonylphenol ether were supplied by Sigma–Aldrich (Milwaukee, USA), 3-(*p*-hydroxyphenyl) propionic acid and 2-(4-hydroxyphenyl)ethylammonium chloride were from Merck (Hohenbrunn, Germany). Ready-to-use HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4), CM5 sensor chips (research grade), Amine Coupling Kit were obtained from Biacore AB (Uppsala, Sweden). Bond Elute NH2 SPE columns (500 mg/3 ml) were supplied by Jones Chromatography (Hengoed, UK). All other chemicals and reagents were purchased from Sigma (Poole, Dorset, UK).

Stock solutions of alkylphenols (1 mg ml^{-1}) were prepared in methanol and stored at −20 °C. Standard solutions in concentration range 0–10 μg ml^{-1} were prepared in HBS-EP buffer and stored at 4 °C.

Production of monoclonal and polyclonal antibodies was described earlier (Franek et al., 2001; Zeravik et al., 2004).

Synthesis of 9-(*p*-hydroxyphenyl)nonanoic acid was described by Mikhura et al. (2000).

An optical biosensor BIACORE Q was obtained from Biacore AB (Uppsala, Sweden) and operated by BIACORE 3.0 control software.

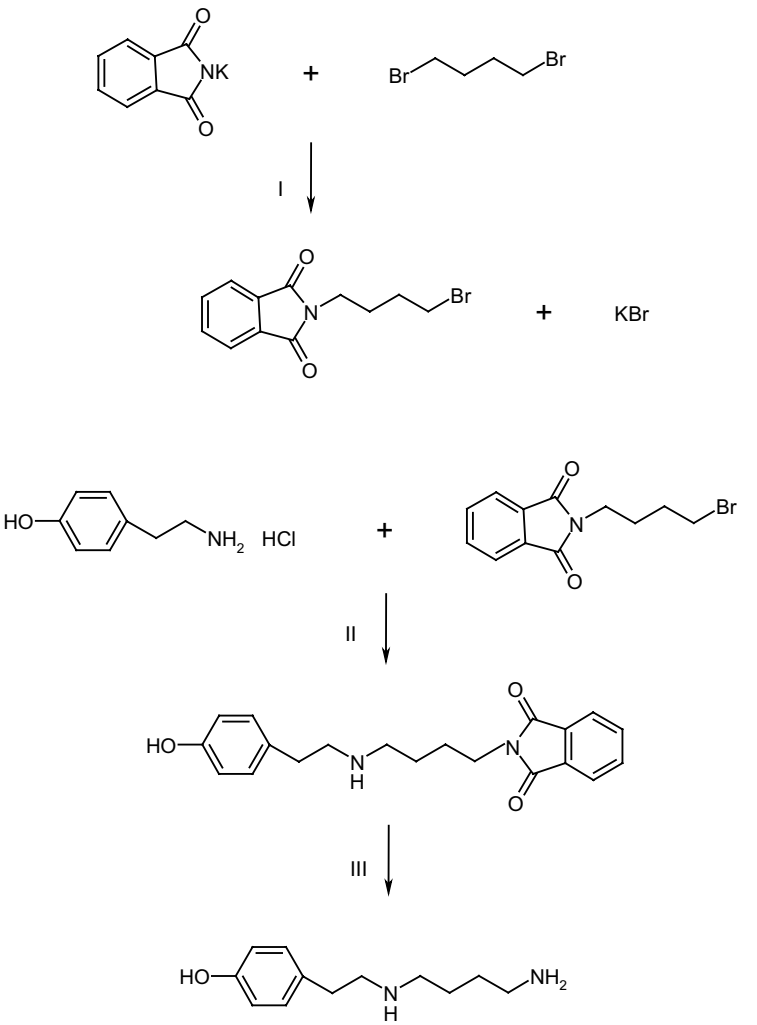
2.2. Synthesis of N-aminobutyl [2-(4-hydroxyphenyl)ethylamine] (Fig. 2)

2.2.1. Synthesis of N-(4-bromobutyl)-phthalimide was performed as described by Mizzoni et al. (1954)

Briefly, a mixture of 65 g (0.35 mol) potassium phthalimide and 200 g (0.93 mol) tetramethylene dibromide was heated with occasional shaking for 12 h. The material which crystallised from supernatant on standing was washed with ethanol and dried under vacuum (m.p. = 80 °C).

2.2.2. Synthesis of N-phthalimidobutyl-2-(4-hydroxyphenyl) ethylamine

2-(4-hydroxyphenyl)ethylammonium chloride (1.0 g, 5.8 mmol) was neutralised in solution 0.61 g (5.8 mmol) $NaHCO_3$ in 10 ml water for 30 min under stirring. Neutralised amine precipitated and water was evaporated under vacuum. A mixture of the neutralised amine and 1.95 g (6.9 mmol, 20% access) *N*-(4-bromobutyl)-phthalimide in 10 ml dry acetonitrile was refluxed for 5 h. Thin layer chromatography (TLC) in the solvent system petroleum ether/methanol/ethyl acetate (30:20:50, v/v)



N-aminobutyl [2-(4-hydroxyphenyl)ethylamine]

Fig. 2. Synthesis of *N*-aminobutyl [2-(4-hydroxyphenyl)ethylamine].

indicated the appearance of the products with $R_f = 0.3$ which slightly reacted with ninhydrin. The reaction mixture was fractioned by column chromatography. A glass column (450 × 35 mm) was filled with silica gel using the same solvent system as a mobile phase. Elution was accomplished by gravity. After solvent evaporation 0.61 g of cream crystalline substance (*N*-phthalimidobuthyl-2-(4-hydroxyphenyl) ethylamine) was obtained (m.p. = 140 °C).

2.2.3. Synthesis of N-aminobutyl [2-(4-hydroxyphenyl)ethylamine]

N-phthalimidobuthyl-2-(4-hydroxyphenyl) ethylamine was dissolved in 15 ml absolute ethanol then 160 μl hydrazine hydrate was added and the reaction mixture was refluxed for 2 h. Then 8 ml 1 N hydrochloric acid was added and the reaction mixture was refluxed for another hour. The mixture was cooled for 1 h at −20 °C and precipitate was discarded. Supernatant was adjusted to pH 9 with 2 N sodium hydroxide and solvent was evaporated under vacuum. TLC in the solvent system petroleum ether/methanol/ethyl acetate (20:50:30, v/v) indicated the appearance of the spot corresponding to the product (amine with long spacer) with $R_f = 0.1$. After preparative TLC 0.16 g pale cream powder was obtained. *N*-aminobutyl [2-(4-hydroxyphenyl)ethylamine] identity was confirmed by NMR. ^{1}H NMR (DMSO-d^6): δ 1.60–1.70 (m, 4H, 2 × CH_2), 2.75–3.00 (m, 8H, 4 × CH_2), 6.71 (d, 2H, aromatic), 7.05 (d, 2H, aromatic), 9.40 (s, 1H, OH). ^{13}C NMR (DMSO-d^6): δ 22.42 (CH_2), 24.04 (CH_2), 30.62 (CH_2), 38.68 (CH_2), 45.77 (CH_2), 48.03 (CH_2), 115.40 (2 × CH, aromatic), 127.22 (C, aromatic), 129.44 (2 × CH, aromatic), 156.21 (C_{quat}-OH, aromatic).

MS *m/z* (%): 209(100), 192(26), 121(3), 107(12). Melting point: >300 °C.

2.3. Immobilisation of 9-(p-hydroxyphenyl)nonanoic acid onto a sensor chip surface

A sensor chip was equilibrated to room temperature. The chip surface was activated by 30 min incubation with 50 μl of a 1:1 (v/v) mixture of 0.05M *N*-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The solution was removed then 50 μl of 1 M ethylenediamine (pH 8.5) added and incubated for 1 h. The chip was washed with HBS-EP buffer (3 × 50 μl). Free carboxyl groups were blocked with 50 μl 1 M ethanolamine hydrochloride (pH 8.5) for 20 min. The chip was washed with 50 μl of HBS-EP buffer. A solution of 5 mg EDC and 2 mg of NHS in 400 μl 10 mM sodium acetate (pH 4.5) was added dropwise to the solution of 2 mg 9-(*p*-hydroxyphenyl)nonanoic acid in 600 μl dimethylformamide (DMF). The mixture (50 μl) was applied onto chip surface and incubated for 2 h. After incubation the solution was removed, the chip was washed with HBS-EP buffer and dried under a stream of nitrogen. When not in use the chips were stored at 4 °C in plastic tubes containing silica gel desiccant.

2.4. Immobilisation of N-aminobutyl [2-(4-hydroxyphenyl)ethylamine] onto a sensor chip surface

A sensor chip was equilibrated to room temperature and the chip surface was activated as described in the previous section. After incubation the chip was washed with HBS-EP buffer and dried under a stream of nitrogen. The solution of 2 mg *N*-aminobutyl [2-(4-hydroxyphenyl)ethylamine] in 1 ml DMF was prepared and 50 μl was applied onto chip surface and incubated for 2 h. After incubation the solution was removed, the chip was washed with HBS-EP buffer and dried under a stream of nitrogen. When not in use the chips were stored at 4 °C in plastic tubes containing silica gel desiccant.

2.5. Extraction of 4-NPs from shellfish

Shellfish samples were homogenised (whole body) and weighed (1.0 ± 0.1) g into glass bottles. 3 ml of hexane was added to each sample and bottles were mixed on a roller mixer for 30 min followed by centrifugation at 3000*g* for 10 min at 10 °C. The solutions were applied onto NH2 SPE columns previously preconditioned by 3 ml of hexane. Columns were washed with 3 ml of hexane and 4-NPs were eluted using 3 ml of diethyl ether. The eluates were evaporated to dryness under a stream of nitrogen at 40 °C. After addition of 200 μl HBS-EP buffer and vortex mixing for 30 s solutions were transferred to glass tubes.

2.6. Biosensor procedure

Mixing of all the reagents, injection and washing were performed automatically by instrument in accordance with the chosen programme. An aliquot of antibodies solution in HBS-EP buffer was mixed with standard solution (or extract diluted 1:2 with HBS-EP buffer) in the ration 1:1 (v/v) (or 4:1 (v/v) for extracts). This mixture was injected onto the chip surface at a flow rate of 50 μl min^{-1} for 2 min. Report points were taken on each sensogram produced before and after sample injection. Chip surface was regenerated by 30 s pulse of regeneration solution (100 mM sodium hydroxide for monoclonal antibodies and 100 mM sodium hydroxide with 10% acetonitrile for polyclonal antibodies). A typical analytical cycle for each sample took about 10 min to complete. Each calibration point or sample was analysed in duplicate.

3. Results and discussion

3.1. Antibodies

In the present study two types of antibodies were used (Zeravik et al., 2004). In direct ELISA the monoclonal antibody was found to be very sensitive to linear forms of 4-alkylphenols and 4-*n*-NP in particular with negligible cross-reactivity to technical 4-NP (0.9%). Characterisation of the polyclonal antibodies produced (Franek et al., 2001) showed a strong tendency to recognise the total amount of 4-alkylphenols (cross-reactivity: 4-*n*-NP, 100%; technical 4-NP, 65.7%; 4-*tert*-OP, 38.2%). Technical NP is comprised of a mixture of ring and branch isomers with 4-NP isomers as a major component (Wheeler et al., 1997). This makes it difficult to identify and determine individual compounds in a mixture. In chromatographic assays all resolved peaks corresponding to different isomers are usually summed without identification to give a total 4-NP concentration (Guenther et al., 2001). Variability of NP mixture also makes it difficult to produce either specific or generic antibodies.

All antibodies used in the present study were obtained against 9-(*p*-hydroxyphenyl)nonanoic acid-protein conjugate (Franek et al., 2001). Using derivatives with shorter alkyl chains as immunogens did not result in production of antibodies with generic specificity to 4-NPs. It was also shown that only homologous system provided the most sensitive assays in indirect ELISA format and the importance of long lateral chain was proven. Similar findings were obtained using a direct ELISA (Zeravik et al., 2004).

3.2. Biosensor assay in buffer

3.2.1. The chip production and generation of calibration curves

9-(*p*-hydroxyphenyl)nonanoic acid (9-HPNA, Fig. 1) was used for immobilisation onto surface of a sensor chip. Both monoclonal and polyclonal antibodies showed good binding with the 9-HPNA chip (Table 1). For both types of antibodies calibration curves in buffer were established. For monoclonal antibody and 4-*n*-nonylphenol as a target substance the influence of flow rate and injection time on assay sensitivity was investigated in detail. Each minute of antibody/analyte mixture injection gave in the region of 250 units of maximum relative response. This enabled a reduction in antibody concentration but did not improve assay sensitivity. An injection time of 2 min was chosen for further developmental work. The flow rates selected were varied from 10 to 100 $\mu l\ min^{-1}$. The higher the flow rate was used the higher the maximum relative response was obtained (Fig. 3). Surprisingly a quite high flow rate of 100 $\mu l\ min^{-1}$ still provided sensitive assays despite the fact that at such flow rates dissociation of antibody from a surface could have had a significant impact on the assay performance. Calibration curves with similar sensitivity were obtained for flow rates from 25 to 100 $\mu l\ min^{-1}$. In contrast, the lower flow rate selected (10 $\mu l\ min^{-1}$) produced the lower sensitivity and the highest background level (Fig. 3). Finally, the following assay conditions were selected for further study: injection time 2 min, flow rate 50 $\mu l\ min^{-1}$ and regeneration for 30 s using 100 mM sodium hydroxide. Technical 4-NP and 4-*tert*-OP showed no inhibition up to 1000 $ng\ ml^{-1}$ concentrations. For the assay a limit of detection of 2 $ng\ ml^{-1}$ and IC_{50} of 15 $ng\ ml^{-1}$ for 4-*n*-NP were achieved.

Table 1
Binding of excess of antibodies with different flow cells of 9-HPNA chips

Chip (%)	Binding with monAb (RU)	Binding with polAb (RU)
9-HPNA 100	3000–4000	5500–6000
9-HPNA 50	1000–1500	2500–3300
9-HPNA 20	500–600	1000–1200
9-HPNA 10	150–250	700–760

Conditions: Monoclonal antibody concentration, 20 $\mu g\ ml^{-1}$; polyclonal antibodies dilution, 1/10; flow rate, 5 μl/min; injection time, 5 min; RU, relative units.

4-*n*-NP and the mixture of branch isomers (technical 4-NP) as target substances were used for assay evaluation with polyclonal antibodies. Similar results (when compared to monoclonal data) were obtained (data not shown). The same assay conditions provided a sensitive assay however a stronger regeneration solution was used to accomplish full chip surface regeneration (100 mM sodium hydroxide with 10% acetonitrile). For both target substances similar cross-reactivity was obtained (Fig. 4). However high background level was observed for technical 4-NP calibration curve. The same effect was noted for 4-*tert*-OP (Fig. 4). The sensitivity of the biosensor assay for both substances (limit of detection 30 $ng\ ml^{-1}$) was better then in indirect ELISA (Franek et al., 2001) but was one order higher compared to monoclonal antibody biosensor assay and direct ELISA results (Samsonova et al., 2003; Zeravik et al., 2004).

3.2.2. Assay optimisation: chip surface and purity of binding protein

From a very practical point of view an assay capable of determining the total amount of 4-NPs is required. Only the polyclonal antibodies tested showed the required generic specificity to the 4-NP group of compounds. The calibration curve obtained during the preliminary assay development work was insufficiently sensitive and further investigations were undertaken to improve the assay characteristics.

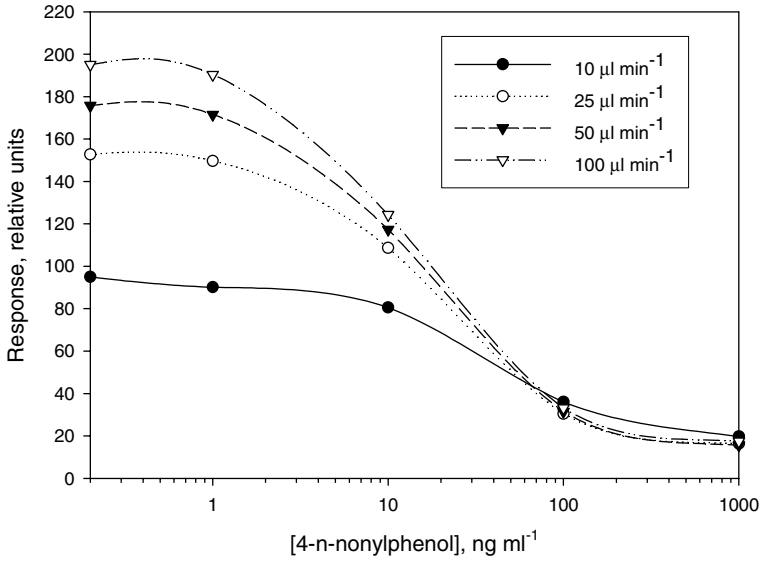


Fig. 3. Influence of flow rate on 4-*n*-nonylphenol biosensor assay with monoclonal antibody. Monoclonal antibody concentration 0.75 μg ml^{-1}, chip 9-HPNA 100%.

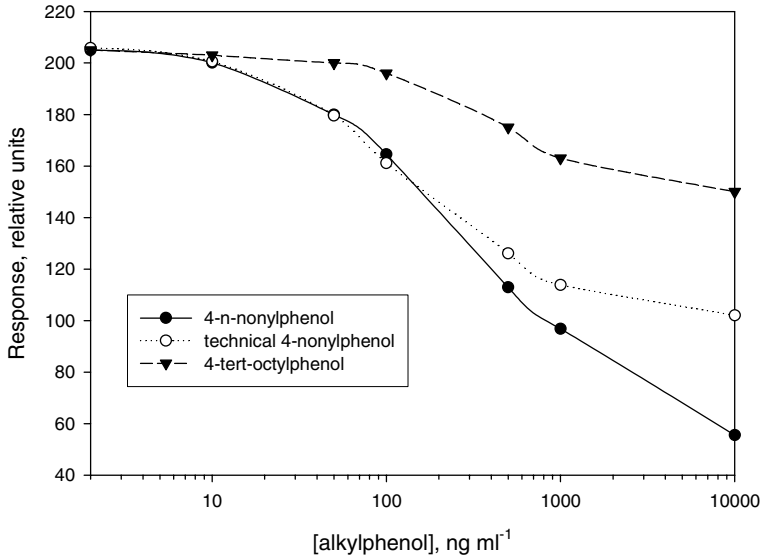


Fig. 4. Calibration curve for different alkylphenols in biosensor assay with polyclonal antibodies. Polyclonal antibodies dilution 1/2000, chip 9-HPNA 100%.

During Biacore biosensor assay development it is common practise to immobilise the maximum possible amount of derivative onto a chip surface. Such surfaces provide the highest binding characteristics with antibodies and increase the chip surface robustness (stability). It was postulated that analogously to indirect ELISA for haptens the amount of immobilised derivative could have an influence on assay performance. To study this possibility a range of sensor chips with differing amounts of immobilized 9-HPNA were prepared (50%, 20% and 10%). These surfaces were compared to the initial chip (100% immobilisation) with regards binding of monoclonal and polyclonal antibodies (Table 1). All chip surfaces were prepared using the same immobilization conditions. Solutions for immobilization were obtained by serial dilutions of the same 9-HPNA stock solution.

It could be seen that the antibody binding pattern corresponded to an amount of the derivative used for immobilization (Table 1). For all chips calibration curves were obtained and compared. Calibration curves for 4-*n*-NP with monoclonal antibody and chips 9-HPNA (100%) and 9-HPNA (50%) were almost identical (Fig. 5(a)). Whereas for polyclonal antibodies the reduced amount of immobilised 9-HPNA helped to decrease the background level observed in the calibration curve for 4-*n*-NP and technical 4-NP and thus improve sensitivity for 4-NP (Fig. 5(b) and (c)). For chip 9-HPNA (20%) assay sensitivity was low in both cases resulting in higher background level for technical 4-NP. Reduction in the amount of the derivative on a chip surface led to the requirement for high working concentrations of antibodies (dilution 1/100) to get a sufficient analytical signal. Therefore for this chip surface non-specific binding had a detrimental impact on assay performance.

The problem of high background levels of calibration curves for technical 4-NP was overcome by using IgG fraction of polyclonal antibodies (Fig. 5(c)). This finding clearly indicated a high level of non-specific binding of serum components was occurring on the chip surface.

In general chips with immobilised 9-HPNA were highly stable and the greater the amount of 9-HPNA which was presented on a surface the longer the time the chip could be used. For example, 500 injections resulted in loss of about 20% surface activity of 9-HPNA chip. Therefore a single chip (4 lanes per chip) had the capability to be used for approximately 2000 injections.

In conclusion, among all produced surfaces the chip 9-HPNA (50%) showed the best assay sensitivity for all combinations of antibodies and target analytes (Fig. 5). This result shows that particular attention should be given to the quantity of analyte immobilised on a surface to gain a fully optimised assay system. High background level of calibration curve for technical mixture of 4-NP can be decreased by using purified polyclonal antibodies in combination with lower amount of immobilised 9-HPNA.

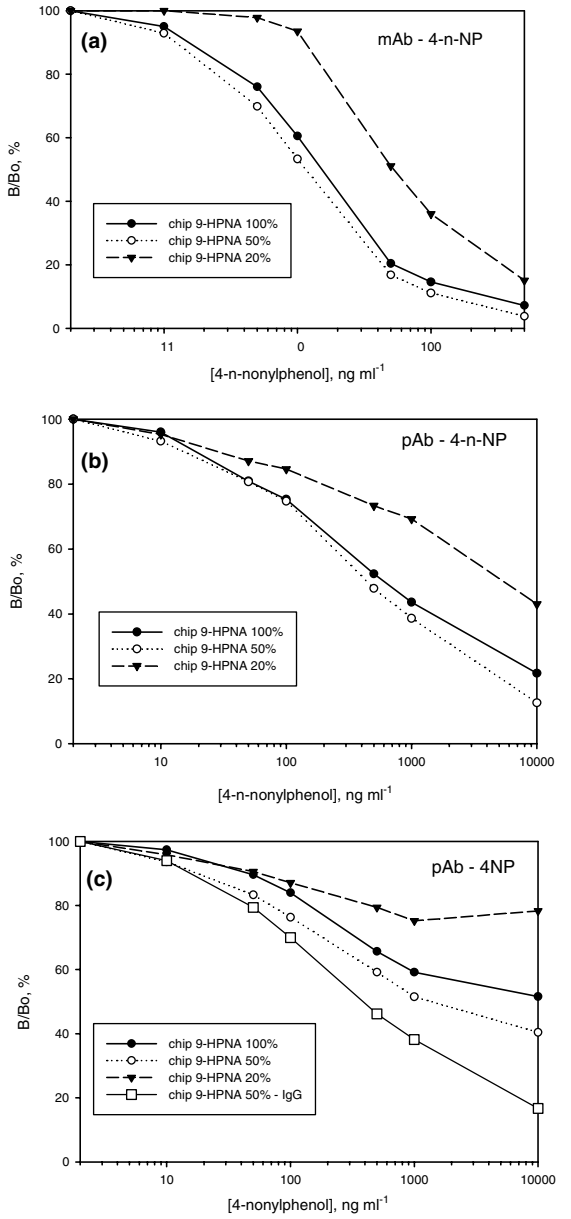


Fig. 5. Calibration curve for different chips and target analytes in biosensor assay. (a) Monoclonal antibody, 4-*n*-NP, (b) polyclonal antibodies, 4-*n*-NP, (c) polyclonal antibodies, technical 4-NP. B/B_o, %, the ratio of the response at the measured concentration of alkylphenol (B) to signal obtained at the absence of the analyte (B_o).

3.2.3. Assay optimisation: standard solutions

Nonylphenols are lypophilic compounds with low solubility in water (about 5 mg l^{-1}) (Ahel and Giger, 1993; Brix et al., 2001). It was postulated that the sensitivity of the assay for technical 4-NP could be improved by the introduction of a small quantity of organic solvent into the reaction mixture. Assays on the Biacore system were found to be very tolerable to the presence of methanol and ethanol (Fig. 6). At the ratio antibody:standard solution 1:1 (v/v) up to 20% methanol or up to 5–10% ethanol could be introduced into a standard solution without a significant decrease of the maximum response. A range of calibration curves with 4-NP standard solutions contained different amount of methanol were obtained and compared (Fig. 7). It was found that the higher amount of methanol was introduced the lower assay sensitivity was observed. A similar, unusual effect of decreasing ELISA sensitivity for 4-*n*-NP in the presence of methanol and dimethylsulfoxide has been reported earlier (Zeravik et al., 2004).

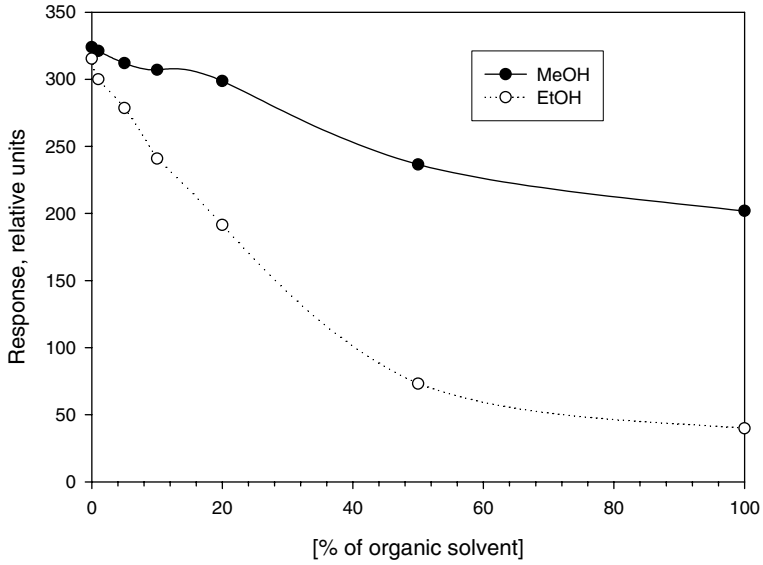


Fig. 6. Influence of organic solvents on a signal at zero concentration of technical 4-NP. Dilution of IgG fraction of polyclonal antibodies 1/500, chip 9-HPNA 50%.

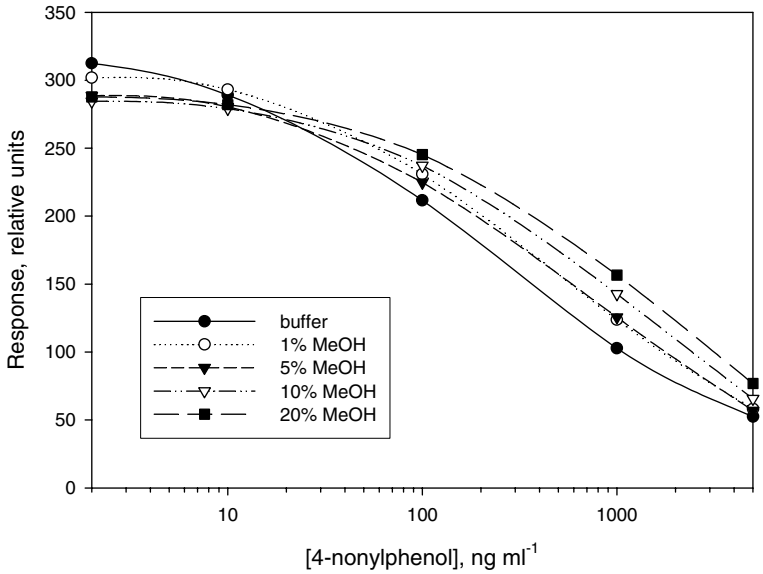


Fig. 7. Influence of methanol content on calibration curves of technical 4-NP. Dilution of IgG fraction of polyclonal antibodies 1/500, chip 9-HPNA 50%.

3.2.4. Design of a chip surface and improvement of the assay sensitivity

One of the means by which biosensor assay sensitivity can be improved is to change the chemistry on the chip surface. It is well known that heterologous system usually provides better sensitivity compared to homologous formats. Polyclonal antibodies showed 11.1% of cross-reactivity to 3-(*p*-hydroxyphenyl) propionic acid (Zeravik et al., 2004) and it was assumed that introduction of this substance with shorter linear chain onto a chip surface could improve assay sensitivity. However, immobilisation of the compound did not give good results and low binding with polyclonal antibodies was observed (about 250 RU).

An alternative means of generating a heterologous system is to change the conjugation site. Commercially

available glycidyl 4-nonylphenol ether containing a reactive epoxy group on the hydroxyl group side was immobilised onto a chip surface. Again however, low binding with antibodies was observed.

Synthesis of 4-NP derivatives with a reactive terminal group suitable for coupling is a difficult task (Mikhura et al., 2000). A new derivative N-aminobutyl [2-(4-hydroxyphenyl)ethylamine] (AHPE) was synthesised with using *N*-(4-bromobutyl)-phthalimide as an auxiliary substance (Fig. 2). The substance was used for the introduction of alkylamine fragments into polyamines structure (Mizzoni et al., 1954). AHPE has a different nature of spacer compared to lipophylic 4-*n*-NP because of an electrophylic amine moiety in the middle of a linear chain (Fig. 2). AHPE was immobilised onto a chip surface and some binding (590–850 RU) was observed with polyclonal antibodies but not the monoclonal antibody which showed strong tendency to recognise 4-phenol derivatives with long lypophilic alkyl chains.

The calibration curve of technical 4-NP for polyclonal antibodies and AHPE chip was found to be more sensitive (limit of detection—5 ng ml^{-1}) compared to homologous surface (Fig. 8). A working dilution of polyclonal antibodies of just 1/100 could be used but there was not a high background level associated with the calibration curve. The calibration curves pattern similar to 9-HPNA chips was obtained for this surface with different flow rates using the same assay conditions. However in this instance chip surface instability was observed. The chip quickly lost 50% of its activity over 200 cycles whilst being regenerated by 100 mM sodium hydroxide. It was found that the chip was substantially more stable using weak regeneration solution (10 mM sodium hydroxide) but full optimisation was not achieved.

3.3. Analysis of shellfish samples

Because of its low solubility 4-NPs can easily accumulate in aquatic organisms (Ahel et al., 1993). These organisms are a good target for environmental monitoring and assessment of water quality (pollution level). Numerous publications showed that 4-NPs are often found in fish and shellfish (Tsuda et al., 2000b; Ferrara et al., 2001; Guenther et al., 2001; Corsi and Focardi, 2002). This makes it difficult to get a true negative sample for immunoassay development. NP free organisms could be cultured at strictly controlled farms but this can make their production very expensive.

Applicability of an assay developed for the analysis of shellfish for 4-NPs residues was examined. The monoclonal antibody assay was used as a model for an extraction procedure development and matrix effect assessment. Moreover, the monoclonal antibody was very specific for 4-*n*-NP which represents only a minor fraction of 4-NPs mixture. In such a system the vast majority of samples can be assumed as being negative. The same assay conditions as for the buffer assay were used, with only the ratio of antibody standard solution (extract) being changed to 4:1 to reduce matrix influence.

Nonylphenol and nonylphenol ethoxilates are usually extracted from fish or shellfish by steam distillation/solvent extraction (Guenther et al., 2001), Soxhlet extraction (Corsi and Focardi, 2002) or solvent extraction followed by SPE clean-up (Tsuda et al., 2000a). A simple

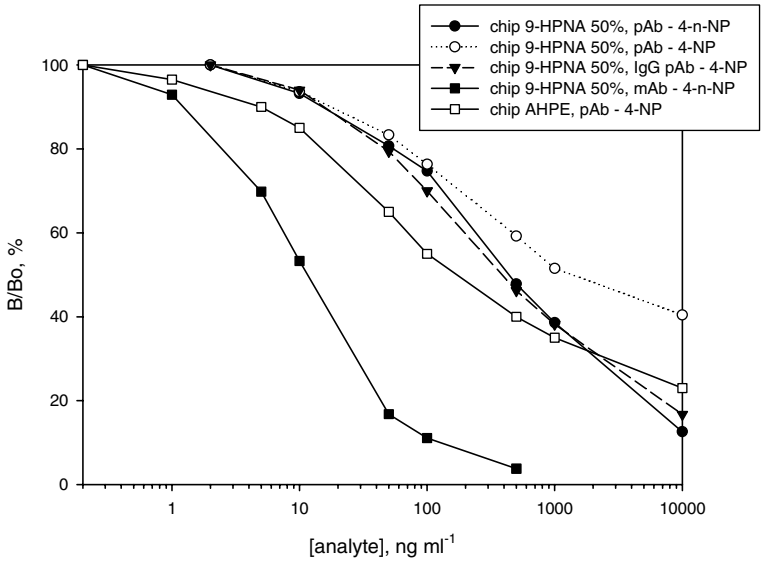


Fig. 8. Calibration curve for different antibody-target substance combinations in biosensor assay.

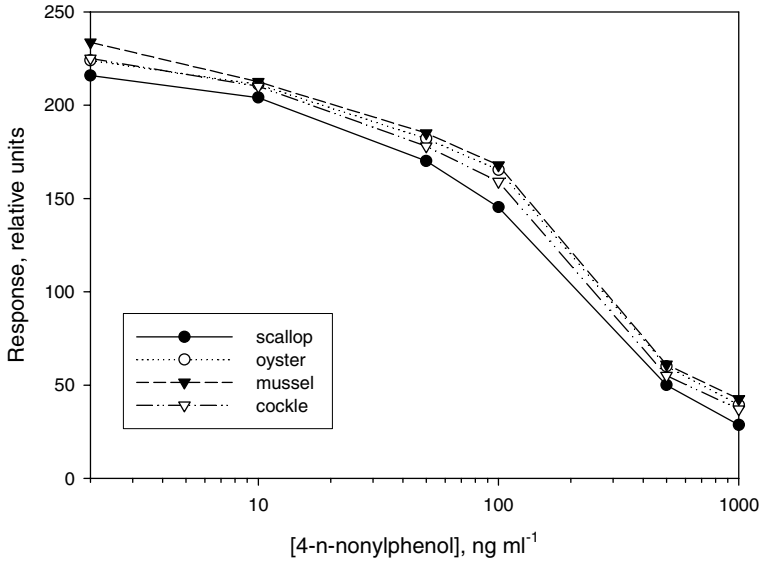


Fig. 9. Calibration curve for 4-*n*-NP in biosensor assay for different shellfish samples. Monoclonal antibodies 1 μg ml^{-1}, chip 9-HPNA 50%.

and rapid extraction procedure was developed. To generate a calibration curves samples of different species (cockles, mussels, oyster, scallop) were homogenized (whole body) and spiked with 4-*n*-NP. The substance was extracted into hexane followed by clean-up on NH2 SPE columns. Several solvents of different polarity showed good washing potential and diethyl ether was chosen as the most suitable in terms of elution speed and evaporation time. The extracts were diluted two times with buffer and directly analyzed in biosensor. Calibration curves generated for cockles, mussels and oyster samples were identical (limit of detection at about 10 ng g^{-1}) whereas for scallop samples a slight decrease (about 5–10%) of absolute response was observed (Fig. 9). The method showed slightly lower sensitivity compared to published chromatographic methods (Tsuda et al., 2000a). The method was not evaluated because of absence of a negative sample. The aim of this part of the study was to establish a suitable extraction method and assess the potential influence of shellfish as a matrix during biosensor based analysis. These results were used as a base for further investigations.

Applicability of the assay developed for 4-NP screening was determined in the following experiment. Thirty one shellfish samples collected from different locations along the Irish coast were analyzed by biosensor using monoclonal and polyclonal antibodies. In the monoclonal antibodies system sensitive to 4-*n*-NP all samples were deemed as negative (i.e. concentrations found were lower than the established limit of detection for the assay). CV for responses of all samples was 4.0%.

Results obtained in the polyclonal antibodies based assay were markedly different. Two different chip surfaces were used (9-HPNA 50% and AHPE). Of the 31 shellfish samples tested 29 showed very similar results, i.e., response units close to each other with CVs between 9 and 12%. The remaining two samples showed a significant reduction in recorded signal. On the basis of these data it was concluded that these two shellfish samples contained a quantity of at least one of 4-nonylphenols. The 9-HPNA (50%) chip for two scallop samples signal at about 70% of mean of all samples was observed. Whereas for the more sensitive assay with AHPE chip the response for these samples was at about 50% below mean. The described study showed that developed method could be applied for shellfish analysis as a preliminary screening method, however verification by chromatographic methods will be required.

4. Conclusion

Careful choice of a derivative for immobilization, amount of immobilized derivative and purity of binding proteins helped to improve assay performance and allowed the development of a range of sensitive biosensor assays for 4-nonylphenols of different specificity. To the authors knowledge this is the first publication which has shown the importance of optimising the amount of immobilized hapten for Biacore biosensor assay development. The assays developed were slightly less sensitive when compared to conventional ELISA but had unquestionable advantages as a fast screening method. Biosen-

sor assay developed showed good potential for shellfish analysis for environmental contaminant monitoring.

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