

Comparison of an Indirect Format ELISA on Modified Graphite and Polystyrene Surfaces against Triazines

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An indirect format enzyme-linked immuno-sorbent assay (ELISA) on graphite rods (\varnothing 0.8 mm \times 20 mm) and, for comparison, on microtiter plates has been developed against terbuthylazine. For this purpose, a series of 2-aminoalkyl-4-chloro-6-terbuthyl-*s*-triazine-2,6-diamine ELISA haptens with alkyl spacer lengths of 2, 4, 6, and 8 CH₂ groups has been synthesized. The graphite rods or the microtiter plates were covered by a polymerized glutaraldehyde network, and the ELISA haptens have been coupled by imino coupling to the free aldehyde groups of that network. ϵ -Aminocaproic acid has been used as an agent to block unspecific binding sites. The ELISA was run in a competitive mode, where the immobilized ELISA hapten and the solute terbuthylazine as a target analyte compete for the solute antibody.

Key words: Indirect Format ELISA, Graphite, Triazines

Introduction

Today enzyme-linked immuno-sorbent assays (ELISAs) are commonly applied to trace level analyses in aqueous systems. Competitive systems are by far dominating for the determination of low molecular weight haptens. In most cases the antibody is immobilized on a suitable surface. In this normal format assay the analyte and a structurally related enzyme-labeled compound compete in the sample solution for the antibody binding sites and thus the enzyme may get into contact with sample solution components which may affect the enzyme. Therefore, indirect formats using immobilized haptens are gaining interest. In most such cases a hapten-protein conjugate is immobilized. However, the synthesis of tracer or coating hapten-protein conjugates often lacks reproducible preparation of these conjugates with respect to coupling densities (Kaur *et al.*, 2004; Wortberg *et al.*, 1995). Therefore, direct immobilization of the hapten should be favoured.

Some papers report direct immobilizations of haptens to surfaces like silanized optical fibre (Fry and Bobbitt, 2001), gamma-irradiated polystyrene (Hofstetter *et al.*, 1997), or hydroxyl succinimide

activated polymer, Affiprep10 (Silvaieh *et al.*, 2002). In Geckeler and Müller (1993) possible polymer materials are discussed. However, only a few papers report competitive ELISAs with direct immobilization of haptens on surfaces. Bier *et al.* (1994) immobilized an atrazine derivative on a SiO₂-TiO₂-waveguide with glutaraldehyde after silanization. In Piehler *et al.* (1996) glass-type surfaces modified by several hydrophilic polymers of different functional properties to immobilize aminocaproic acid atrazine are used, and in Trau *et al.* (1997) 2,4-D (2,4-dichlorophenoxyacetic acid) was immobilized to the surface of quartz capillaries utilizing poly-L-lysine. A glutaraldehyde network was used for immobilizing several atrazine derivatives on polystyrene microtiter plates (MTP) (Holthues *et al.*, 2001, 2005). A thiolated pyrene derivative was immobilized on a gold surface in an amperometric immunosensor for the determination of benzo[a]pyrene (Liu *et al.*, 2000).

In particular, surfaces other than MTP are of interest with regard to applications in the field of biosensors. In the present communication the development of an indirect ELISA for terbuthylazine, a common herbicide, on graphite rods is reported. The assays for two different antibodies and

4 different coating haptens are compared with assays of the same format in MTP.

Material and Methods

Antibodies

Two different monoclonal antibodies have been used here. The lyophilized antibodies (2 mg each) were dissolved in 200 μ l sterile phosphate buffered saline (PBS) buffer (80 mmol l⁻¹, pH 7.6). The monoclonal antibodies P6A7 and 4A54 were a kind gift from T. Giersch and B. Hock (Technical University of Munich, Weihenstephan, Germany), and are described in detail elsewhere (Giersch *et al.*, 1993). P6A7 is highly specific for the determination of terbuthylazine (Giersch *et al.*, 1993), 4A54 is highly sensitive to a series of *s*-triazines (Winklmair *et al.*, 1997), including terbuthylazine, since it was prepared through a multi-hapten immunogen consisting of 10 different haptens linked to one carrier protein.

Apparatus

Adjustable pipettes [Finpette 40–200 μ l and Finpette 200–1000 μ l (Labsystems, Helsinki, Finland), Eppendorf pipette 0.5–10 μ l, and Eppendorf Multipette (Eppendorf, Hamburg, Germany)] were used to dispense the liquids. Data evaluation has been performed by Kaleidagraph 3.5 (Synergy Software, Reading, PA, USA). ELISAs were performed in 96-well flat-bottom polystyrene microtiter plates (MTPs, Maxisorb No. 442404, Nunc, Wiesbaden, Germany). A Dynatech MR5000 (Dynatech Laboratories, Virginia, USA) ELISA reader was used. The software Biolinx 2.21 (Dynatech Laboratories) was used to control the reader. Pencil rods (graphite rods) were a kind gift from Staedler (Nuremberg, Germany).

Chemicals

Organic reagents for synthesis were purchased from Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany). Terbuthylazine was obtained from Fluka-Riedel de Häen (Seelze, Germany). Silica gel 60 (0.063–0.1 mm) and aluminium-backed silica-gel 60 F254 thin-layer chromatography (TLC) plates were from Merck. Tween 20 was obtained from Serva (Heidelberg, Germany). Horseradish peroxidase (HRP) (lyophilized, 250 U/mg) was purchased from Boehringer (Mannheim, Germany). Goat anti-mouse

antibody (IgG), goat anti-mouse IgG-peroxidase conjugate and bovine serum albumin (BSA) were obtained from Sigma. Glutaraldehyde (GA) (25% in water) was purchased from Baker (Griesheim, Germany). All other chemicals were of analytical grade.

Experimental

General

ELISA was carried out in the competitive indirect mode:

- 1.) A suitable ELISA hapten was immobilized on a carrier surface (MTP or graphite pencil rods, 0.8 mm \varnothing , 20 mm length).
- 2.) The target analyte and the ELISA hapten competed in a solution to which a mouse antibody against the analyte was added.
- 3.) After a washing step the bound amount of mouse antibody was determined by a peroxidase (POD) labeled goat anti-mouse antibody (GaM-POD).
- 4.) The amount of bound anti-antibody was determined by the peroxidase induced reaction and the photometric detection of the reaction product at 450 nm.

All these steps have been thoroughly optimized, with all details given by Fouad (2003).

ELISA haptens

Since in a competitive ELISA the relative affinity of the analyte and of the ELISA hapten towards the antibody strongly affects the sensitivity of the ELISA, several bridge homologous ELISA haptens have been synthesized starting from cyan-

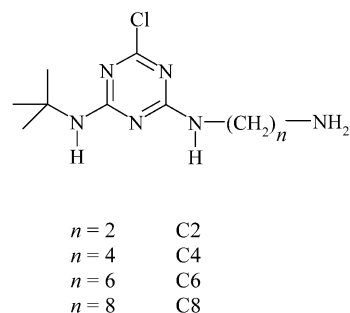


Fig. 1. Structure of the synthesized haptens 4-chloro-2-ethyl-6-terbutyl-*s*-triazine-2,6-diamine (C2), 2-butyl-4-chloro-6-terbutyl-*s*-triazine-2,6-diamine (C4), 4-chloro-2-hexyl-6-terbutyl-*s*-triazine-2,6-diamine (C6), and 4-chloro-2-octyl-6-terbutyl-*s*-triazine-2,6-diamine (C8).

uric chloride through dichloroterbutylazine to yield the alkyl-series 2-aminoalkyl-4-chloro-6-terbuthyl-*s*-triazine-2,6-diamine, following methods described elsewhere (Holthues *et al.*, 2005). The purity of all compounds was confirmed by TLC and proton nuclear magnetic resonance spectroscopy (^1H NMR), respectively. The structure of the haptens is shown in Fig. 1.

2,4-Dichloro-6-terbuthylamino-*s*-triazine (dichloroterbutylazine)

To a well stirred solution of cyanuric chloride (0.1 mol in 80 ml acetone) at 0 °C, 0.1 mol of terbuthylamine was added drop-wise, so that the temperature did not exceed 5 °C. Then 50 ml of a 2 N Na_2CO_3 -solution were added and the temperature was kept between 0–5 °C. The reaction mixture was then filtered off, and the solid product was dried in a desiccator. Re-crystallization three times from petroleum ether (40/65) gave white crystals, yield 65%, m.p. 130 °C. – $R_f = 0.65$ (EtOH/ $\text{H}_2\text{O}/\text{NH}_3$ 8:1:1, UV). – ^1H NMR (CDCl_3): $\delta = 1.7$ (s, 9H, 3 CH_3), 5.9 (s, 1H, NH).

2-Aminoalkyl-4-chloro-6-terbuthyl-*s*-triazine-2,6-diamine

A solution of 1.2 mmol dichloro-terbutylazine in 8 ml ethanol was added drop-wise over 60 min to a well stirred solution of 12 mmol of the corresponding diaminoalkane in 8 ml ethanol while keeping the temperature at 30–35 °C. The mixture was filtered and the volume was slightly reduced under reduced pressure. The product was isolated immediately by preparative column chromatography on silica gel using $\text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O}/\text{NH}_3$ (25% in H_2O) (8:1:1 v/v/v) as basic eluent. Those fractions, which contained the product were identified by TLC and combined. The solvent was removed under reduced pressure and the residue was dissolved in methanol and filtered. Then methanol had to be removed under reduced pressure. Rests of solvent were removed in a nitrogen stream. A white solid remained which was dried in a vacuum desiccator.

4-Chloro-2-ethyl-6-terbuthyl-*s*-triazine-2,6-diamine (C2)

4-Chloro-2-ethyl-6-terbuthyl-*s*-triazine-2,6-diamine was synthesized starting with di-chloro-terbutylazine and 1,2-diaminoethane. A white solid was obtained, m.p. 141 °C. – $R_f = 0.4$ (EtOH/ $\text{H}_2\text{O}/\text{NH}_3$

8:1:1, UV, ninhydrin). – ^1H NMR (DMSO): $\delta = 1.6$ (s, 9H, 3 CH_3), 2.9–3.1 (t, 2H, CH_2), 3.4–3.6 (m, 4H, CH_2 and NH_2), 7.7 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding of D_2O , bands for NH_2 and NH groups disappeared.

2-Butyl-4-chloro-6-terbuthyl-*s*-triazine-2,6-diamine (C4)

2-Butyl-4-chloro-6-terbuthyl-*s*-triazine-2,6-diamine was synthesized starting with di-chloro-terbutylazine and 1,4-diaminobutane. A white solid was obtained, m.p. 106 °C. – $R_f = 0.45$ (EtOH/ $\text{H}_2\text{O}/\text{NH}_3$ 8:1:1, UV, ninhydrin). – ^1H NMR (DMSO): $\delta = 1.5$ (s, 9H, 3 CH_3), 1.7–1.9 (br, 4H, 2 CH_2), 2.8–2.9 (br, 2H, CH_2), 3.3–3.5 (m, 4H, CH_2 and NH_2), 7.7 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D_2O , bands for NH_2 and NH groups disappeared.

4-Chloro-2-hexyl-6-terbuthyl-*s*-triazine-2,6-diamine (C6)

4-Chloro-2-hexyl-6-terbuthyl-*s*-triazine-2,6-diamine was synthesized starting with di-chloro-terbutylazine and 1,6-diaminohexane. A white solid was obtained, m.p. 89 °C. – $R_f = 0.48$ (EtOH/ $\text{H}_2\text{O}/\text{NH}_3$ 8:1:1, UV, ninhydrin). – ^1H NMR (DMSO): $\delta = 1.2$ (s, 9H, 3 CH_3), 1.3–1.5 (br, 8H, 4 CH_2), 2.5–2.6 (t, 2H, CH_2), 3.1–3.4 (m, 4H, CH_2 and NH_2), 7.5 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D_2O , bands for NH_2 and NH groups disappeared.

4-Chloro-2-octyl-6-terbuthyl-*s*-triazine-2,6-diamine (C8)

4-Chloro-2-octyl-6-terbuthyl-*s*-triazine-2,6-diamine was synthesized starting with di-chloro-terbutylazine and 1,8-diaminooctane. A white solid was obtained, m.p. 76 °C. – $R_f = 0.5$ (EtOH/ $\text{H}_2\text{O}/\text{NH}_3$ 8:1:1, UV, ninhydrin). – ^1H NMR (DMSO): $\delta = 1.3$ (s, 9H, 3 CH_3), 1.4–1.7 (br, 12H, 6 CH_2), 2.5–2.6 (t, 2H, CH_2), 3.0–3.4 (m, 4H, CH_2 and NH_2), 7.5 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D_2O , bands for NH_2 and NH groups disappeared.

Assay procedure

ELISA haptens were immobilized on MTP and graphite rods using a GA network. The assay on MTPs was carried out as described previously (Holthues *et al.*, 2001). Each well of the MTP was treated by 200 μl GA solution (6.25% v/v in 0.05 M carbonate buffer). Incubation was for 2 h at room

temperature. Graphite rods have been incubated for 4 h in a 400 μl reaction vessel, each with 200 μl GA solution, as described with the MTPs.

After washing with distilled water coating with the ELISA haptens (C2 to C8, respectively) was performed through imino coupling of the ELISA haptens to the free aldehyde groups of the GA polymer network using 200 μl coating hapten solutions prepared in PBS buffer at pH 8. Reaction was over night at room temperature.

A second coating with 250 μl ϵ -aminocaproic acid, 0.5 M in PBS buffer at pH 7.5, has been applied in order to block unspecific antibody or anti-antibody binding sites yet available on the GA polymer network, or even on the polystyrene or graphite carrier.

For the competition step 100 μl of a standard terbuthylazine solution in PBS buffer (pH 7.5) mixed with 100 μl antibody solution in PBS buffer (pH 7.5) have been incubated for 2 h at room temperature. After a washing step 200 μl of the secondary anti-antibody GaM-POD dilution in PBS (pH 7.5) + BSA (0.06% w/w) buffer were added and then incubation was for 2 h at room temperature. The final enzyme reaction was in 200 μl of the substrate solution and was stopped after 30 min by 50 μl 2 N H_2SO_4 . The detection was by absorption at 450 nm in an ELISA reader.

Pre-incubation of the analyte and the antibody prior to incubation with the ELISA hapten for a certain time has been checked for the P6A7 antibody. For this purpose, a mixture of 100 μl of the terbuthylazine standards with 100 μl of the antibody solution was allowed to pre-incubate for 1 h. The subsequent competition step with the immobilized ELISA hapten on MTP or graphite rods was for 1 h.

Data evaluation and error considerations

The ELISA reader results have been evaluated in the usual way and normalized plots of % B/B_0 are presented:

$$\% B/B_0 = (A - A_{\text{ex}})/(A - A_0), \quad (1)$$

where A_{ex} and A_0 are the absorptions of the considered analyte sample at excess of the analyte and in absence of the analyte, respectively. The so-called four-parameter curve was used to fit the sigmoidal calibration curves (Rodbard, 1974):

$$Y = (a - d)/(1 + (x/c)^b) + d. \quad (2)$$

Here Y is the dose response absorption, a is the response at zero dose, c is the midpoint of test, most often called IC_{50} value, d is the response at infinite dose of the analyte, x is the dose of the analyte, and b is the slope of the sigmoidal curve at the IC_{50} value.

R^2 as a measure for the fit quality was in all cases larger than 0.992, mostly between 0.995 and 0.997.

All assay signals in this communication are means of four replicates in MTPs and three replicates with graphite rods. The errors reported here may be compared to the IUPAC guidelines (Krotzky and Zeeh, 1995), which recommend for ELISAs that the coefficient of variation of the IC_{50} value and of the limit of detection should not exceed 15% and 25%, respectively. The reproducibility of the IC_{50} value at the same day and under different conditions should not exceed 12.5%, and for different days not 25%.

Results

Effect of the antibody dilution and of the concentration of the ELISA hapten coating solution

Since the sensitivity of a competitive ELISA strongly depends on each step, careful optimization of all the reaction partners is essential. In a first step the effect of the hapten coating concentration as well as the antibody dilution were checked by measuring A_0 and A_{ex} . The relative difference of these two values should be high to ensure low errors in the calibration curve. As an example Figs. 2 and 3 show the effects of different antibody dilutions for the C6 hapten on MTP.

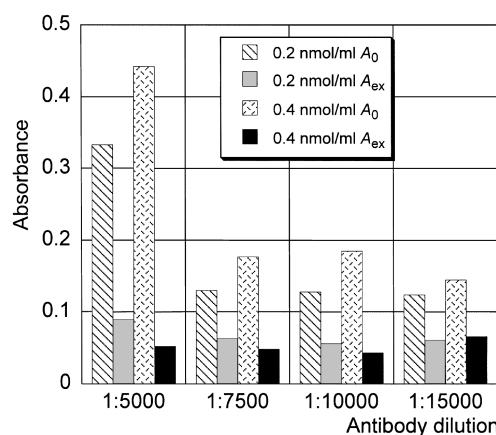


Fig. 2. On the ELISA optimization: A_0 and A_{ex} pairs as dependent on the 4A54 antibody dilution and the C6 hapten concentration. Absorbance at $\lambda = 450$ nm.

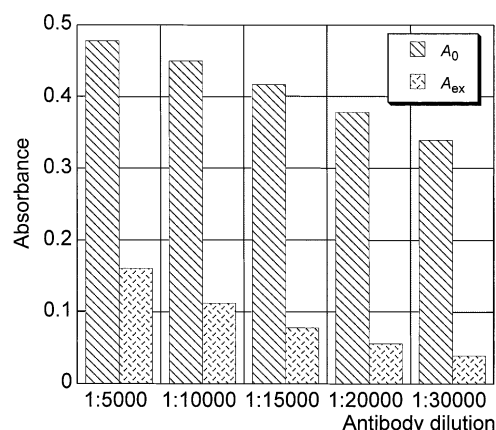


Fig. 3. On the ELISA optimization: A_0 and A_{ex} pairs as dependent on the P6A7 antibody dilution and the C6 hapten concentration. Absorbance at $\lambda = 450$ nm.

For the antibody 4A54 a lower dilution factor is required than for the antibody P6A7 which applies for both surfaces. Typically the antibody dilution for P6A7 was 1:20000 to 1:30000 for MTP (data not shown) and 1:15000 for graphite, for 4A54 1:5000 for MTP (data not shown) and 1:600 to 1:1000 for graphite (data not shown). The optimized hapten concentrations were 0.2 nmol/l to 0.4 nmol/l for MTP for both antibodies. In case of the graphite surface the optimized hapten concentration was 1 nmol/l for all haptens, with the antibody 4A54. In addition in the case of C6, the hapten concentration dependence was found to be very small; hapten concentrations of 0.2 nmol/l and 1 nmol/l gave the same relative difference between A_0 and A_{ex} .

Effect of the ELISA hapten spacer length on the IC50 value

The spacer length of the ELISA hapten may influence the IC50 values and therefore the different haptens were tested in an ELISA using the optimized conditions for both surfaces.

Fig. 4 shows the calibration curves for the ELISA on MTP for the two considered antibodies 4A54 and P6A7. In both cases the IC50 values were lowest for the C2 hapten. For C2 and P6A7 the IC50 value was 10.8 $\mu\text{g/l}$, for 4A54 0.17 $\mu\text{g/l}$ being clearly lower than for C4, C6 and C8. For graphite as surface the results for both antibodies are summarized in Table I. For all haptens a concentration of 1 nmol/l was used. Here C2 also showed the lowest IC50 value compared to the

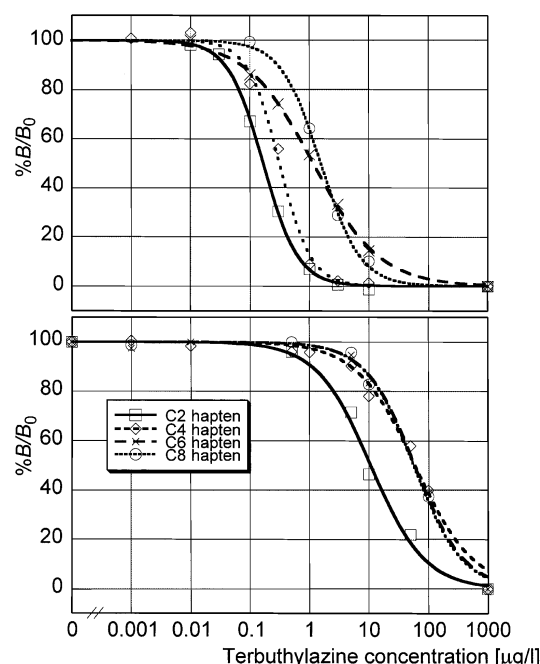


Fig. 4. Spacer length-dependent calibration curves on MTP, shown as normalized absorption response $\%B/B_0$ against the terbutylazine concentration. Upper: with the 4A54 antibody at 1:5000 dilution; hapten concentrations were 0.4 nmol/l; lower: with the P6A7 antibody at 1:30000 dilution; hapten concentrations were 0.2 nmol/l.

Table I. IC50 values for ELISAs with immobilized different chain length, ELISA haptens C2 to C8, immobilized on graphite. Hapten concentration: 1 nmol/l for 4A54; 0.2 nmol/l for P6A7.

Hapten	IC50 value* [$\mu\text{g/l}$]	Antibody	Antibody dilution
C2	0.43 ± 0.06 ($n = 3$)	4A54	1:600
C4	0.54 ± 0.08 ($n = 2$)	4A54	1:1000
C6	2.9 ± 0.4 ($n = 3$)	4A54	1:1000
C8	3.0 ($n = 1$)	4A54	1:1000
C2	23 ± 7 ($n = 3$)	P6A7	1:15000
C4	46 ± 9 ($n = 2$)	P6A7	1:15000
C6	54 ± 9 ($n = 3$)	P6A7	1:15000
C8	75 ± 13 ($n = 3$)	P6A7	1:15000

* Mean \pm standard deviation from n independent calibration curves at different days.

other spacer lengths, being 0.43 $\mu\text{g/l}$ for 4A54 and 23.0 $\mu\text{g/l}$ for P6A7. In the case of P6A7 the IC50 value increased with increasing spacer length of the hapten. The coefficients of variation of the IC50 values which were obtained on different days were within the IUPAC guidelines.

Comparing the two antibodies 4A54 showed for all haptens and surfaces lower IC₅₀ values than P6A7. Comparing the two surfaces, graphite based assays resulted in slightly higher IC₅₀ values than MTP based.

Effect of pre-incubation

Pre-incubation was tested to improve the IC₅₀ value. In all cases C6 was used as hapten and P6A7 as antibody. The antibody dilution was 1:30000 for MTP and 1:10000 for graphite as a surface. A hapten coating concentration of 0.2 nmol/l was applied in all cases.

For MTP the IC₅₀ value could be improved slightly from 58 µg/l in the standard assay to 25 µg/l when using pre-incubation. However, in the case of graphite as surface pre-incubation remarkably improved the IC₅₀ value from 52 µg/l to 8.5 µg/l.

Discussion

ELISAs using the indirect format with immobilization of the hapten have to be carefully optimized in regard to the hapten coating concentration and the antibody dilution, because both will influence the IC₅₀ value. As the immobilized hapten and the free hapten, the analyte, compete for the antibody binding sites, the lower the amount of bound hapten is the less analyte can be detected. However, extremely low hapten coating concentrations result in absorbance values in the assay, which are too low to clearly discriminate between the different analyte concentrations.

High antibody dilutions are favoured, because of low values of A_0 , but the A_{ex} value should not decrease below 0.1.

For the two antibodies 4A54 and P6A7 the optimized dilutions are different due to their binding constants. P6A7 is highly specific for the determination of terbuthylazine (Giersch *et al.*, 1993). An affinity constant of $4 \cdot 10^8$ l/mol can be deduced from the results reported by Piehler *et al.* (1997). 4A54 is highly sensitive to a series of *s*-triazines with a reported affinity constant of $(4.3 \pm 0.9) \cdot 10^{10}$ l/mol for terbuthylazine (Winklmaier *et al.*, 1997). Since the affinity constant of P6A7 is lower than that of 4A54, P6A7 requires lower dilution factors which holds for assays on MTP as well as on graphite. With regard to the hapten coating concentrations graphite required an up to fivefold higher concentration and as a consequence the an-

tibody dilution was lower than on MTP for 4A54 and P6A7, respectively. Applying the optimized hapten coating concentrations and antibody dilutions the IC₅₀ values for both surfaces were comparable, showing that graphite is a suitable surface for an ELISA with directly immobilized haptens. Terbuthylazine derivatives having different spacer lengths were used as coating haptens and the IC₅₀ values obtained in the ELISA were compared. For both antibodies and both surfaces the assay employing C2 gave the lowest IC₅₀ value. For P6A7 and graphite as surface the IC₅₀ values increased from C2 via C4, C6 to C8. Since Holthues *et al.* (2005) have shown, that different spacer length haptens should only be compared with respect to their IC₅₀ values, if working in the affinity limit, this finding should not be overstressed. To improve the IC₅₀ value pre-incubation techniques are discussed in literature (Zettner and Duly, 1974; Weller, 1992). Using this sequential incubation of antibody and hapten (Holthues, 1998) could decrease the IC₅₀ value by a factor of two, compared to an ELISA without pre-incubation, for a competitive ELISA with immobilization of atrazine derivatives to MTP. In the present report pre-incubation was checked for the antibody P6A7 and C6 as coating hapten and the IC₅₀ value decreased by a factor of two in the case of MPT, but for graphite the IC₅₀ value decreased markedly by nearly a factor of six.

Conclusion

Graphite rods as surface could be successfully applied in a competitive ELISA for terbuthylazine using direct immobilization of the hapten. After optimization of the assay parameters the IC₅₀ values were comparable with the values obtained in ELISAs on MTP. Using the pre-incubation technique the IC₅₀ value could be improved in particular remarkably on graphite rods. These results using graphite will open up the use of this material in the field of immunosensors with immobilized haptens.

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- Bier F. F., Jockers R., and Schmidt R. D. (1994), Integrated optical immunosensor for *s*-triazine determination: regeneration, calibration and limitations. *Analyst* **119**, 437–441.
- Fouad D. (2003), Comparison of ELISA and Immuno-electrode Techniques for the Determination of Trace Levels of Triazines in Aqueous Media. Thesis, Assiut University, Assiut.
- Fry D. R. and Bobbitt D. R. (2001), Hapten immobilization for antibody sensing using a dynamic modification protocol. *Talanta* **55**, 1195–1203.
- Geckeler K. E. and Müller B. (1993), Polymer materials in biosensors. *Naturwissenschaften* **80**, 18–24.
- Giersch T., Kramer K., and Hock B. (1993), Optimization of a monoclonal antibody-based enzyme immunoassay for the detection of terbuthylazine. *Sci. Total Environ.* **132**, 435–448.
- Hofstetter O., Hofstetter H., Then D., Schurig V., and Green B. S. (1997), Direct binding of low molecular weight haptens to ELISA plates. *J. Immunol. Methods* **210**, 89–92.
- Holthues H. (1998), Das Heterologieprinzip bei Immunoassays – Untersuchungen zu einer kontrovers diskutierten Hypothese. Thesis, Mainz University, Mainz.
- Holthues H., Pfeifer-Fukumura U., Hartmann I., and Baumann W. (2001), An immunoassay for terbutryn using direct hapten linkage to a glutaraldehyde network on polystyrene surface of standard microtiter plates. *Fresenius' J. Anal. Chem.* **371**, 897–902.
- Holthues H., Pfeifer-Fukumura U., Sound I., and Baumann W. (2005), Evaluation of the concept of heterology in a monoclonal antibody-based ELISA utilizing direct hapten linkage to polystyrene microtiter plates. *J. Immunol. Methods* **304**, 68–77.
- Kaur J., Singh K. V., Raje M., Varshney G. C., and Suri C. R. (2004), Strategies for direct attachment of hapten to a polystyrene support for application in enzyme-linked immunosorbent assay (ELISA). *Anal. Chim. Acta* **506**, 133–135.
- Krotzky A. J. and Zeeh B. (1995), Immunoassays for residue analysis of agrochemicals: proposed guidelines for precision, standardization and quality control. *Pure Appl. Chem.* **67**, 2065–2088.
- Liu M., Li Q. X., and Rechnitz G. A. (2000), Gold electrode modification with thiolated hapten for the design of amperometric and piezoelectric immunosensors. *Electroanalysis* **12**, 21–26.
- Piehl J., Brecht A., Geckeler K. E., and Gauglitz G. (1996), Surface modification for direct immunoprobes. *Biosensors Bioelectronics* **11**, 579–590.
- Piehl J., Brecht A., Giersch T., Kramer K., Hock B., and Gauglitz G. (1997), Affinity characterization of monoclonal and recombinant antibodies for multianalyte detection with an optical transducer. *Sensors Actuators B* **38–39**, 432–437.
- Rodbard D. (1974), Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20**, 1255–1270.
- Silvaieh H., Schmid M. G., Hofstetter O., Schurig V., and Gubitz G. (2002), Development of enantioselective chemiluminescence flow- and sequential-injection immunoassays for alpha-amino acids. *J. Biochem. Biophys. Methods* **53**, 1–14.
- Trau D., Theuerl T., Wilmer M., Meusel M., and Spener F. (1997), Development of an amperometric flow injection immunoanalysis system for the determination of the herbicide 2,4-dichlorophenoxyacetic acid in water. *Biosensors Bioelectronics* **12**, 499–510.
- Weller M. G. (1992), Strukturelle und kinetische Untersuchungen zur Entwicklung und Optimierung von Hapten-Enzymimmunoassays (ELISAs) am Beispiel der Bestimmung von Triazinherbiziden. Thesis, TU München, Weihenstephan.
- Winklmaier M., Weller M. G., Mangler J., Schlosshauer B., and Niessner R. (1997), Development of a highly sensitive enzyme-immunoassay for the determination of triazine herbicides. *Fresenius' J. Anal. Chem.* **358**, 614–622.
- Wortberg M., Kreissig S. B., Jones G., Rocke D. M., and Hammock B. D. (1995), An immunoarray for the simultaneous determination of multiple triazine herbicides. *Anal. Chim. Acta* **304**, 339–352.
- Zettner A. and Duly P. (1974), Principles of competitive binding assays (saturation analysis). II. Sequential saturation. *Clin. Chem.* **20**, 5–14.