New detection method for atrazine pesticides with the optical waveguide Mach–Zehnder immunosensor

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Abstract

Concentrations of analytes can be determined within a few minutes using on-line analysis of the immunobinding kinetics in a solid phase immunoassay. This approach has been applied to the detection of atrazine. Atrazine is detected, at concentrations around the European Community limit (0.1 µg/l) by a competitive assay. To this end, the two channels of a Mach–Zehnder waveguide sensor are used simultaneously in a difference measurement. The advantage of this way of measuring is discussed with the atrazine measurements.

Keywords: Immunosensor; Pesticides; Antibody binding kinetics; Mach–Zehnder sensor; Waveguide sensor

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1. Introduction

In the last few years it has become clear that the widespread agricultural use of pesticides have contaminated the drinking water and ground water. Since pesticides accumulate in animal tissue and high concentrations may cause diseases, there is a considerable interest in the development of new sensitive detection techniques to detect these pesticides in water. The European Water Act requires that drinking water contains less than 100 ng/l of an individual pesticide and less than 500 ng/l of total pesticides. Till now pesticides are often detected with classical analytical techniques like liquid chromatography or gas chromatography–mass spectroscopy [1–3]. Usually these techniques need sample pre-treatments which make

the detection complex and time-consuming. As a consequence large amounts of sample are used for methods that require sophisticated laboratory equipment. For this reason in recent years immunochemical ELISA techniques have been developed [4,5]. These ELISAs are now commercially available [6].

The immunochemical approach for pesticide detection can also be used in combination with other sensor techniques; Optical (Surface Plasmon Resonance sensors [7], Grating Coupler sensors [8], Reflectometric Interference sensors [9]), and Piezo-electric devices [10]. Among the class of optical sensors the Mach–Zehnder interferometer (MZI) sensor shows the highest sensitivity [11,12]. However, it is not the theoretical sensitivity of the device that in a practical situation determines the lower limit for pesticide detection. This is determined by sample handling and temperature drift [9]. The MZI sensor

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has an intrinsic reference channel. With this reference channel it becomes less sensitive to variations in sample handling and temperature drift. For this reason it is the most promising device for pesticide detection. It also offers the ability of real time measurements. With an analysis of the kinetics of the immunobinding reaction samples can be measured in a short time [13]. In this paper we report on this novel kinetic assay particularly suitable for MZI applications. With this assay water can be screened without difficult information processing within 10 min on triazines, like atrazine, around the European Community guideline for drinking water (0.1 $\mu\text{g/l}$).

2. Materials and methods

2.1. Chemicals

The triazine derivative 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexane-carboxylic acid) for hapten–bovine serum albumin (BSA) conjugate production was kindly provided by Prof. Hock, Technical University of München. The hapten–BSA conjugate production was performed according to the CMC procedure [14], with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-4-toluene-sulfonate (CMC, Sigma) and BSA (Sigma). It was found by UV absorption spectroscopy that on the average 17 triazines were bound to one BSA molecule. Atrazine (Riedel de Haen) was directly diluted in distilled water, filtered and measured by UV absorption spectroscopy at a concentration of 0.06 g/l. This standard was further diluted in phosphate buffered saline (PBS)

at desired concentrations before the measurements. The monoclonal atrazine antibody K4E7, produced, purified, and tested by Giersch [4], was provided by Prof. Hock.

2.2. Equipment

The planar waveguide MZI sensor is a device developed in our group and it is shown in Fig. 1. In this paper we give only a very short description of the principle of operation of the MZI; details can be found elsewhere [11,12]. Light from a HeNe laser is split in two beams with a glass cube and coupled into the planar waveguide with a lens. Depending on the local refractive index at the surfaces 1 and 2, respectively, beams 1 and 2 will be retarded. The resulting phase difference $\Delta\phi$ between the beams is proportional to the difference in mass bound to the surfaces 1 and 2, respectively; $\Delta\phi$ is measured by mixing the two beams and observing the changing interference pattern. With this device extremely low mass changes at the sensor surface can be detected. A lower detection limit of 0.1 pg/mm^2 per hour is found, which is a significant improvement compared to the value quoted in our earlier work [12,15]. This mainly stems from the improved phase feedback system and the better mechanical stability of the instrument [11].

On the sensorchip a laminar flow cuvette with a 100 μm thick flow channel is mounted to increase the reaction speed at the sensor surface and to improve the reproducibility of the sample injection. A peristaltic pump was used to propel the samples with an average flow rate of 1.3 $\mu\text{l/s}$ through the cuvette.

Fig. 1. Planar waveguide Mach-Zehnder set-up. For details see [8,9].

Fig. 2. The inhibition assay for atrazine detection. For details see text.

2.3. Preparation for inhibition assay

We have remarked earlier [16] that, even with a lower detection limit of 0.1 pg/mm^2 per hour in mass change at the surface, the measurement of small molecules, like atrazine (mol. wt. $\sim 250 \text{ Da}$) binding to a receptor sensor surface, still needs an enhancement step to obtain reasonable optical changes at this surface. Here this enhancement step is performed by measuring with an inhibition assay, which is illustrated in Fig. 2. Instead of measuring low molecular mass atrazine molecules binding to an antibody receptor surface, high molecular mass antibodies (mol. wt. 150 kDa) which bind to an atrazine modified surface are measured. In an inhibition assay, antibodies in a known concentration are pre-mixed with the unknown atrazine sample. Atrazine will bind to part of the antibodies in the sample, thereby lowering the active antibody concentration available for binding to the immobilised atrazine. The measured binding response is dependent on this concentration, and thus dependent on the unknown atrazine concentration.

In order to obtain a reasonable strong binding between the substrate and BSA-atrazine the surface has to be hydrophobic [17]. After cleaning with a plasma O_2 the Si_3N_4 surface was spin coated with dichlorodimethylsilane (contact angle after spinning $90 \pm 5^\circ$). Subsequently 10^{-6} M BSA-atrazine was allowed to adsorb to the hydrophobic surface. The specificity of the formed receptor surface was tested

with $1 \times 10^{-7} \text{ M}$ of anti-apotransferin IgG, which showed no response as expected.

After this last test the inhibition assay was used to screen PBS in the presence of pesticides around the EC limit of 100 ng/l . To be able to perform these measurements the samples were pre-mixed for 1 h with $5 \times 10^{-10} \text{ M}$ of anti-atrazine antibodies. This concentration was chosen to obtain enough sensitivity around the EC limit. In view of the affinity constant ($K \sim 10^8 - 10^7 \text{ M}^{-1}$) this concentration was calculated to be the best compromise between differential sensitivity and absolute number of bound molecules. This mixing is performed in BSA pre-coated glasswork to reduce adsorption losses.

3. Results and discussion

The immunochemical screening of atrazine in water can be done by determining the equilibrium response on the anti-atrazine mixed with atrazine, but this is a slow method which easily can take hours. It was shown before by Bier et al. [8] and Karlsson et al. [13], that with other methods that analyse the response kinetics of the reactions, much faster measurements can be done. This was done by a dynamic slope measurement. To be able to perform such a measurement the sample has to be applied in a reproducible way to the sensor surface. In this way other effects like flow speed that also influence the reaction rate in diffusion limited reactions are reduced.

The dependence of the slope on the anti-atrazine concentration has been measured to obtain a good calibration for the measurements. The receptor surface was prepared as described before. The result of this series of measurements can be found in Fig. 3.

From Fig. 3 we can see that the rate of the binding reaction has a linear dependence on the applied antibody concentration. An advantage of these dynamic slope measurements is that more measurements can be done with the same receptor surface without saturating it, because only a small amount of the available binding sites on the surface is used during one measurement. A complete binding curve of a $1 \times 10^8 \text{ M}$ anti-atrazine binding was used to determine how often a receptor surface could be reused without introducing systematic errors as a result of the irreversible saturation of the receptors at the surface. A $1 - \exp(-t/\tau)$

Fig. 3. Dependence of the slope of the response on a concentration of anti-atrazine added to a BSA–atrazine modified surface. The line is a least-square fit to the data. More sets of measurements were done on different sensors. The data of one set is indicated with one kind of symbol. Fit $Y = A + BX$, with: $A = 8 \times 10^{-5}$ sd 2×10^{-5} , $B = 1.6 \times 10^5$ sd 1×10^4 , correlation = 0.97, $n = 16$.

curve was fitted to the measured binding curve. In this way a typical τ of 1×10^4 s is found. Multiple measurements on the same surface can be done as long as $t < \tau$. In the measurements discussed in this paper, this condition was always fulfilled.

As already mentioned the Mach–Zehnder sensor has two channels. In fact this device performs a difference measurement between events occurring in channel 1 and those in channel 2. The first advantage of this is that common-mode effects that usually limit the performance of optical evanescent wave sensors can be kept small; thus temperature fluctuations or variations in composition of solutions, are relatively unimportant. The second advantage is that analysing samples can be compared directly with a reference sample. This comparison can be performed by injecting simultaneously the analysing sample in the sensor channel and a reference sample in the reference channel. In Fig. 4 such a measurement is depicted: (A) is the response on an analysing sample containing 4×10^{-11} M atrazine in channel 1 and PBS in channel 2, (B) is the response on the reference sample with 4×10^{-10} M atrazine in channel 2 and PBS in channel 1, and (C) is the response on the analysing sample in channel 1 and the reference sample in channel 2. It is clear that in Fig. 4C the difference in response of the sensor on the analysing sample to the reference sample is measured. The positive slope

Fig. 4. The kinetic difference measurement for fast pre-screening of water on atrazine at concentrations around 4×10^{-10} M (100 ng/l): (A) PBS sample with atrazine concentration (I) in the sensor channel 1 and 'clean' PBS in the reference channel 2; (B) reference sample with atrazine concentration (II) in the reference channel 2 and 'clean' PBS in the sensor channel 1; (C) PBS sample with atrazine concentration (I) in the sensor channel 1 and the reference concentration (II) in the reference channel 2. (I: 4×10^{-11} M, II: 4×10^{-10} M). The surface is coated with BSA–atrazine and 5×10^{-10} M anti-atrazine is mixed for 90 min with the atrazine samples before adding to the sensor surface. Before the measurements $\Delta\phi$ was arbitrarily set to zero.

Fig. 5. The response of the slope direction when the atrazine concentration in the PBS sample is higher or lower than in the reference sample. Before the measurements $\Delta\phi$ was arbitrarily set to zero.

of the response tells us directly that in the analysing sample the atrazine concentration is lower than 4×10^{-10} M (note that in this inhibition assay a lower response is expected for a higher atrazine concentration). The switching between negative and positive slopes as a result of the change in concentration difference between sample and reference sample

can be seen very clearly in Fig. 5. A sample containing 1×10^{-10} M atrazine in channel 1 gives a response with a positive slope, whereas a 1×10^{-9} M solution gives a response with a negative slope. Measuring the direction of the slope can be done fast; even at low analyte concentration only 10 min are required. In this way many water samples can be analysed in a short time to find out if the atrazine concentration is above the limit or not. It should be noted that all the measured responses showed two different response domains. After injection first a fast step like response is measured dependent on bulk differences between the samples (a typical measured step $\Delta\phi = 0.02 \times 2\pi$ could be caused by a temperature difference of $\approx 0.01^\circ\text{C}$ between the samples). The second part of the response is much slower and is dependent on the concentration of atrazine. The result of difference measurements on 12 'blind' water samples performed within 1 h can be seen in Fig. 6. In these measurements one person prepared atrazine solutions, varying from 10^{-11} to 10^{-7} M, whereas another person independently decided on the basis of the MZI results whether a particular sample was below or above the reference concentration of 4×10^{-10} M. Although these preliminary experiments do not permit a detailed statistical

Fig. 6. Pre-screening of 12 samples on atrazine around a reference concentration of 4×10^{-10} M (100 ng/l). The surface is coated with BSA-atrazine and 5×10^{-10} M anti-atrazine is mixed with the PBS samples for 90 min before adding to the sensor surface.

analysis, it is obvious from the data in Fig. 6, that in more than 90% of the samples a correct decision is made.

In principle the slope value $d\phi/dt$ gives a quantitative evaluation for the exact atrazine concentration. However, currently the drift in the baseline caused by contamination of the flow system and temperature differences between the samples preclude the use of the calibration curve for this purpose.

4. Conclusions

We have shown that atrazine can be detected, with a label-free competitive immunoassay performed on the planar waveguide MZI sensor. Particularly the kinetic difference measurement shown in this article is very promising. It is a fast method (<10 min) with a limiting sensitivity around 100 ng/l (EC limit for triazines). In fact this number is determined by the drift that we observe during measurement. This fast semi-quantitative slope direction measurement is especially suitable for pre-screening applications. The pre-screening of water can reduce the number of samples that should be analysed with classical complex and time-consuming analytical techniques like liquid chromatography or gas chromatography–mass spectroscopy.

Also other small molecules like aflatoxins and hormones, for which growing interest exists in the food industry, can be detected with the presented method, provided antibodies are available for the specific molecules of interest.

In future new measurements will be done with 'real' waste water samples to investigate whether a 'real' environmental monitoring is possible. Also the basics of the kinetics of the immunobinding reaction will be further investigated to optimise the parameters and to improve the reliability and the sensitivity of the device to obtain a real quantitative sensor.

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