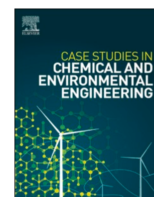
Case Studies in Chemical and Environmental Engineering 5 (2022) 100199

Contents lists available at ScienceDirect

Case Studies in Chemical and Environmental Engineering

journal homepage: www.sciencedirect.com/journal/case-studies-in-chemical-and-environmental-engineering



Case Report

Highly sensitive homogeneous-heterogeneous nanogold-based microimmunoassays for multi-residue screening of pesticides in drinking water

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A R T I C L E I N F O

Keywords:
Water monitoring
Pesticides
Biosensing
Immunoassay
Warning bio-systems

A B S T R A C T

A homogeneous-heterogeneous immunoassay based on the use of antibody modified gold nanoparticles, 5 and 50 nm in diameter, is developed with and without signal amplification. The assays involve the capture of the target analytes in the homogeneous phase and subsequent detection of the immunoreaction product, following a heterogeneous scheme performed on a regular DVD. The analytical approach was evaluated developing multiplexed competitive immunoassays for the determination of residues of the pesticides chlorpyrifos and azoxystrobin as model targets. The results revealed that homogeneous immunocapture strategy improves considerably the assay performance, giving better assay sensitivity when compared to the standard heterogeneous immunoassay format. Under the best conditions, the least detectable level for chlorpyrifos and azoxystrobin were 0.1 μg/L. The immunoassays were also highly selective, showing little or no cross-reactivity with other structurally similar compounds. The immunocapture approach was assessed by the analysis of water. The analytical sensitivity was compared with that of reference chromatographic methods, and recovery results agreed. The good recoveries obtained (mean values ranging between 80% and 125%) make this strategy a suitable screening biosensing methodology for either environmental monitoring or laboratory quantification of pesticide residues without sample treatment in a maximum time of 65 min at lower cost.

1. Introduction

The current demand in environmental monitoring for fast and sensitive results within short amount of time in order to take adequate actions makes the immunoassay a powerful analytical tool. The immunological methods are very useful for pollutant determination in aqueous samples because very low concentrations can be detected without the need for previous laborious and time-consuming sample preparation. The most commonly used immunoassays for pesticide detection in environmental samples, such as water, are based on the heterogeneous format [1]. These assays are sensitive and provide broad dynamic ranges of 2–3 orders of magnitude, however, they suffer from multiple time-consuming incubation and washing steps, usually lasting 1–2 hours in traditional ELISA format. An alternative approach is the homogeneous technique that permits to eliminate diffusion-dependent processes, separation and washing steps and this way to accelerate and simplify the assay [2]. The most widely applied homogeneous immunoassay for pesticide detection in water samples is fluorescence polarization immunoassay [3]. The biggest advantage of this type of the test is that the analysis does not require the mechanical separation of the resulting compounds; therefore, the quantitative determination of an analyte of interest can be performed in a few minutes. Biosensing techniques can be also conducted in homogeneous format using colloidal and magnetic particles [4,5], as well. Many different immunoassays based on this concept were developed, such as for detection of thiacloprid [6], imidacloprid [7], carbofuran [8], and diclofenac in wastewater [9]. Also, other homogeneous immunoassays for pesticide detection were developed, such as entrapping lysosomes for atrazine detection [10], or time-resolved fluoroimmunoassay to detect carbofuran [11]. However, these techniques show limitations. The sensitivity

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https://doi.org/10.1016/j.cscee.2022.100199
Received 31 January 2022; Received in revised form 14 March 2022; Accepted 16 March 2022
Available online 24 March 2022
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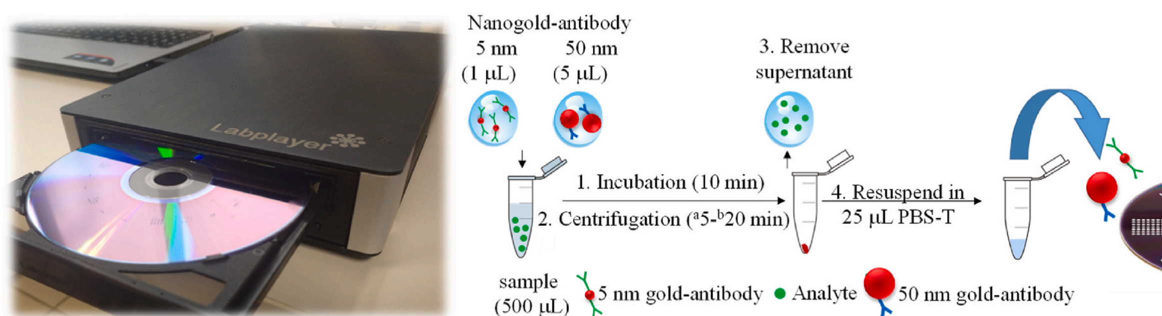

Fig. 1. A) The modified DVR drive used as detector (labplayer). B) Scheme of the immunocapture procedure. The centrifugation conditions vary depending on the assay format. [a]Assay B without signal amplificacion. [b]Assay A with silver enhancement amplification.

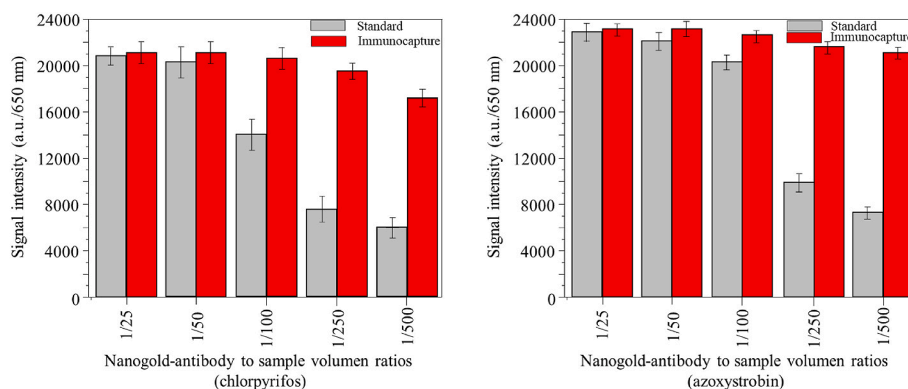

Fig. 2. Signal intensity profile for different nanogold-antibody to sample volume ratio after the immunocapture process for chlorpyrifos and azoxystrobin.

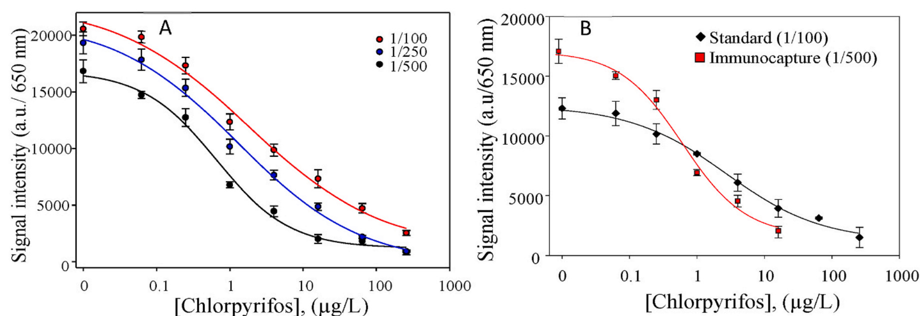

Fig. 3. A) Calibration curves for the competitive assays using the immunocapture approach with silver amplification for different nanogold-antibody to sample volume ratio. B) Curves for the standard and the immunocapture based immunoassays.

is usually inferior when compared to that of ELISA [12]. The homogeneous approach is susceptible to interference from light scattering and endogenous fluorophores in samples, and from tracer binding to sample matrix components [13]. For this reason, most immunoassays rely upon a solid phase to perform the separation between bound and free antibodies.

As it has been demonstrated in the COVID pandemic, in many fields, there is increasing need for fast and simple to use techniques, assays are expected to evolve toward the quantification of analytes at low concentration range in a short time [14].

The use of metal nanoparticles as labels has attracted considerable interest. For example, magnetic nanoparticles were used as capture, separation and detection species [15], showing huge potential in immunoassays. However, gold nanoparticles have gained more interest, because of the easy preparation, functionalization, bioconjugation and the detection versatility [16,17].

In this context, the use of antibody modified gold nanoparticles overcome the drawbacks of both homogeneous and heterogeneous techniques. Herein, the nanogold-antibody conjugates play a dual role as they serve as a capture probe and as a reporter of the analytical signal. As a proof of concept, two immunoassays are developed, following an immunocapture scheme, implemented in an analytical system based on compact-disk technology [18,19] to determine chlorpyrifos and azoxystrobin residues in water as a proof of concept demonstration.

2. Materials and methods

2.1. Chemicals

Gold nanoparticles (5 and 50 nm), ovalbumin, tween 20, trizma base and silver enhancer solutions (A and B) were obtained from Sigma Aldrich (Madrid, Spain). Antibodies against azoxystrobin and chlorpyrifos were obtained as previously described [20,21]. Azoxystrobin (AZB) and chlorpyrifos (CLP) standards were purchased from Dr.

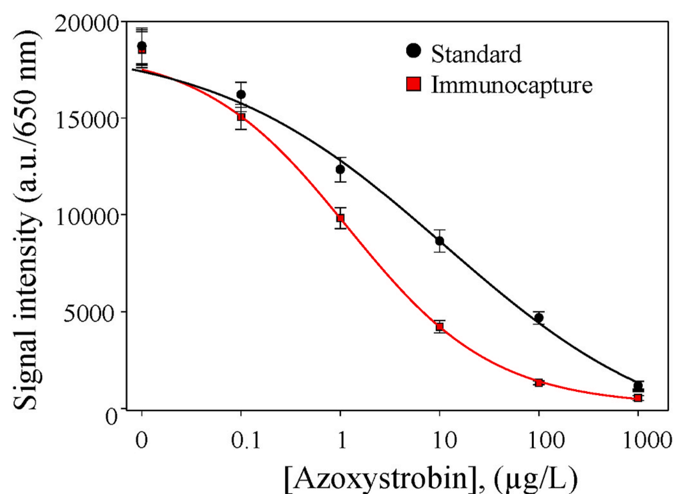

Fig. 4. Calibration curves for azoxystrobin using the immunocapture approach without silver amplification.

Ehrenstorfer (Augsburg, Germany) and Syngenta AG (Basel, Switzerland), respectively. Table S1 shows the chemical structures of the analytes and the haptens used in this work. Stock solutions were prepared in dimethyl sulfoxide and stored at 4 °C. Coating conjugates were prepared with ovalbumin following the active ester method.

PBST, PBS 1x containing 0.05% (v/v) Tween 20 was used as a working and washing buffer. Coating buffer was 50 mM carbonate-bicarbonate, pH 9.6.

2.2. *Nanogold-antibody conjugates*

The antibody modified gold nanoparticles were prepared as previously described [22]. The colloidal gold nanoparticles (OD = 1) used in this study were of 5 and 50 nm in diameter. The as-prepared nanogold-antibody conjugates were stored in 20 mM Tris containing 1% BSA (pH = 8.5). The optical densities obtained were 10 and 80 for 5 and 50 nm gold nanoparticles, respectively. The colloidal suspensions were stored at 4 °C for further experiments.

2.3. *Immunocapture protocol*

The immunoassay aims at the quantification of chlorpyrifos and azoxystrobin residues in water, following an immunocapture approach and optical detection. First, coating conjugate solutions (OVA-triclopyr at 20 µg/L and OVA-AZB at 10 µg/L) were arrayed onto the polycarbonate surface of standard DVDs (CD Rohling-up GmbH, Saarbrücken, Germany), in microarray format, using a non-contact printing device (AD 1500 BioDot, Inc., Irvine, CA, USA) at 25 °C and a relative humidity of 60%. After printing, the disk was incubated for 16 h at 4 °C. Before running the assay, the disk was thoroughly washed with PBST, rinsed with deionized water, and dried by centrifugation at 1000 rpm. For the assay using 5 nm nanogold-antibody conjugates (A), the protocol was as follows. First, 500 µL of sample (with and without analyte) were mixed with 1 µL of nanogold-antibody conjugate (OD = 10) and left to incubate for 10 min. Next, the mixture was centrifuged (20 min, 15,000 rpm), supernatant was discarded and pellet resuspended in 25 µL of PBS-T buffer. The protocol of the assay using 50 nm nanogold-antibody conjugates (B) was as follows. First, 500 µL of sample were mixed with 5 µL of nanogold-antibody conjugate (OD = 80) and left to incubate for 10 min. Next, the mixture was centrifuged (5 min, 8000 rpm), supernatant discarded and pellet resuspended in 25 µL of PBS-T buffer. The resuspended solutions were placed on a coated standard DVD and incubated during 25 min. After that time, the disk was washed with PBST. Silver enhancer solution was only used for the assay A. The reaction was stopped after 10 min by washing the disk with distilled water. Then, the disk was scanned using a modified DVD drive (Fig. 1A) as previously described [23]. The analog signals are related to the optical density of the insoluble precipitate, which is indirectly proportional to the concentration of target analyte in the sample. The analytical performances (EC50, limit of detection and quantification) were calculated with a non-linear logistic four-parameter regression model.

2.4. *Case study. Júcar-Turia channel water source*

Twelve water samples taken from the Júcar river at different points close to the drinking water treatment plant of "La Presa" (Valencia, Spain) were chlorpyrifos and azoxystrobin free, with a pH between 7.7 and 8.3 and used as blanks. The samples were aliquoted and stored at 4 °C prior to use. A mixture of the targeted compounds at different levels

Table 1
Results obtained for the immunocapture approach (IC) and chromatographic[b] analysis (CA) (GC-MS or HPLC-MS) of spiked water samples.

Sample	Added (µg/L)	Found (µg/L)		Recovery (%)		Added (µg/L)	Found (µg/L)		Recovery (%)	
	1					2				
		IC	CA	IC	CA		IC	CA	IC	CA
AZB	1	1.1 ± 0.2	0.78 ± 0.12	110	78	0.5	0.6 ± 0.1	0.37 ± 0.12	120	74
CLP	2	2.3 ± 0.4	1.34 ± 0.41	115	67	1	1.0 ± 0.3	0.65 ± 0.16	100	65
Sample	**3**					**4**				
AZB	0.5	0.4 ± 0.1	0.51 ± 0.03	80	102	1	1.2 ± 0.4	0.93 ± 0.09	120	93
CLP	2	2.2 ± 0.2	1.39 ± 0.19	110	70	0.5	0.5 ± 0.1	0.47 ± 0.09	100	94
Sample	**5**					**6**				
AZB	1	0.9 ± 0.2	0.87 ± 0.16	90	87	0.5	0.5 ± 0.1	0.47 ± 0.09	100	94
CLP	0.25	0.2 ± 0.1	0.26 ± 0.01	80	104	0.25	0.2 ± 0.1	0.28 ± 0.03	80	112
Sample	**7**					**8**				
AZB	4	4.1 ± 0.5	3.28 ± 0.53	102	82	0.5	0.4 ± 0.1	0.48 ± 0.06	80	96
CLP	1	1.0 ± 0.2	0.93 ± 0.08	100	93	0.5	0.4 ± 0.1	0.56 ± 0.06	80	112
Sample	**9**					**10**				
AZB	0.25	0.3 ± 0.1	0.18 ± 0.06	120	72	0.25	0.3 ± 0.1	0.25 ± 0.09	120	100
CLP	0.5	0.6 ± 0.2	0.37 ± 0.07	120	74	1	1.0 ± 0.1	0.83 ± 0.17	100	83
Sample	**11**					**12**				
AZB	4	4.8 ± 0.6	3.57 ± 0.47	96	89	2	1.9 ± 0.6	2.83 ± 0.29	95	142
CLP	2	2.5 ± 0.5	2.04 ± 0.18	125	102	0.5	0.4 ± 0.2	0.56 ± 0.06	80	112

were used to spike the samples. Before the analysis, the samples were first conditioned by mixing 9 parts by volume of water with 1 part by volume of 10-fold concentrated PBS-T, pH 7.4. The nanogold-antibody conjugate solution was added to this mixture with known amounts of chlorpyrifos and azoxystrobin, covering the complete analytical range. Standards and samples were run in three replicates. For comparison purposes, the spiked samples were analyzed in parallel by high-performance liquid chromatography/tandem mass spectrometry (LC/MS [2]) and by solid-phase extraction-gas chromatography/mass spectrometry (SPE-GC/MS) (Details in Supporting Information).

3. Results

3.1. Selection of the conditions for homogeneous immunocapture approach

The conceptual principle of the immunocapture approach is schematized in Fig. 1A. The antibodies attached to gold nanoparticles act as capture species to bind specifically the target molecules in solution. As the immunoreaction event occurs much faster in the liquid phase, rather than in a heterogeneous format, the target molecules bind faster to the conjugate.

The nanogold-antibody to sample volume ratio was the most important parameter studied. For these experiments, 1.0 μL of 5 nm nanogold-antibody conjugate was used. The optical density of the studied dilutions ranged between 0.02 and 0.4 as the sample volume varied from 25 to 500 μL. For comparison purposes, the assay was also performed using the same 5 nm nanogold-antibody conjugate dilutions directly dispensed on the disc without the immunocapture step. Fig. 2 presents the results.

As it can be observed, the higher the sample volume, the lower signals were obtained for the standard assay. A remarkable change was observed for the immunocapture assays for dilution 1/500 (which corresponds to OD = 0.02 a. u.), the signal significantly increased from 5000 to 17000 a. u, and from 7000 to 21500, for chlorpyrifos and azoxystrobin assay, which corresponds to a signal increase percentage of 183 and 207%, respectively.

3.2. Competitive assays using immunocapture step

The competitive calibration curves for chlorpyrifos are presented in Fig. 3A. As it could be expected, the lower the optical density (1/500), the better the sensitivity obtained.

The sensitivity (IC50) of the assay was 1.8 μg/L, 1.6 μg/L and 0.6 μg/L, for the 1/100, 1/250 and 1/500 dilutions, respectively. A standard immunoassay without immunocapture step was also conducted to compare the sensitivity. This assay consisted in mixing the nanogold-antibody conjugate with the sample and then directly applying the solution onto the coated disk. The nanogold-antibody conjugate was used at 1/100 dilution. The results are presented in Fig. 3B. As seen, the best sensitivity was obtained for the immunocapture approach. The IC50 was 0.6 μg/L and the detection limit 0.1 μg/L. The dynamic range of this approach was 0.2–3.6 μg/L (Table S2). The standard immunoassay was significantly less sensitive (2.7 μg/L), showing a dynamic range from 0.2 to 15.9 μg/L. The same behaviour was observed for the azoxystrobin assay, whose sensitivity (IC50 = 0.3 μg/L) increased 3 fold compared to that obtained without the immunocapture step (0.9 μg/L).

The immunocapture approach was also studied without signal amplification (assay B). For that, 50 nm nanogold-antibody conjugates were used. As a first step, different optical densities of the gold-antibody conjugate were tested. As it was already reported [14], it is necessary to use high optical density nanogold-antibody conjugate solutions to get high signals without amplification step. Therefore, the volume of the antibody modified gold nanoparticles was 5.0 μL, whereas the sample volume ranged between 50 and 500 μL. These conditions were tested for the samples in absence of target and with 1.0 μg/L of azoxystrobin as a proof of concept demonstration. Fig. S1 shows the results. As it is observed, signal inhibition percentage caused by 1.0 μg/L of azoxystrobin were 21, 33, 41 and 52% for 1/10, 1/25, 1/50 and 1/100 dilutions, respectively. As it was already stated, the lower the antibody concentration used, the higher the sensitivity achieved. These results were confirmed by performing the calibration curve. Also, to compare the results, a standard assay was performed. Fig. 4 presents the competitive curves. The sensitivity shown by the standard immunoassay was 11.9 μg/L while applying the immunocapture concept, the sensitivity considerably improved till an IC50 value of 1.2 μg/L, one order of magnitude more sensitive, reaching a limit of detection of 0.1 μg/L.

Cross-reactivity studies were also performed. For that, different concentrations of several analytes were tested. The results revealed that there was not cross-reactivity within the working ranges (see Table S3), corroborating the selectivity of the developed immunoassays.

3.3. Analysis of water samples

The suitability of the immunocapture approach, using the 5 nm nanogold-antibody conjugates was evaluated by the analysis of twelve spiked Jucar-Turia channel water samples. Table 1 shows the results.

As can be observed, 92% of the spiked concentrations were successfully determined (80% ≤ R ≤ 120%), and the results were in good agreement with those obtained with the reference methodologies. Besides, no false positives were detected when blank samples were analyzed.

4. Conclusions

A promising approach to improve the sensitivity of the immunoassays based on an immunocapture concept, using gold-antibody conjugates was developed. The approach allows improving significantly the sensitivity for the detection of small organic pollutants, compared with the standard immunoassay. The method is very versatile as antibodies of different nature can be used. Thus, detection of several targets, starting from small molecules and ending with large biomolecules could be performed by adapting the assay to the desired analytical scenario.

Apart from an increase in sensitivity, this approach has several other advantages over existing methods. First, the low sample and nanogold-antibody conjugate consumption make it very competitive to standard immunoassays where high amounts of reagents are required. In addition, high versatility as it performs using different gold sizes, though the best results in terms of sensitivity were reached with the 5 nm gold nanoparticles. Other types of nanoparticles such as colloidal silver or gold nanorods could be used and detection performed on more suitable platform such as Blu-ray disk, showing the flexibility of the concept. In addition, the use of a compact disk as analytical platform increases the high-throughput capabilities because many samples are analyzed in parallel. This technology is of great analytical interest for biosensing applications where antibody modified nanoparticles may be involved.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

S.M awards grants PID2019-110713RB-I00 funded by MCIN/AEI/10.13039/501100011033 and by “ERDF A way of making Europe”. PROMETEO/2020/094, Proyecto “88/2021” “Nominativa Acción 2021–12. Dr. A. Abad-Fuentes kindly provided the anti-azoxystrobin antibody and the corresponding coating conjuagte.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cscee.2022.100199.

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