Detection of Parathion Pesticide by Quartz Crystal Microbalance Functionalized with UV-Activated Antibodies

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ABSTRACT: Photonic immobilization technique (PIT) has been used to develop an immunosensor for the detection of parathion. An antibody solution has been activated by breaking the disulfide bridge in the triad Trp/Cys-Cys through absorption of ultrashort UV laser pulses. The free thiol groups so produced interact with gold lamina making the antibody oriented upside, that is, with its variable parts exposed to the environment, thereby greatly increasing the detection efficiency. PIT has been applied to anchor polyclonal antiparathion antibodies to the gold electrode of a Quartz Crystal Microbalance (QCM) giving rise to very high detection sensitivity once the parathion is made heavier by complexion with BSA (bovine serum albumin), this latter step only required by the mass based transducer used in this case. The comparison of the sensor response with irradiated antibodies against different analytes shows that the high degree of antibody specificity is not affected by PIT nor is it by the complexion of parathion with BSA. These results pave the way to important applications in biosensing, since the widespread occurrence of the Trp/Cys-Cys residues triads in proteins make our procedure very general and effective to detect light analytes.

The high toxicity of pesticide residues and their bioaccumulation effects in human body underpins the research for fast response biosensors with high sensitivity and specificity for relatively light molecules. Parathion (IUPAC name O,O-diethyl O-4-nitrophenil phosphorothioate) is an example of such compounds extensively used in agriculture as acaricide. As all the organophosphate insecticides, it is a selective inhibitor of acetylcholinesterase, an essential enzyme for nerve function in insects, humans, and many other animal species, thereby resulting highly toxic. The accumulation of this molecule leads to respiratory distress and muscular problems. Once absorbed, the parathion acts indirectly on the acetylcholinesterase since it is oxidized by enzymes of the host in its more reactive form, the paraoxon, which is capable of irreversible covalent binding the acetylcholinesterase. The use of parathion is forbidden in the European Union.

Since the toxicity manifests its effects only several hours after exposure it is of paramount importance the availability of a tool allowing real-time analysis in environmental monitoring and particularly over agricultural waste. The lowest limit of detection (LOD) for parathion, and more in general measured for organophosphates, in aqueous solution has been achieved by Walker et al.3 using a photonic crystal device. They developed a polymerized crystalline colloidal array (PCCA) photonic crystal sensing material that reaches a detection limit of 0.001 ppt. The crystals were coupled with acetylcholinesterase, which is irreversibly bonded by the organophosphate compound. The experiments were performed exposing the PCCAs to sample solutions as large as 100 mL thereby requiring up to 30 min for the analysis. Disadvantages of these devices include complex nanofabrication processes and the high cost of commercially modified substrates.

Amperometric devices allow rapid and low cost detection of organophosphates. A sensitive method for the detection of parathion has been developed by Zen et al.,4 who reached a LOD of 50 nM (0.9 ppb) using a Nafion-coated glassycarbon electrode. Other detectors involve electrodes functionalized using organophosphorus hydrolase. Mulchandani et al.5 reached a LOD of 20 nM (0.4 ppb) for both methyl-parathion and paraoxon, whereas Sacks et al.6 efficiently detected parathion at nanomolar concentration. It is worth to mention that electrochemical devices are effective tools for analysis of aqueous solutions providing that the molecules to be detected are electroactive. In addition, this kind of sensors can be easily influenced by other oxidizable species that may well be present in a real sample.

Because of their cost-effectiveness, flexibility, and reliability, quartz crystal microbalance (QCM) based sensors have received an increasing interest in recent years7,8 with wide applications to liquid samples. For instance, Bi and Yang5 detected pesticides in aqueous environment using a QCM based method. They used molecular imprinted monolayers (MIMs), obtained from hexadecanethiol self-assembling on the

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QCM gold electrode, to detect imidacloprid and thiacloprid in celery juice reaching a LOD of 1 \( \mu \text{M} \) by using an extremely sensitive QCM apparatus with a resolution of 0.1 Hz. Marx et al.\textsuperscript{10} developed a sol–gel functionalization method based on MIP (molecularly imprinted polymer) films for the detection of parathion. They applied this technique for the development of both cyclic voltammetry devices and QCM based sensors, but despite their high sensitivity, sol–gel-based functionalizations can be easily affected by cross-sensitivity phenomena.

The sensor specificity is inherently warranted when antibodies are used as linkers, but in this case, the surface functionalization becomes a crucial phase in the realization of the sensor, the antibody orientation being one of the main issues.\textsuperscript{11} Usually a biological molecule moves into aqueous environment and its functions are characterized by parameters, which can change significantly when the same molecule is, instead, immobilized. So, there is a strong interest in the research of new immobilization and functionalization techniques which allows better sensitivity and lower LOD as witnessed by the vast literature on this topic.\textsuperscript{12,13}

The most suitable functionalization procedure depends both on the nature of the biological sensitive element and on the transduction principle. Besides that, functionalization of gold laminates enlists many efforts in view of their use not only in QCM-based immuno-sensors\textsuperscript{14–17} but also in surface plasmon resonance (SPR).\textsuperscript{18–20} Moreover, gold is an inert metal having a low tendency to oxidize and is biocompatible and easily cleaned by chemical treatments such as piranha solution (a mixture of sulfuric acid and hydrogen peroxide). The stability of gold–sulfur interaction is one of the mechanisms exploited to immobilize molecules on a support, since the only requirement is the availability of a thiol group. To this end, it is possible both to use a chemical marker of the molecule, usually easy to realize by chemical synthesis, or to exploit existing thiol groups within proteins and peptides.\textsuperscript{21}

UV induced immobilization is a novel technology that results in spatially oriented and spatially localized anchoring of biomolecules onto thiol-reactive surfaces as demonstrated by Neves-Petersen et al.\textsuperscript{22} The reaction mechanism behind this immobilization technique involves the photonic activation of disulfide bridges, that is, the light-induced breakage of disulfide bridges in proteins through UV illumination of nearby aromatic amino acids.

In a previous paper, we have demonstrated that no pretreatment to the surface is necessary, the presence of this structural characteristic being the only condition required to apply this approach that we will refer as photonic immobilization technique (PIT).\textsuperscript{23} The mechanism of photonic activation of the Trp/Cys–Cys triads and the crystallographic structure of a type G immunoglobulin (IgG)\textsuperscript{24} are shown in figure 1. Basically, the UV photon energy is absorbed by Tryptophan and then transferred to the close Cys-Cys thereby forming free reactive thiol groups which interact with thiol-reactive surfaces (like gold plates or surfaces treated with alkanethiols). Thus, the “driving force” for a possible side-up orientation of the antibody onto the gold surface is provided by the open disulfide bridges; these are produced in a selective way because the occurrence of a solvent accessible triad Trp/Cys–Cys, which is required by PIT, is limited to interdomain regions.\textsuperscript{22}

The UV radiation may reduce or vanish the biological activity of proteins and enzymes, causing also structural changes in the biomolecules,\textsuperscript{25} but PIT preserves the native structure and the functional properties of the immobilized proteins while favoring the proper orientation of the biomolecule on the support,\textsuperscript{23} and this is achieved by avoiding any chemical and thermal treatment.

One of the advantages of PIT relies in the wide field of application since the closely spaced triad of residues Trp/Cys–Cys is present in all members of the immunoglobulin superfamily. Every IgG has twelve intradomain disulfide bridges near a tryptophan residue, one of them being present in every domain of the protein. It is likely to induce the opening of these disulfide bonds through UV irradiation of the near aromatic residue. With the breaking of these disulfide bridges there is an increase of free thiol groups, which can react with a gold or thiol-rich surface. The same geometry of the Trp/Cys–Cys triad is observable in structures of several IgSF (immunoglobulin superfamily) molecules as well as in TCRs (T cell receptors), MHCs (major histocompatibility complex),\textsuperscript{26} cell surface antigens (CD4 and CD8), and various cell-adhesion molecules. It is suggested that the conservation of the geometrical relationship between this three aminoacids is due to the role played by tryptophan in stabilizing or protecting the disulfide bridge.\textsuperscript{27}

The proof of principle of PIT reported in our previous paper concerned the detection of murine immunoglobulin by QCM based immuno-sensor for which the sensitivity improved only by a factor of 2.\textsuperscript{23} This result is in agreement with the findings reported in a recent paper by Trilling et al.\textsuperscript{28} who show that the effect of the antibody orientation on the capture of the analyte is strongly dependent on the mass of the analyte. In particular, lighter analytes are more sensitive to the side-up orientation of the antibodies, so that PIT can render effective several techniques (e.g., QCM) otherwise impractical.

In this paper, we demonstrate how PIT improves the sensitivity of a QCM by about 1 order of magnitude compared to that achieved when the antibodies are tethered randomly oriented. The main drawback of a mass-based transducer like QCM in detecting light molecules (parathion weighs only about 300 Da), is the small frequency shift associated with their tethering to the antibody. We overcame this problem through a procedure that makes the molecules heavier so that a LOD of 4 ppb was achieved with an analysis requiring less than 10 min. We also demonstrate that PIT does not affect the specificity

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**Figure 1.** UV photonic activation of a generic IgG (1IGY). (a) The protein solution is irradiated. (b) One UV photon is absorbed by a tryptophan side-chain which transfers the energy to the near cysteines. (c) The disulfide bridge opens and the thiol groups so produced can effectively interact with the gold surface.
features of the antibody as the test with similar compounds have shown.

**EXPERIMENTAL SECTION**

**Chemicals.** Antiparathion polyclonal antibodies were purchased from antibodies-online.com as rabbit serum (ABIN113883). Protein A agarose (Pierce) from Thermo Scientific has been used for the antibodies purification, whereas bovine serum albumin (A2153), parathion (45607), bisphenol A (239658), p-nonylphenol (46018), dichlorvos (45441), and paraoxon (36186) were purchased from Sigma-Aldrich. Since these compounds are highly toxic, all the samples were prepared in the fume hood. Other materials we have used are PBS 1× buffer solution pH 7.4, Helix water, sulfuric acid 98%, and hydrogen peroxide 30%.

**Experimental Apparatus.** The QCM is a μLibra from Technobiochip, Italy. The frequency variation of the balance in the cell is displayed on a connected computer by a producer-released software. The quartz crystals are from ICM Manufacturer, Oklahoma city (U.S.A.). They are AT-CUT quartz with a fundamental frequency of 10 MHz. The crystal diameter is 1.37 cm while the diameter of the gold lamina is 0.68 cm. The electrodes are cleaned using the Piranha solution (3:1 ratio between concentrated sulfuric acid and 30% hydrogen peroxide solution).

The fluidic apparatus consists of a GILSON peristaltic pump, Tygon silicone tubes with different diameters (0.51 mm for input into the cell and 0.64 mm for output) and the cell which contains the crystal placed on the electronic console for the measurement of frequency oscillation. The volume just above the electrode is approximately 40 μL and the fluidic apparatus is designed so that the whole replacement of the solution takes about 80 s when the flow rate is 3.3 μL/s. This was tested by measuring the time required by PBS to remove completely a colored solution (Flavin adenine dinucleotide) from the cell. The permanence of BSA above the electrode entails that this interaction is formed as a consequence of unspecific interactions. This is sketched in figure 2 where the BSA and the analyte are represented as big green and small orange balls, respectively. To check the complex formation between parathion and BSA we measured the BSA fluorescence spectrum (∆λ = 278 nm) observing a strong reduction of the yield as a function of the parathion concentration. This quenching effect of parathion on BSA fluorescence shows the interaction between these two partners.

The UV light source used is a custom femtosecond PHAROS laser system having a high tunable pulse repetition rate (Light Conversion, Ltd., www.lightcon.com). UV laser pulses having a length of approximately 120 fs are obtained coupling the laser with a harmonic generator stage (HIRO), which provides the conversion to 515, 343, or 258 nm wavelengths of the IR fundamental radiation.

**UV Activation of the Antibody Solution.** Aliquots of 1 mL containing 5 μg of antiparathion polyclonal antibody, have been irradiated by means of the femtosecond laser system previously described. The laser source operates, in this case, at a pulse repetition rate of 10 kHz and delivers 250 mW of average power at λ = 258 nm (resulting in energy per pulse of 25 μJ). These irradiation conditions guarantee the maximum efficiency in disulfide bond breaking as demonstrated by Della Ventura et al. through the Ellman’s assay.

**QCM Protocol.** The experimental protocol consists of the following steps: (1) Reaching of the basal frequency stabilization by washing the electrode surface with 1× phosphate buffer (PBS) pH 7.4. (2) UV-assisted adsorption or passive adsorption of antiparathion polyclonal antibodies. (3) Blocking the remaining gold free surface with bovin serum albumin (BSA) solution (50 μg/mL) to avoid nonspecific interactions. (4) Flowing of parathion solution in the fluidic circuit that interacts with BSA solution still inside the volume above the electrode. The complex so formed is subsequently recognized by the antibody (see Figure 2). (5) Final washing step with PBS 1× to eliminate weakly bonded antigen.

Figure 3 shows typical QCM outputs obtained when the antibodies are irradiated (black continuous line) and not-irradiated (red dashed line). To be noted that the first drop in the frequency shift (∼300 Hz, step 2 of the protocol) is the same in both cases (irradiated and not irradiated) so that we can assume that PIT does not change the amount of antibody

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**Figure 2.** Antigen binding on the functionalized gold electrode. The big green spheres are the BSA macromolecules which are complexed with the smaller antigens (orange spheres). The model is not in scale.

**Figure 3.** QCM responses of irradiated (black solid line) and not-irradiated (red dashed line) antibody samples with a protein concentration of 5 μg/mL. The first drop at ∼50 s corresponds to the antibodies tethered to the electrode and is not affected by PIT, whereas the second drop at ∼350 s is given by an antigen solution (parathion 51.5 μM) conveyed to the cell and is much larger when PIT is used. The vertical dashed lines show the steps described in the text.
tethered to the electrode. This is in fair agreement with the surface density measurements reported by Peluso et al. who studied the effects produced by orientation on both full-sized antibodies and Fab fragments, finding comparable surface densities between aligned and not-aligned antibodies in three different cases (Mab602, Mab208, and Mab9647).30

When BSA reaches the electrode (step 3) no frequency shift is observed demonstrating that the surface is fully covered by antibodies. Subsequently, the analyte is conveyed to the electrode (step 4) and a completely different response is observed whether PIT is used or not. Such a difference is kept even when the electrode is washed by means of PBS (step 5).

RESULTS AND DISCUSSION

Sensitivity and LOD. The response of the QCM is proportional to the mass tethered to the electrode31 so that the frequency shift \( \Delta f(P) \), \( P \) being the concentration of parathion, is a measure of the amount of analyte bound to the antibodies. As reported in the Experimental Section, when the parathion reaches the volume above the electrode, the BSA solution is still there in high concentration thereby allowing the complex formation. The BSA molecules (approximately 66 kDa) bind the organophosphate compound by means of unspecific interactions and plays a “ballast” role, making the analyte “heavier” and detectable by a balance. In fact, even if all the antibodies on the electrode bound one parathion molecule (\( \sim 300 \text{ Da} \)), the QCM frequency shift would be only few hertz and, thus, not detectable by our device. This has been verified by washing the fluidic circuit, thus removing the BSA, and checking that the parathion is not detectable any more, even if PIT is used. It is worth to stress that this unconventional labeling of the analyte does not affect the features of the sensor which is fully insensitive to BSA once the antibodies are tethered to the electrode. Figure 3 shows that when BSA is conveyed to the QCM the crystal oscillation frequency keeps constant (see the signal in the interval 150–330 s). Thus, with a relatively high concentration of BSA in the fluidic circuit we have compared the response of QCM to irradiated (PIT) and nonirradiated antibodies obtaining the results shown in figure 4.

It is readily seen that no significant signal is measurable when the parathion concentration is lower than 50 \( \mu \text{M} \) (15 \( \mu \text{g/mL} \)) if the antibodies are not irradiated, the signal being already in the saturation region in the opposite case. By considering the law of mass action and given the free diffusion conditions of our experiment, the analysis of the interaction kinetic between the analyte and the antibody leads to a Michaelis–Menten type equation:

\[
\Delta f(P) = \frac{(\Delta f)_{sat} P}{P + K_M}
\]

(1)

where \( (\Delta f)_{sat} \) contains the instrument response and \( K_M \) is the so-called Michaelis–Menten constant. The former parameter includes the number of antibodies tethered on the balance as well as their effectiveness in capturing the analyte, that is, their orientation, whereas the latter provides estimation for the range of linearity of the realized sensor. The fitting of the experimental results obtained with irradiated antibodies by eq 1 (solid line in figure 4) provides \( (\Delta f)_{sat} = 151 \pm 5 \text{ Hz} \) and \( K_M = 2.2 \pm 0.7 \text{ \( \mu \text{M} \)} \). The uncertainty in our measurements is due to instrumental limitations in the performances of our QCM, as well as to the fluctuations in some steps of the procedure. Although we expect a remarkable improvement of the reproducibility by an up-to-date QCM as well as an improved fluidic circuit setup, currently we consider a conservative frequency error of 15 Hz (see also the inset in figure 4). Thus, a minimum frequency change \( (\Delta f)_{min} = 15 \text{ Hz} \) is required for a measurement to be significantly different from zero. An evaluation of the lower LOD can, then, be obtained by inverting eq 1

\[
LOD \approx \frac{(\Delta f)_{min} K_M}{(\Delta f)_{sat}} \approx 2 \times 10^{-7} \text{M} \approx 60 \text{ ng/mL}
\]

(2)

Since the concentration of water is about 55 \( \text{M} \) a LOD better than 4 ppb for parathion in water results from our functionalization technique applied to a QCM.

By considering the response at the concentration of 5.15 \( \mu \text{M} \) (see figure 4) we can estimate an increase in the sensitivity by at least 1 order of magnitude when PIT is used. Such a result can only be attributed to the side-up orientation of the antibodies and is much more significant than that achieved with heavier analytes,33 in agreement with the mass dependence effects of antibody orientation recently reported by Trilling et al.28

Specificity. To test the sensor specificity, the same experimental procedure has been used for four compounds, progressively similar to parathion, from the chemical and structural point of view. They are bisphenol A (4,4’-(propane-2,2-diyl)diphenol), p-nonylphenol, dichlorvos (2,2-dichlorovinyl dimethyl phosphate), and paraxoan (diethyl 4-nitrophenyl phosphate). At concentrations of approximately 1.7 \( \mu \text{M} \), where parathion exhibits a significant response (see inset of Figure 4), all the four compounds showed no detectable frequency shift when they were tested separately. Thus, we tested the sensor response with a mixture of the four compounds at an even higher concentration (3.5 \( \mu \text{M} \)) thereby mimicking a real complex matrix. The results are reported in Figure 5a, where the last steps of the protocol are highlighted. The frequency shift measured when only the four compounds are in the solution is just few hertz (mix), but the frequency goes back to the previous value when PBS is made to flow (blue dotted line).
The parathion alone at 3.5 μM gives rise to a frequency shift of approximately 90 Hz (red dashed line). The addition of the four compounds (Pt + mix) produces a slightly larger shift (solid black line) that disappear after purging with PBS. Thus, the concentrations were increased by 2 orders of magnitude (170 μM) compared to the initial one (1.7 μM), but despite such high concentration, the QCM responses were always lower than those provided by parathion in the saturation regime of the dose–response curve. It is also noticeable to look at the sensor response as a function of “similarity” with the parathion, reported in Figure 5b. Such data are internally consistent showing once again that the recognition capability of antibodies is unaffected by PIT.

**CONCLUSIONS**

The side-up orientation of antibodies is the most effective way to improve the sensitivity of immunosensors designed for light molecules and becomes a necessary condition when QCMs are going to be used. By applying the novel photonic immobilization technique to antibodies to be tethered to the gold electrodes of a QCM, we have been able to achieve a sensitivity that allows the detection of a light molecule like parathion (291.26 Da) in water with a LOD lower than 4 ppb (60 ng/mL). This value has been reached with a QCM capable to detect frequency shift of approximately 10 Hz and, although the resulting LOD is already competitive with those obtained with similar devices, we anticipate a significant improvement if an up-to-date QCM capable to detect frequency shift as low as 0.1 Hz is used. Thus, in principle, by adopting PIT, as well as the complexon with a heavy molecule like BSA, we expect to be able to reach LOD much better than 1 ppb. This kind of immunosensor is inherently highly specific and provides fast response (only few minutes are required for the whole analysis). Moreover, in view of its flexibility and portability, such a device is suitable for in situ analysis. On the basis of our microscopic interpretation of the experimental findings and given the widespread presence of the triad Trp/Cys-Cys in all the antibodies, PIT can be considered as a general technique to anchor oriented antibodies on thiol reactive surfaces thereby paving the way to use this cost-effective technology for the detection of a variety of analytes.

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**Notes**

The authors declare no competing financial interest.

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