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Development of a photosystem II-based optical microfluidic sensor for herbicide detection

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ABSTRACT

Herbicides are highly toxic for both human and animal health. The increased application of herbicides in agriculture during the last decades has resulted in the contamination of both soil and water. Herbicides, under illumination, can inhibit photosystem II electron transfer. Photosynthetic membranes isolated from higher plants and photosynthetic micro-organisms, immobilized and stabilized, can serve as a biorecognition element for a biosensor. The inhibition of photosystem II causes a reduced photoinduced production of hydrogen peroxide, which can be measured by a chemiluminescence reaction with luminol and the enzyme horseradish peroxidase. In the present work, a compact and portable sensing device that combines the production and detection of hydrogen peroxide in a single flow assay is proposed for herbicide detection.

1. Introduction

The detection of environmental hazardous chemicals using sensor technology has been rising in demand [1–3]. Chemicals such as herbicides, fungicides and insecticides may exist in harmful levels and pose an environmental threat. Even low levels of contaminants can cause adverse effects on humans, plants, animals and ecosystems. The application of herbicides has increased appreciably during the past few decades, resulting in the massive pollution of water and soil. About 40% of herbicides (derivatives of phenylurea, triazine, diazine and phenolic types) that are currently in use inhibit the light reactions of photosynthesis (photosynthetic herbicides); usually by targeting photosystem II (PSII)-dependent electron flow [4].

At present, the modern methods of herbicide detection are HPLC, GC–MS and bioassays. These methods require expensive equipment, shipment of samples to the laboratories and highly qualified personnel for the analysis [5,6].

Photosystem II is a multisubunit complex located in the thylakoid membranes of algae, cyanobacteria and higher plants. It uses light energy to catalyze a series of electron transfer reactions resulting in the splitting of water into molecular oxygen and protons. Isolated thylakoid membranes produce the superoxide free radical ion and hydrogen peroxide as a product of the dismutation of O^{2-} [7]. This reaction is stimulated by autooxidizable electron acceptors of photosystem I and occurs in the presence of the natural electron acceptor system, ferredoxin and NADP, following the reduction of NADP [8].

Triazine, diazines, phenolic and urea herbicides are the ones with the highest effect on chloroplasts. These compounds inhibit photosynthetic process by binding to the D1 protein of photosystem II [9]. Photosynthetic herbicides block electron transport by the displacement of the bound plastoquinone called QB from its binding site on the D1 protein [10]. This protein is an essential part of the PSII reaction centre and together with the homologous protein D2 binds all the prosthetic groups involved in the main PSII electron transfer pathway [11,12].

Immobilized chloroplasts and thylakoids have been used to detect herbicides either by testing inhibition of the Hill reaction, inhibition of DCPIP photoreduction or change in chlorophyll fluorescence [2,13–16]. These observations have initiated interest in

Abbreviations: ABTS, 2,2′-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid); CCD, charged coupled device; DCPIP, 2,6-dichlorophenolindolphenol; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HRP, horseradish peroxidase; LED, light emitting diode; LOD, limit of detection; MES, 2-morpholinoethanesulfonic acid; NADP, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; PTFE, polytetrafluoroethylene.

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developing biological sensors to detect low levels of herbicides in water and soil using PSII.

Magnetic particles have been utilized extensively in diagnostics and other research applications for the capture of biomolecules and cells. Many assays and separations have been adapted to a magnetic bead format to take advantage of the benefits of microspheres and magnetic separation [17–21]. The most reported biosensing configurations based on beads are biosensors and bioreactors integrated into bead injection analytical systems [22–24].

In this study, we describe the production and preliminary characterization of an optical (chemiluminescence) PSII microfluidic sensing device for herbicide monitoring. The proposed detection principle is the chemiluminescence-based monitoring of the concentration of photosynthetically produced H_2O_2, which can be disrupted by herbicides.

The photosystem II complex and the HRP enzyme are immobilized on magnetic beads, which are in turn magnetically entrapped. The sensor unit is able to perform the assays and optically stimulate photosystem II within the unit and detect the HRP-catalyzed chemiluminescence of luminol/hydrogen peroxide. The system combines the production and detection of hydrogen peroxide in a single flow assay by combining all the individual steps in a compact and portable device.

2. Experimental

Polystyrene amino-magnetic beads (5% (w/v); 3.35 μm of diameter) were purchased from Spherotech Inc. (USA). The Carboxyl Terminated Beads (10% (w/v); 3.2 nm of diameter, made of polystyrene copolymer/iron oxide, 55:45) were from Europa Bioproducts (UK). All reagents are of analytical grade and were purchased from Sigma Chemical Co. All buffers were prepared using nanopure water.

2.1. Micro-system principles and design

The detection principle used in this system is chemiluminescence-based monitoring of the concentration of photosynthetically produced H_2O_2, which can be disrupted by herbicides. The sensor unit is able to perform the assays and optically stimulate photosystem II within the unit and detect the enzyme-mediated chemiluminescence of luminol/hydrogen peroxide. The sensor comprises of a short fluidic channel with two "active" regions including: (i) immobilized PSII and (ii) immobilized HRP to catalyze luminol/hydrogen peroxide chemiluminescence. Initial design consists of a flow channel constructed of machined Perspex sandwiching a laser-cut elastomer spacer/flow channel in which regions of appropriate reagents will be magnetically entrapped (Fig. 1).

The sensor consists of: (i) a pump in order to force the sample mixed with luminol through the sensor (after pre-concentration and sample "clean-up" if necessary), i.e. a simple flow-injection arrangement; (ii) a light source and delivery optics to illuminate the PSII region, an Agilent HMLP-C117 "high brightness LED" (peak wavelength 645 nm, with luminous intensity 300 mcd) and (iii) a suitable detector module and collection optics for the detection of chemiluminescence from the hydrogen peroxide detection region, e.g. a photomultiplier tube (PMT) or a staircase avalanche photodiode (SAPD) modules to detect luminescence of luminol at about 431 nm.

2.2. μ-Fluidic system fabrication

The micro-system was manufactured in-house at Cranfield University, following the design and principles described. The sandwiching blocks were machined from Perspex (polymethylmethacrylate), while fluidic channels were made from silicon elastomer using a mask as well as black rubber. Each Perspex block has the following dimensions: $H \times W \times L = 20\,\text{mm} \times 20\,\text{mm} \times 60\,\text{mm}$.

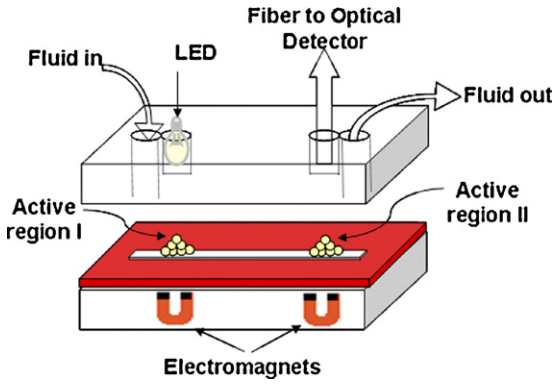

Fig. 1. Schematic representation of the micro-system, with the two Perspex blocks that sandwich the fluidic channel (in red) wide apart. The "active" regions are also shown, with the magnetic beads immobilized into the magnetic field of the magnets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The fluidic channel has a height of only 2 mm (for the silicone channel) and 0.3 mm (for the rubber channel). For the rubber fluidic channels, a CO_2 laser was used for cutting the structures, which allowed rapid prototyping of μ-fluidic gaskets/structures, coupled with increased accuracy compared to the silicone ones. Alternative channel structures were used in order to optimize capture and functional activity of HRP-coated magnetic beads. Currently, the fluidic channels are 300 μm thick and 1000 μm wide.

2.3. Particle-based biochemistry

The two "active" regions, PSII and HRP, which are actually non-permanent, are immobilized on magnetic beads, which are in turn immobilized (magnetically) on the "active" regions. The unit was designed taking into consideration all the steps of detection in advance. A protocol of the steps includes the following.

Beads with attached HRP enter the fluidic channel, and are immobilized by magnetic forces on the region II. Beads with attached PSII enter the fluidic channel, and are immobilized by magnetic forces on the region I. A water sample (pre-mixed with luminol) enters the fluidic channel, and is illuminated, on the region I. The sample with H_2O_2 produced by PSII flows towards region II, where the chemiluminescence reaction takes place, and the light produced is, through the optical fiber, detected by the detector. The removal of the magnetic forces and the flow of buffer/water result in the removal of the beads, and the sensor can be used again.

2.4. Luminol chemiluminescence batch H_2O_2

Different concentrations of luminol and HRP were added to 1 ml polystyrene cuvettes, and were placed in the detection region of the spectrophotometer. A sample of Tris–HCl buffer, 10 mM, pH 8.5 with various H_2O_2 concentrations was manually pipetted. To ensure that the crucial first few seconds of the reaction were monitored, the recording of light was started before the addition of the sample, while the ambient light was maintained at the minimum possible.

The chemiluminescence was transduced to an electric signal by a portable SD2000 CCD luminometer (Ocean Optics, Netherlands) or a bench-top VARIAN spectrophotometer. The chemiluminescence intensity profiles were recorded and the maximum intensity was used to plot the graphs.

For samples of thylakoids producing H_2O_2, the procedure was similar. Aliquots of thylakoid membranes were diluted accordingly with Tris–HCl buffer, 10 mM, pH 8.5 and introduced in a glass pipette by aspiration. The glass pipette was then illuminated. Luminol (30 mM), HRP (150 U ml^{-1}) and H_2O_2 (30 μM–3 M) were prepared freshly in Tris–HCl buffer, 10 mM, pH 8.5, and were pipetted in a polystyrene cuvette, that was placed in the appropriate location in or next to the light-reading apparatus. Once the illumination of the glass pipette containing the thylakoids was terminated, its contents were rapidly transferred into the cuvette, to allow instantaneous mixing. The reaction of the H_2O_2, produced by the thylakoids during the illumination, with luminol and the HRP, resulted in the characteristic light which is the product of this reaction. The light-reading apparatus was used to measure the light produced from the very start of this process.

2.5. Immobilization of HRP on magnetic beads

For the immobilization of HRP on the beads, a protocol supplied by Cortex Biochemicals was used (http://www.cortexbiotech.com), with some modifications. One milliliter of MagaBeads-Carboxyl Terminated was washed twice in 10 ml of MES buffer (50 mM, pH 6.1). Volume of particles was adjusted to a 10 mg ml^{-1} concentration. Then a same volume of EDC (100 nM) was added and allowed to react for 15 min at room temperature with continuous mixing. The microsphere particles were washed twice in phosphate–citrate buffer and resuspended in 5 ml of the same buffer. Five milliliters of HRP (1 nM) were added and allowed to react at room temperature for 2 h with constant mixing. The remaining active sites of the MagaBeads were blocked in a solution of 0.03% (w/v) glycine in MES buffer for 30 min at room temperature. The particles were washed four times with 2 ml of MES buffer each time, using a magnet to sediment particles. After a final washing with 2 ml sodium acetate buffer, 0.1 M, pH 4.0, the microspheres were ready to use.

Two types of HRP were used such as HRP-1 (type II, 148 U mg^{-1}) and HRP-2 (type VI, a highly purified and stabilized, 300 U mg^{-1}). EDC (100 nM), HRP (1 nM), glycine (0.03%, w/v) were prepared just before each immobilization assay, in MES buffer, 50 mM, pH 6.1. HRP-1 and HRP-2 were purchased from Sigma Chemical Co.

For the HRP activity assay, one tablet of ABTS was dissolved in 100 ml of 50 mM phosphate–citrate buffer and 25 μl of 30% H_2O_2 was added. One hundred microliters of ABTS–H_2O_2 were added to 100 μl of sample in each well of a white microtitre plate with a clear bottom. Immediately after initiation of the reaction, absorbance at 405 nm was measured every 10 min.

2.6. Flow assay for hydrogen peroxide with HRP immobilized on magnetic beads

The flow system (Fig. 1) consisted of one peristaltic pump, delivering a 10 mM luminol and a H_2O_2 sample pre-mixed at a continuous flow rate of 2.5 ml min^{-1}. Luminol and H_2O_2 were freshly prepared every day in Tris–HCl buffer, 10 mM, pH 8.5. PTFE tubing (0.8 mm i.d.) was used to connect the flow system components. Firstly, the beads (1 mg) were flown in the flow system by a peristaltic pump. A permanent magnet was used to attract the beads as they were flowing, thus immobilizing them in a specific area of the channel, underneath the area "viewed" by the optical fiber (50 μm of diameter, for detection in wavelength: 200–800 nm) (Avantes, Netherlands). The magnet was not moved during the experiment, in order to ensure that the beads were not carried away by the continuing flow. The reaction was initiated once the pre-mixed luminol–H_2O_2 reached the area of the flow channel that was covered with the beads. The chemiluminescence was transduced to an electric signal by a SD2000 portable CCD luminometer (Ocean Optics, Netherlands). The chemiluminescence intensity profiles were recorded and the maximum intensity was used to plot the graphs.

2.7. Thylakoid membranes isolation protocol

Thylakoid membranes were isolated from fresh spinach leaves (*Spinacea oleracea* L.) using the procedure described by Touloupakis et al. [15]. One hundred grams of leaves were washed with distilled water, dried on filter paper and homogenized in 200 ml of extraction buffer containing 20 mM Tricine, pH 7.8, 300 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA and 0.2% (w/v) bovine serum albumin (BSA). The homogenate was filtered through six layers of cheesecloth and centrifugated at 7500 × *g* for 20 min. The pellet was suspended in a buffer containing 50 mM Tricine, pH 7.8, 70 mM sucrose, 5 mM $MgCl_2$ and centrifugated at 7500 × *g* for 20 min. Finally, the obtained pellet (thylakoid membranes) was resuspended in a buffer containing 50 mM Tricine, pH 7.8, 70 mM sucrose and 5 mM $MgCl_2$. Aliquots at [Chl] = 3 mg ml^{-1} were placed in Eppendorf tubes and kept at −80 °C. The total chlorophyll content was calculated according to Porra [25].

2.8. Immobilization of thylakoid membranes on magnetic, amine polystyrene beads

Five hundred microliters of water suspension of magnetic aminopolystyrene beads (5%, w/v) were washed with 1.0 ml of PBS buffer (KH_2PO_4 1.8 mM, KCl 7 mM, NaCl 15 mM, Na_2HPO_4 10 mM, pH 7.2) for three times. The pellet was suspended in 1.0 ml of a glutaraldehyde solution (glutaraldehyde dissolved in PBS buffer to a final concentration of 10% (w/v)). The reaction was carried out with continuous mixing at 30 °C for 24 h. The microsphere suspension was washed three times with 1.0 ml of PBS buffer to remove the excess of glutaraldehyde. Pellet was resuspended in 0.5 ml of 15 mM MES, pH 6.5 (wash/coupling buffer). Then, 200 μl of thylakoid membranes ([chlorophyll] = 2.75 mg ml^{-1}) and 300 μl of 15 mM MES, pH 6.5, 70 mM sucrose, 5 mM $MgCl_2$ were added at 4 °C in the dark. To allow the coupling between the thylakoids and glutaraldehyde, the heterogeneous reaction was carried out at 4 °C for 2 h under continuous mixing. Finally, 100 μl of BSA (quenching solution) was added to 400 μl of immobilized thylakoids. The beads were washed three times with 1.0 ml of PBS buffer in order to remove the excess glutaraldehyde.

2.9. Fluorescence efficiency

The stability of the photosynthetic material, before and after immobilization in beads, was tested by measuring changes in fluorescence yield with a 650 nm light every 10 min after a dark period. Under exciting light, the fluorescence yield rapidly rises and then slowly decreases. The F_v/F_m ($F_m - F_0)/F_m$ parameter is the maximum quantum yield of PSII and reflects the potential quantum efficiency of PSII as a sensitive indicator of plant photosynthetic performance [26]. All fluorescence measurements were performed by using the Plant Efficiency Analyser (Hansatech Instruments Ltd., UK) at room temperature.

3. Results and discussion

3.1. Chemiluminescence batch assay for H_2O_2 produced by thylakoid membranes

Luminescent signal is seen with illuminated thylakoid membrane preparations. The signal is proportional to the light intensity

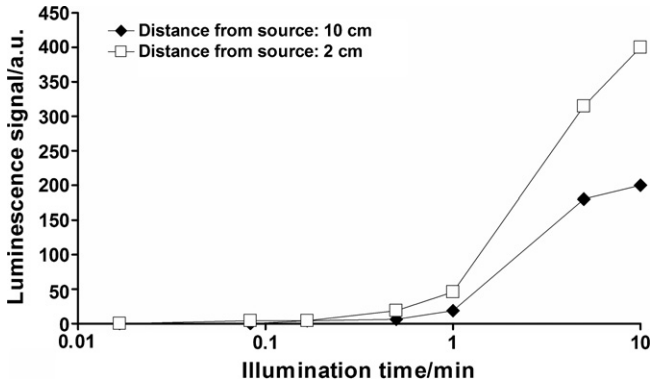

Fig. 2. Light produced by the chemiluminescence reaction of light-induced hydrogen peroxide from thylakoids illuminated for different times and distances. [HRP] = 20 U ml^{-1}, [luminol] = 100 μM, [chlorophyll] = 3.15 mg ml^{-1} with bench-top detector, at pH 8.5.

and duration. The effect of illumination time and distance on luminescence emission is shown in Fig. 2. The same signal is absent in the presence of DCPIP, hydroquinone or catalase, all of which act as either hydrogen peroxide inhibitors or mediators of the electron transport in the photosynthetic cycle. The presence of the latter group of additives means that the electrons never reach the hydrogen peroxide producing complex of the thylakoid, therefore, inhibiting any signal. At the same time, H_2O_2 spiked samples, i.e. thylakoid samples illuminated normally with added known hydrogen peroxide concentrations, give an additive luminescence response, suggesting that the hydrogen peroxide is not consumed by a catalase-type activity in the thylakoid preparation.

Both atrazine and diuron herbicides cause concentration-dependent inhibition of the chemiluminescent signal, and hence the H_2O_2 production by illuminated thylakoids (Fig. 3). The limits of detection (LODs) for these herbicides are: 3.0E−08 for atrazine and 1.0E−08 for diuron. An example of individual luminescence assay for diuron can be seen in Fig. 4.

3.2. Chemiluminescence batch assay for H_2O_2

For the detection of H_2O_2, four concentrations of luminol (10 mM, 1 mM, 100 μM and 50 μM) and four concentrations of HRP (50 U ml^{-1}, 10 U ml^{-1}, 5 U ml^{-1} and 1 U ml^{-1}) were tested in all possible combinations. At higher concentrations of H_2O_2 (mM

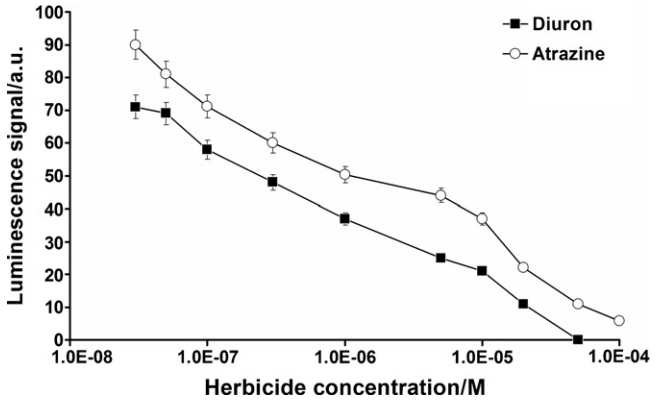

Fig. 3. Light produced by the chemiluminescence reaction of light-induced hydrogen peroxide from illuminated thylakoids in the presence of diuron or atrazine, with [HRP] = 20 U ml^{-1}, [luminol] = 100 μM, [chlorophyll] = 3.15 mg ml^{-1} with bench-top detector, at pH 8.5.

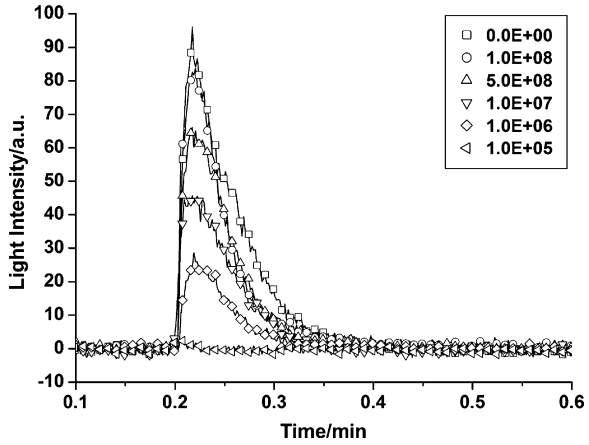

Fig. 4. Light produced by the chemiluminescence reaction of light-induced hydrogen peroxide from illuminated thylakoids in the presence of diuron, with [HRP] = 20 U ml^{-1}, [luminol] = 100 μM, [chlorophyll] = 3.15 mg ml^{-1} with bench-top detector, at pH 8.5.

region), the reaction acts like a typical "glow-type" chemiluminescence reaction expanding over minutes. At concentrations lower than micromolar, the result is typically a flash for less than 1 s. Having identified the peak light production of luminol at 431 nm, measurements of light intensity over time were performed, at a wavelength of 431 ± 20 nm. Such a large "window" was chosen in order to allow the maximum detectable light and, as the reaction is performed in the dark, any light detected would only be from the chemiluminescence reaction.

Calibration curves for all the previously mentioned combinations were plotted, and the LODs were calculated as 5σ values. Calibration curves were obtained for both the maximal light output and the integrated light output (data not shown). Among all the calibrations, the most sensitive detection was in the sample employing luminol 100 μM and HRP 5 U ml^{-1}, with an LOD: 1.34E−06 (data not shown).

3.3. Immobilization of thylakoid membranes on magnetic beads

Thylakoid membranes were immobilized on magnetic beads. Activity of immobilized thylakoid membranes was determined by fluorescence induction analysis. The F_v/F_m parameter represents the maximum quantum yield of PSII. The F_v/F_m parameter of the photosynthetic material, before the immobilization in beads, was 0.7. At 25 °C the observed half-life of thylakoid membranes activity was 53 h (Fig. 5). The data in Fig. 5 represent the average of three experiments with a relative standard devi-

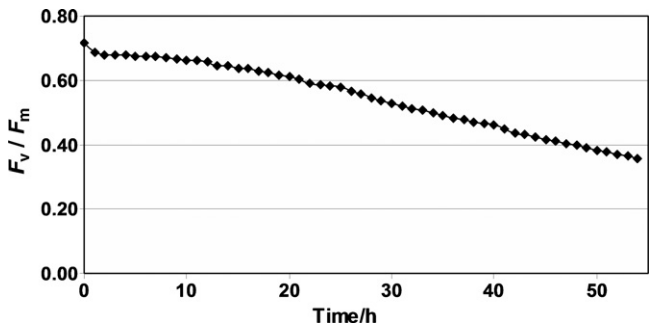

Fig. 5. Fluorescence induction analysis of immobilized thylakoid membranes on magnetic beads. Activity is monitored as F_v/F_m ratio in function of time in storage in the dark at 25 °C.

ation of 6.67. From a chlorophyll calculation it was found that the amount of the immobilized photosynthetic material was 0.3 μg (chlorophyll) mg^{-1} beads.

3.4. Immobilization of HRP on magnetic carboxyl-modified beads

HRP (HRP-1 and HRP-2) were immobilized in active form on magnetic carboxyl-modified beads. The amount necessary to achieve a complete activation of all the carboxyl groups on the beads was 100 nmol of EDC per 1 mg of beads. The optimized immobilization protocol included several washing steps (especially between the EDC and the HRP incubations) and different washing buffers. A step of washing the beads with sodium acetate buffer, 0.1 M, pH 4.0, after the HRP immobilization was included. To evaluate the effect of this, two different routes were taken, one with and one without the sodium acetate buffer washes. It should be noted that, for experimental integrity, the beads that were not washed with sodium acetate were instead washed with MES buffer, which was the buffer used for the washes throughout the experiment, thus bringing the total washes of the two routes to the same number. After the different washing steps, both bead lots were washed three times with the buffer of choice, in this case phosphate–citrate, which was the one favored by the ABTS assay protocol. The beads with the sodium acetate washes had slightly lower HRP activity than the ones washed only with MES. More specific, the rate of activity was lower for the sodium acetate (0.132 OD min^{-1}) compared to the MES buffer (0.150 OD min^{-1}) although the overall substrate conversion at the end of 10 min of the two bead lots was the same (data not shown). Throughout the immobilization procedure, all the in-between washes were also assayed with ABTS, in order to ensure that the multiple washing steps were actually washing out any excess of unbound HRP, and that the final product did not have any residual free HRP (data not shown).

Initially, an HRP with a lower hemin content and therefore activity was used (HRP-1). This HRP-1 was more prone to deactivation in various environments. A different HRP was then used (HRP-2) which is further purified and chemically stabilized to protect the primary amines and to maintain activity at low pH and higher temperature. Using HRP-1, 1 mg of beads had HRP activity equal to 0.1 purpurogallin units according to the ABTS assay (1 unit will oxidize 1 μmol of ABTS per minute at 25 °C, pH 5.0). With HRP-2, 1 mg of beads had activity equal to 0.375 units. A final alteration of the protocol was the washing out of the excess EDC, before the HRP was incubated with the beads. That was performed by following the protocol suggested by Mueller, whereby the excess unbound EDC would bind to HRP that would otherwise be immobilized to the beads [27]. The results with this alteration showed a dramatic increase in activity; 1 mg of beads had 1.2 units. According to the marked difference in the resulting activities between the two HRPs, as indicated by the ABTS assay, only HRP-2 was used in all the experiments shown either in free-form or immobilized.

The theoretical maximum number of HRP molecules bound to a bead can be calculated assuming a monolayer cubic close packing of HRP on the bead surface. The "monolayer coating" assumption is reasonable since the formation of a multilayer enzyme coating may take place, in fact, as a consequence of protein crosslinking. This requires the activation of the carboxyl groups of the enzyme; with the coating chemistry employed here however, only the carboxyl groups of the beads are activated, minimizing the possibility of a multilayer. In the case of the 3.2 μm of diameter beads, with the spherical diameter of HRP to be 4.6 nm, the theoretical maximum value of 441.67×10^3 HRP molecules per bead is obtained [28]. From further calculations, it was found that the amount needed for a monolayer for 3×10^9 beads, which is the amount of beads in 1 mg, would be 88 μg HRP, which is double the amount suggested by the protocol and used through the experiments. As for the immobilization experiments only half the amount of HRP was used, thus it can be assumed that only a partial monolayer has been achieved.

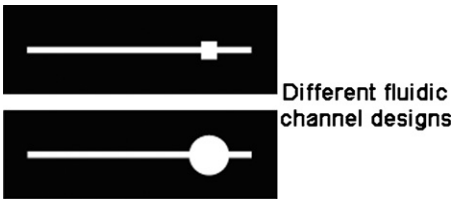

Fig. 6. The rubber channels employed in the experiments. The region used for the entrapment of the magnetic beads is the circular area (channel B) and the rectangular area (channel A).

Different storage buffers were also tested. The activity of free HRP prepared and stored for 1 week was greatly reduced while the activity of HRP immobilized on beads irrespective of the used buffer as a storage medium remained roughly unchanged.The activity of free HRP prepared and stored for 1 week

3.5. Flow assay for hydrogen peroxide with HRP immobilized on magnetic beads

Beads with immobilized HRP were magnetically entrapped in the fluidic device, in order to perform the H_2O_2 assay with luminol pre-mixed with the sample in flow. The light produced was detected with a portable detector.

Samples of different H_2O_2 and luminol concentrations were premixed just prior to the experiments with a T-mixer. Two different fluidic channels were used for the experiments, as shown in Fig. 6. The circular area of channel B is the area covered by the beads, and attracted by the magnet (area = 200 mm^2). For flow channel A, the rectangular area, 16 mm^2 covered by beads was smaller. In both cases 1 mg of beads was used, so the only difference was in the spreading of the beads and not the actual amount of HRP.

The light produced by the reaction was measured and the results are shown in Fig. 7. The channel B, allowing the beads to spread over a larger surface area, allowing for more HRP to be employed in the reaction, gave an increased response, as well as a lower LOD of 100 μM, compared to channel A, that had the beads stacked on a smaller area, as the HRP on the beads was not fully used, as a lot of the beads were covered by others, not allowing the luminol–H_2O_2 to reach them. Therefore, the key to the maximization of the possible chemical interaction between the H_2O_2, luminol and HRP lied in the ability to spread out the HRP bound on the beads in a two-dimensional plane.

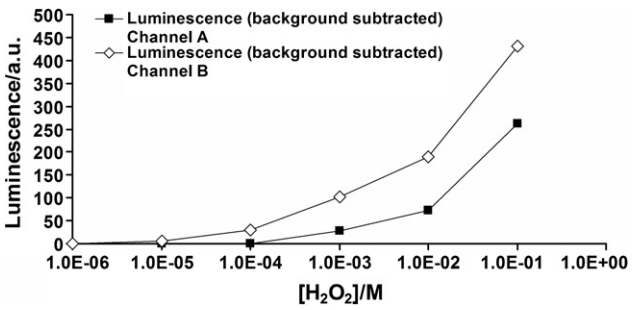

Fig. 7. Light output from different concentrations of H_2O_2. [luminol] = 10 mM at pH 8.5, with buffer 100 mM Tris–HCl. The HRP was immobilized on beads and 1 mg of beads were accommodated in two different channels. In channel A the beads were immobilized on area of 16 mm^2, in channel B they employed a surface area of 200 mm^2.

4. Conclusions

There is a growing interest for the generation of rapid, inexpensive assays to screen for the presence of herbicides. In this work, we have developed photosystem II-based optical microfluidic sensor for herbicide detection. The sensor is easy to handle and sensitive enough to provide preliminary environmental analysis screening the samples that require more detailed laboratory analyses.

The production of hydrogen peroxide by thylakoid membranes extracted from higher plants was investigated and detected under illumination with concentrations increasing in a time- and light intensity-dependent manner. The presence of herbicides in the thylakoid samples reduces the hydrogen peroxide measured in a concentration-dependent manner.

Thylakoid membranes and HRP were immobilized on magnetic beads, which were in turn magnetically immobilized in the device. The luminol–HRP–H_2O_2 chemiluminescence reaction was investigated in respect to H_2O_2 as the reactant of interest, and a calibration curve was obtained.

The integration of the two-step reaction has been achieved by designing and constructing a microfluidic device, which consists of a flow channel constructed of machined Perspex sandwiching a laser-cut elastomer spacer/flow channel in which regions of appropriate reagents are immobilized.

The sensor unit is able to perform the herbicide assays; optically stimulate photosystem II within the unit and detect the HRP-catalyzed chemiluminescence of luminol/hydrogen peroxide, which can be disrupted by herbicides.

The large number of approved active ingredients in agriculture (approximately 600 chemicals) makes difficult to obtain accurate and actual information on herbicide application in different countries. The real samples are present in a complex mixture containing active ingredients and their metabolites [29]. Our preliminary results indicate that use of real water matrix (control not containing herbicides) does not inhibit PSII activity and in some cases can induce an activation by 5–21% measured as oxygen evolution (data not shown). Therefore, the developed biosensor can be applied in real samples and better reflect the real physiological impact of active compounds because even low concentrations of pollutants affect living organisms by altering physiological processes. A deep study on real samples is in progress and will be presented in a future publication.

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