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Biomaterials based on photosynthetic membranes as potential sensors for herbicides

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ABSTRACT

In this study, ultrathin film multilayers of Photosystem II-enriched photosynthetic membranes (BBY) were prepared and immobilized on quartz substrates by means of a Layer by Layer procedure exploiting electrostatic interactions with poly(ethylenimine) as polyelectrolyte. The biomaterials thus obtained were characterized by means of optical techniques and Atomic Force Microscopy, highlighting the fact that the Layer by Layer approach allowed the BBYs to be immobilized with satisfactory results. The activity of these hybrid materials was evaluated by means of optical assays based on the Hill Reaction, indicating that the biosamples, which preserved about 65% of their original activity even ten weeks after preparation, were both stable and active. Furthermore, an investigation of the biochips' sensitivity to the herbicide terbutryn, as a model analyte, gave interesting results: inhibition of photosynthetic activity was observed at terbutryn concentrations higher than 10^{-7} M, thus evidencing the potential of such biomaterials in the environmental biosensor field.

1. Introduction

The importance of photosynthesis is common knowledge, but today new vistas are opening in which photosynthetic organisms could also be useful for biotechnological applications (Baldini et al., 2003; Croisetiere et al., 2001; Giardi and Pace, 2006; Koblìzek et al., 2002; Piletskaya et al., 1999). Photosynthetic organisms include not only higher plants and algae, but also photosynthetic bacteria; any of these organisms could potentially be used to obtain photosynthetic materials. Such biomaterials are of great interest to researchers working on biosensing and environmental applications, due to their high level of sensitivity to several classes of toxic species, ranging from herbicides to heavy metals.

The reasons for the high sensitivity of photosynthetic materials towards herbicides lie in the action mechanisms typical of many such toxic compounds. Two classes deserve to be cited, namely the triazine-derivatives and the phenylurea-derivatives, which are able to inhibit the electron flux through the Photosystem II (PSII) inside the thylakoid membrane, by linking to the Q_B site and substituting the electron carrier plastoquinone Q_B (Kidd and James, 1991). In addition, these herbicides are considered as dangerous substances for human health: both triazines and phenylureas are indicated as possibly carcinogenic compounds by EU official bodies and the EPA (Wackett et al., 2002), whose regulations currently fix the limits for pesticide concentration in drinking water at 0.1 μg/L for any individual pesticide, and 0.5 μg/L for total pesticides (Giardi and Pace, 2006).

Clearly, methods for monitoring the levels of such toxic species are very important, especially in water or ecosystems close to the areas where pesticides are, or have been, used. Conventionally, the analytical techniques employed to assess the level of contamination by pesticides have been gas and/or liquid chromatography, with traditional or mass spectrometry detectors. These techniques are powerful tools for detecting pesticides, even though they are difficult to design for rapid and in situ analyses. To this end, new unconventional methods, such as bioassays and biosensors, have been developed in recent years (Del Carlo and Compagnone, 2010).

There is a considerable body of data in the literature regarding the possibility of exploiting photosynthetic materials to detect the presence of toxic compounds by means of assays in solution. On the one hand, for example, it has been reported that chloroplasts and thylakoid membranes extracted from spinach leaves can be suspended in suitable aqueous buffers and then used as sensing tools for optical detection of atrazine, terbutryn and diuron, as well as of copper and mercury bivalent cations (Campàs et al., 2008; Ventrella et al., 2009, 2010). On the other hand, studies evaluating photosynthetic efficiency in the presence of toxic compounds, have employed Clark-type electrodes recording oxygen evolution rate changes (Koblìzek et al., 2002), or electrochemical techniques

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based on amperometry detecting electron flux variations through the thylakoid membranes (Croisetiere et al., 2001).

The use of assays in solution is one of the established approaches for applying photosynthetic materials in the environmental field, while another successful approach relies on the preparation of biomaterial-modified substrates, suitable for biosensing applications.

Recently, biosensors involving photosynthetic materials have been developed, and different responses have been obtained depending on: the type of photosynthetic materials (native or engineered bacterial/plant cells, membranes or proteins); the procedure for immobilizing the biocomponents (see below); the analytical detection technique adopted (amperometric, optical). Improvements in even one of the three above-mentioned factors, could clearly be of great interest in the field of herbicide detection.

In particular, to date, various methods for immobilizing photosynthetic materials have been employed, such as physisorption onto different substrates (Frense et al., 1998; Naessens et al., 2000), inclusion in a natural or synthetic gel matrix (Koblìzek et al., 2002) or chemical covalent binding/cross-linking, although the materials produced by these methods have so far tended to become less active over a period of time (Rouillon et al., 2006).

A very promising technique for the deposition of biological materials onto substrates is the Layer by Layer (LbL) method (Crespilho et al., 2006; Decher et al., 1992). It allows the adsorption of alternate anionic and cationic layers of polyelectrolytes and/or proteins (which are generally negatively charged) onto solid supports by exploiting electrostatic interactions. This technique has great potential, since it can be applied to different kinds of materials (Oliveira et al., 2002; Raposo et al., 1997). The versatility of this approach could allow the creation of several hetero-structures with specialized functions. In addition, the adsorption processes do not depend on the substrate size and topology, and increasing the number of deposited layers results in a linear increase in film thickness. Several examples are reported in the literature accounting for the immobilization of proteins by this procedure, although only few examples deal with photosynthetic materials and these examples focus on the photosynthetic bacteria Reaction Center (Kong et al., 1999; Mallardi et al., 2007).

In this study, for the first time, quartz (Qz) substrates were functionalized by PSII-enriched thylakoid membrane fractions (BBY) obtained from spinach leaves employing a LbL method and using poly(ethylenimine) (PEI) as polyelectrolyte to modify the substrate. The prepared hybrid inorganic-biomaterial was characterized by means of AFM and optical techniques in order to obtain information on the surface distribution and to evaluate the biocomponent's activity. In particular, the bioactivity of the hybrid materials was evaluated by adapting the Hill Reaction optical assay to solid substrates. The Hill assay is widely employed to establish the integrity of the electron transfer chain efficiency for photosynthetic samples in solution (Hill, 1937), but to the authors' knowledge this is the first time it has been applied to a solid substrate. The stability of the hybrid materials was evaluated in terms of ageing effects, and the response of the prepared biochips in the presence of the herbicide terbutryn, at different concentrations, was investigated in view of possible biosensoristic applications.

2. Materials and methods

2.1. Chemicals

Triton X-100, [2-N-morpholine]ethane-sulphonic acid (MES), NaCl, $CaCl_2$, $NaHCO_3$, 2,6-dichlorophenol indophenol (DCIP), terbutryn, isopropanol, methanol, hydrogen peroxide (30%), sulphuric acid (96%) and Poly(ethylenimine) (PEI, 50% (w/v) in H_2O, MW ~ 750000 Da) were purchased from Sigma–Aldrich (St. Louis, MO); acetone (99.8%) was purchased from Carlo Erba Reagents (Milano, Italy). Qz $100 \times 100 \times 1$ (mm) slides (fused silica) were purchased from EOT (Polcenigo-Pordenone, Italy) and small slides were cut with dimensions of about 30×10 (mm) for the experiments reported in this paper.

2.2. Isolation of PSII enriched membrane fractions

BBY membranes were isolated from market spinach leaves following Hankamer's procedure (Berthold et al., 1981; Hankamer et al., 1997). The concentration of the BBY samples was quantified as chlorophyll concentration, in mg mL^{-1} (Arnon, 1949). The photosynthetic materials were stored at −80 °C after freezing in liquid nitrogen.

2.3. Preparation of PEI–BBY multilayers

The immobilization of the BBYs onto Qz slides was achieved exploiting the LbL procedure. The Qz slide was previously treated with cleaning solvents for 10 min, respectively methanol, isopropanol and acetone. Afterwards the Qz substrate was negatively charged by dipping it for 4 min inside a "piranha" solution, i.e. oleum H_2SO_4/30% H_2O_2 in the ratio 3:1, then rinsed with Milli-Q water and dried by nitrogen stream. In the next step, a layer of positively charged PEI was adsorbed by 10 min incubation of the Qz slide in a stirred solution of 1.6 mg/ml PEI in Milli-Q water. A negatively charged BBY layer was then adsorbed onto the PEI-modified slide, by a 10 min incubation in a suspension of 0.015 mg/ml (chlorophyll concentration) BBY in an aqueous buffer (MNCB) containing MES (25 mM), NaCl (10 mM), $CaCl_2$ (5 mM) and $NaHCO_3$ (10 mM) at pH 6.5, under stirring conditions at 4 °C. The slide was subsequently rinsed with Milli-Q water and dried with nitrogen stream. In such a way, the first BBY layer, called "layer 1", was immobilized and further layers were obtained by repeating the steps reported above, until suitable PEI–BBY multilayers had been produced. The dried PEI–BBY multilayers on Qz were stored at 4 °C.

2.4. Absorption measurements

UV–vis absorption spectra were recorded using a Varian CARY 5000 spectrophotometer (Varian, Palo Alto, USA). For optical characterization of the biosamples in aqueous suspension, 1 cm path length quartz cells were used, while for measurements on the biomodified Qz chips, a suitable support was employed.

2.5. Photochemical assays

In this study, the electron transfer efficiency was determined by means of the Hill Reaction optical assay, i.e. DCIP photoreduction by an electron donor (in the case of BBYs, water) catalyzed by the biomaterials studied (Hill, 1937; Ventrella et al., 2009). Absorbance measurements were carried out at 600 nm. A set of four 13 W fluorescent tubes (Osram, Munich, Germany), each producing basic cool white light, was used to induce DCIP reduction in saturating conditions; the photon flux density of the white light was 880 μmol m^{-2} s^{-1}. This assay was employed not only for the analysis of the BBY suspensions, but also for that of the BBY modified Qz supports; in both cases DCIP was 60 μM and MNCB buffer was used.

2.6. Atomic Force Microscopy (AFM) characterization

Height mode AFM investigations were performed on the biomodified substrates in air, at room temperature, by means of

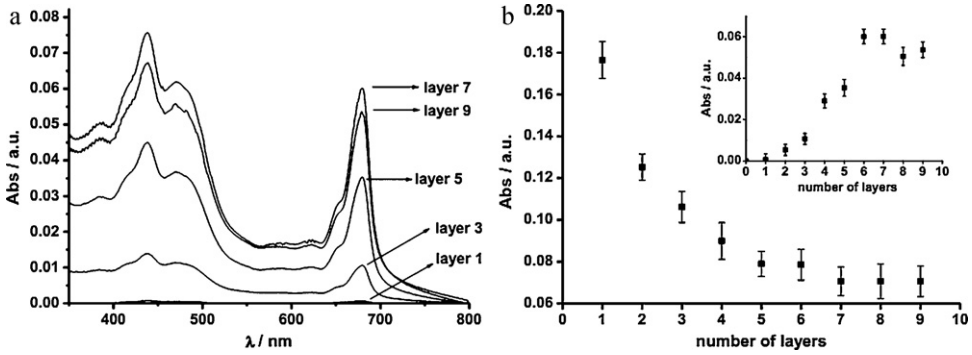

Fig. 1. Characterization of PEI–BBY multilayers by UV–vis absorption spectroscopy. Panel "a": Spectra of BBYs immobilized onto Qz at increasing numbers of layers. Panel "b": absorption peaks at 680 nm for the BBY solution used for the immobilization, as a function of the number of layers. Inset: absorption peaks at 680 nm of immobilized BBYs as a function of the number of layers. Reported data represent mean values ± standard deviations obtained from three replicates.

a PSIA XE-100 SPM system in AFM mode and cantilevers with silicon nitride tips were used, operating in non-contact mode at a 1 Hz scan rate.

A silicon SPM sensor for non-contact AFM (PSIA), having a force constant of 42 N m^{-1} and a resonance frequency of 320 KHz was used.

AFM images were processed by using a XEI Program to flatten the topographic micrographs, and remove the slope and curvature artefacts produced by the scanning process.

3. Results and discussion

3.1. Preparation and characterization of PEI–BBY multilayers

As reported in Section 2, following the LbL procedure, PEI–BBY multilayers were assembled onto Qz substrates; the support material was chosen in function of the analytic technique used to monitor the immobilization process and the activity of the biomaterials deposited. Indeed, in this work, the hybrid materials were analysed step by step, by means of UV–vis absorption in order to verify the effective loading of the biocomponent, rich in chlorophyll pigments.

In the UV–vis absorption spectra (Fig. 1a), the typical absorption bands of BBY at about 680 and 438 nm (corresponding to chlorophyll *a*) and the shoulders in the range of 450–550 nm (due to carotenoids and luteine) are clearly visible; it should be noted that PEI is transparent in the examined wavelength range. It can be seen from Fig. 1 that increasing the number of layers increases the absorption signal intensity, suggesting a higher quantitative loading of the biocomponent.

Nevertheless, an excessive layer number (higher than 6–7), was found to produce no further increment in the absorption bands, as is more easily observable in the inset of Fig. 1b, where the absorption peak values of PEI/BBY multilayers at 680 nm are reported as a function of the BBY layer number. These observations were confirmed by the UV–vis spectra of the BBY suspension employed for transferring the biosamples onto the substrate (Fig. 1b). Therefore 6–7-layers can be considered as the optimal number to obtain the immobilization of the greatest amount of BBYs. This was found to be sufficient to produce intense optical signals.

Further confirmation of the immobilization of the BBY membrane fragments was achieved by Attenuated Total Reflectance-Fourier Transform IR Spectroscopy (ATR-FTIR). The ATR-FTIR spectra of the PEI–BBY multilayers (see Supporting information) showed the Amide I and Amide II absorption bands at 1653 cm^{-1} and 1541 cm^{-1} respectively, that are typical of protein structures, as well as a band at 1738 cm^{-1}, indicating the presence of lipid structures.

The topography of BBYs on the biomodified supports was investigated by non-contact mode AFM. An image taken of a PEI–BBY 7-layer modified slide, obtained on 5 μm area, is shown in Fig. 2a, together with the corresponding height profiles (Fig. 2b). The patchy and dense distribution of structures, observed even at 40 μm (images not shown), can be safely attributed to BBY samples since no comparable signals were observed for Qz supports modified with only PEI (see Supporting information).

As reported in the line profile (Fig. 2b), the *z*-axis dimensions of these structures are approximately 20 nm, 40 nm, even reaching 80 nm, as identified by the increasing brightness in Fig. 2a. Such height values are in good agreement with the size of the stacked grana fragments of our biosamples. Even though it is not possible to evaluate accurately the lateral dimensions, we can assume the latter are within a few hundred nanometres, in accordance with the average diameter of grana disks, reported to be in the range of 350–600 nm (Arvidsson and Sundby, 1999; Kirchhoff et al., 2008; Staehelin and Van der Staay, 1996).

Interestingly, a 3D magnification of a 2 μm area (Fig. 2c) shows steep increases in height to different plateaus. According to the literature, a value of about 10–11 nm could be expected for a single grana fragment (i.e. a single bilayer fragment), and most BBY membranes consist of a stack of two grana disks; therefore, the height levels observed here could represent a stack of two, four and eight grana fragments, respectively (Nield et al., 2002; Kirchhoff et al., 2008). The variability in height observed is certainly due to the different degree of fragmentation of grana thylakoids occurring during the BBY preparation.

3.2. Functionality and stability of the immobilized BBYs

Once the PEI–BBY multilayers had been prepared, measurements were carried out to test whether BBY activity was preserved by observing the electron transfer efficiency through a Hill Reaction based optical assay (see Section 2).

This method, as mentioned above, is usually employed in solution, and has been adapted here, for the first time to solid biofunctionalized substrates.

The decrease in absorbance intensity at 600 nm for a solution containing oxidized DCIP and a freshly prepared 7-layer PEI–BBY biochip showed that the biochip was active after preparation, since the immobilized BBY layers were found to be capable of performing DCIP reduction. The dependency of the "specific electron transfer efficiency" for PEI/BBY multilayers on the number of deposited layers was verified; the parameter indicated as "specific electron transfer efficiency" was calculated as the ratio between the absorbance change at 600 nm (from the Hill assay) and the absorbance of the biochip at 680 nm, in order to exclude any effect

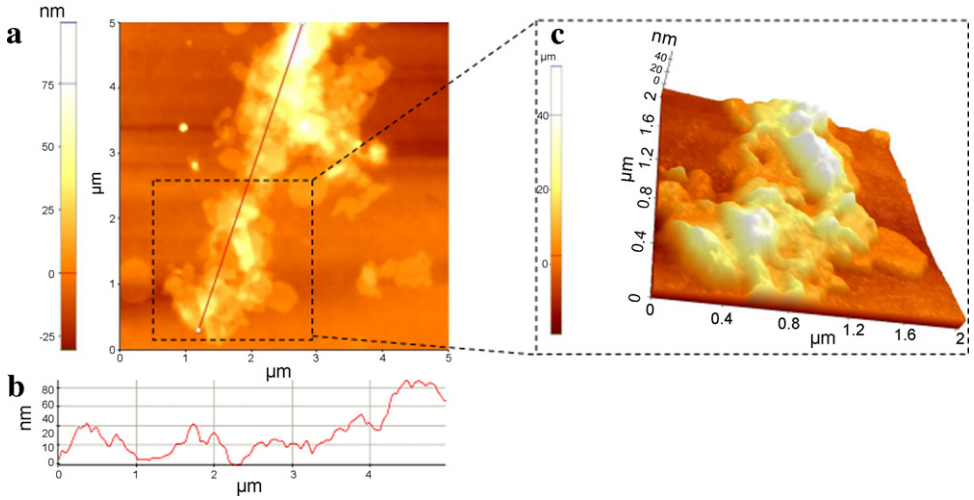

Fig. 2. Morphological characterization. Panel "a": AFM image of the BBY multilayers (7-layers) at a resolution of 5 μm. Panel "b": height profile corresponding to the section indicated by the line in panel "a". Panel "c": 3D magnification of a 2 μm area.

on the chip responses due to possible small variations of absorbance (i.e. of amount of immobilized biomaterial) in the different biochips used for this experiment. As result, no remarkable activity was found until layer 4 was deposited, while for a higher number of layers, the activity was found to increase as a function of the amount of immobilized BBYs (Fig. 3). It can be deduced that the optimal number for deposited layers is 7, since for higher numbers of layers no increment in the absorption signals (i.e. amount of immobilized biomaterial) was observed.

In order to evaluate the loss of activity produced by the BBY multilayer preparation with respect to BBY solutions before immobilization, Hill Reaction optical assays were performed on a BBY suspension characterized by the same absorbance as the immobilized samples. The results showed that after immobilization the activity was preserved at 85–90%.

The stability of the BBY multilayer preparation was then evaluated by comparing the activities of chips stored for 1, 4 and 10 weeks with freshly prepared chips. The results, reported as percentages of the residue electron transfer efficiency, are shown in Fig. 4.

It can be seen that the biochips showed a high percentage of activity during the first week, and interestingly a considerable activity (about 65%) was observed even 10 weeks after preparation. It is worth noting that this stability value is considerably higher than those obtained immobilizing photosynthetic materials by means of other techniques (Rouillon et al., 2006). Moreover, it should be highlighted that not only did the general aspect of the PEI–BBY multilayer absorbance spectrum not change throughout the period of observation, but a decrease in the maximum intensity values was observed only during the first week. Afterwards, no significant change in the absorbance values was noticed, evidence that the immobilization procedure here reported allows the preparation of relatively stable samples without an excessive loss of biomaterial during storage period.

3.3. Sensing responses to a model analyte

The BBY modified Qz supports were used to test their ability to sense the presence of herbicides belonging to the class of triazine-derivatives; specifically, for these tests the herbicide terbutryn was employed.

In order to find the optimal conditions for the tests, three BBY chips were flooded for different incubation periods, i.e. for 10, 30 and 60 min, respectively, in suitable beakers containing terbutryn solutions at the same concentration; after the incubation with terbutryn, the electron transfer efficiency for each chip was evaluated

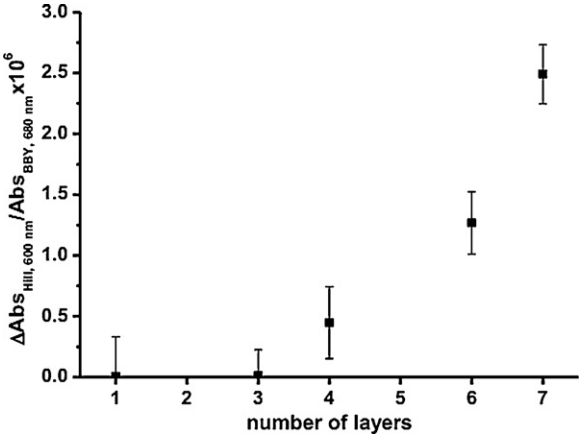

Fig. 3. Effect of the number of layers on PEI–BBY multilayer activity. Dependency of the "specific electron transfer efficiency" of a PEI–BBY multilayer slide on the number of immobilized layers, after 30 min illumination assay. Reported data represent mean values ± standard deviations obtained from three replicates.

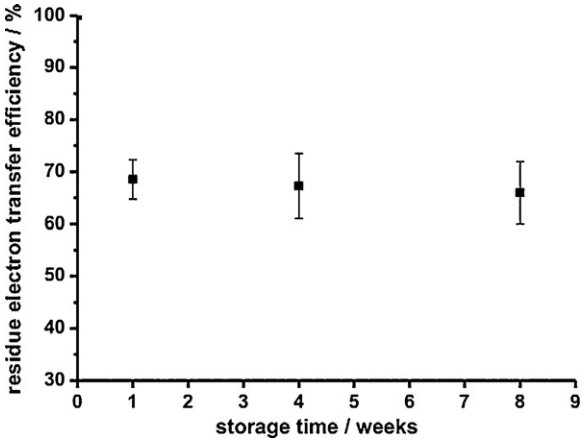

Fig. 4. Stability of the PEI–BBY 7-layer slides. Residue electron transfer efficiency, at different sampling times after preparation, calculated as a percentage of the value obtained from freshly prepared PEI–BBY 7-layer slides. Reported data represent mean values ± standard deviations obtained from three replicates.

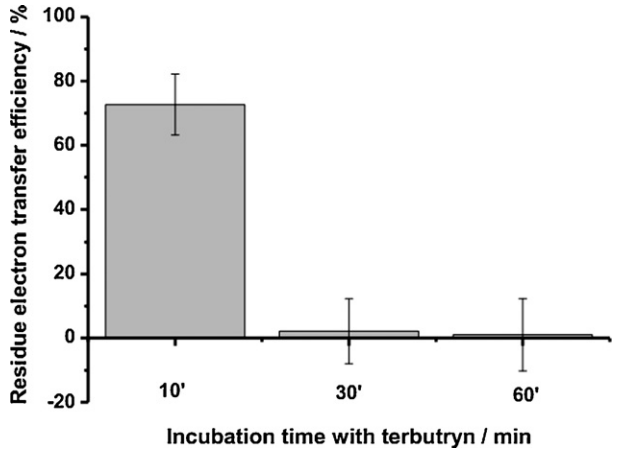

Fig. 5. Effect of incubation time with terbutryn. Residue electron transfer efficiency of a PEI–BBY 7-layer slide immersed in a solution containing 10^{-4} M terbutryn, as a function of incubation time, calculated as a percentage of the value obtained from a PEI–BBY 7-layer slide not treated with terbutryn. Reported data represent mean values ± standard deviations obtained from three replicates.

by means of the Hill Reaction optical assay, and compared with that of a BBY chip which had not been incubated with terbutryn.

The relevant activity was plotted in a graph (see Fig. 5) as a function of incubation time with the herbicide: it can be clearly observed that an incubation time of 10 min did not produce any remarkable inhibitory effect onto the BBY chips, while 30 min were found to be sufficient for a complete inhibition of the electron transfer process.

Following this, the dependency of the BBY chips' response on terbutryn concentration was evaluated. The graph in Fig. 6 shows the "specific electron transfer efficiency" of BBY chips plotted against the logarithm of the terbutryn concentration, in the range of 10^{-10}–10^{-4} M.

As can be easily noted, the higher the herbicide concentration, the more PEI–BBY activity was inhibited. The chips allowed terbutryn to be detected at concentrations higher than 1.58×10^{-7} M.

This value was obtained as follows: let y and x be considered as the dependent and independent variables in Fig. 6; after recording the assay responses for five blank replicates, the average blank value (y_B) was found to be equal to 2.41 (with the units reported in Fig. 6). The not-detectable interval was considered as $y_B \pm 3S_B$, where S_B represents the contribution of the blank standard deviation, equal to 0.13 for the blank here in. In such a way the dependent variable value of the detection limit (y_{lod}) was found to be equal to 2.02. The linear regression obtained by fitting the four experimental points relevant to concentrations 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M (10^{-10} M was not considered since clearly out of the dynamic range of the graph) was used to find $x_{lod} = -6.80$, that is the logarithm of the minimum concentration detectable, and so the relevant detection limit was found to be 1.58×10^{-7} M.

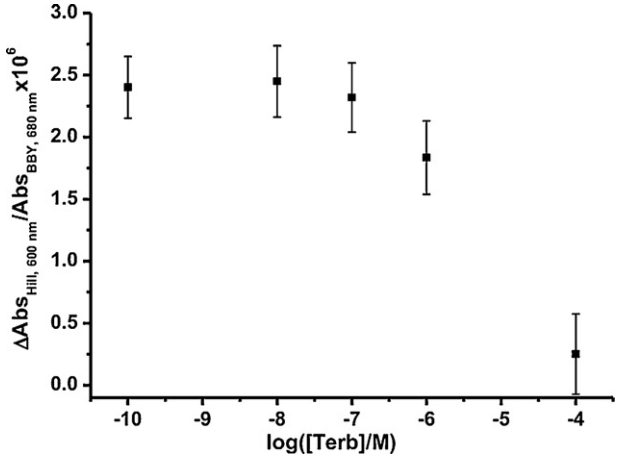

Fig. 6. Effect of terbutryn concentration. Dependency of the "specific electron transfer efficiency" of a PEI–BBY 7-layer slide on the terbutryn concentration, after a 30 min incubation. Reported data represent mean values ± standard deviations obtained from three replicates.

Although this detection limit value is higher than those obtained by conventional methods or innovative methods based on genetically engineered photosynthetic proteins (Giardi et al., 2009), it is comparable to those obtained by non-conventional techniques and sensors involving native photosynthetic materials (Del Carlo and Compagnone, 2010).

4. Conclusions

In this study, the LbL procedure was used for the immobilization of BBYs onto Qz slides, obtaining biomaterials with different numbers of layers; PEI was employed as polyelectrolyte. The PEI–BBY multilayers were found to be well covered and at the same time the biological activities of the BBYs used, estimated by means of optical assays as electron transfer efficiencies were found to remain high immediately after immobilization. In addition, the biomaterials were found to preserve remarkably high activity values (about 65% of their original values) even ten weeks after preparation. These results are of considerable interest since they are, to our knowledge, the first examples of LbL performed on thylakoid biomembranes. The use of membranes, instead of proteins, could prove very useful in numerous biosensor applications, since the purification of membrane proteins and enzymes, i.e. the sensing part of a biosensor, not only requires great loss of time and money, but is also more difficult to perform, compared with the extraction of the membranes containing such proteins. The PEI–BBY multilayers were tested as a tool for sensing herbicides belonging to the class of triazine-derivatives, and an inhibition optical assay was performed by incubating the biomaterial, in the presence of terbutryn, at different concentrations. Interesting responses were found at terbutryn concentrations higher than 10^{-7} M, a value that is still higher than the official legal limits for this herbicide in drinking water, but in competition with the values detectable by other non-conventional methods based on biosensors that exploit native photosynthetic materials.

The PEI–BBY multilayers could even be useful for laboratories not equipped with electrochemical facilities, but having spectrophotometric instruments, since rapid optical (colorimetric) assays could be performed with the BBY biochips. Furthermore, this study could provide useful information to researchers wishing to perform immobilization of other, different, "protein enriched biomembranes" by means of the LbL technique.

In future, the authors intend to evaluate parameters that could improve the quality of BBY immobilization and the detection limit value, in order to obtain more resistant, stable and sensitive biochips, for instance using photosynthetic membranes from genetically engineered organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.05.043.

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