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COMMUNICATION

A whole-cell amperometric herbicide biosensor based on magnetically functionalised microalgae and screen-printed electrodes†

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We report the fabrication of an amperometric whole-cell herbicide biosensor based on magnetic retention of living cells functionalised with magnetic nanoparticles (MNPs) on the surface of a screen-printed electrode. We demonstrate that *Chlorella pyrenoidosa* microalgae cells coated with biocompatible MNPs and retained on the electrode with a permanent magnet act as a sensing element for the fast detection of herbicides. The magnetic functionalisation does not affect the viability and photosynthesis activity-mediated triazine herbicide recognition in microalgae. The current of ferricyanide ion was recorded during alternating illumination periods and biosensor fabricated was used to detect atrazine (from 0.9 to 74 μM) and propazine (from 0.6 to 120 μM) (the limits of detection 0.7 and 0.4 μM, respectively). We believe that the methodology presented here can be widely used in fabrication of a number of whole cell biosensors since it allows for efficient and reversible cells immobilisation and does not affect the cellular metabolism.

Introduction

Whole-cell biosensors are regarded as powerful toxicity screening tools, mostly because the use of living cells as bioreporters provides effective and fast assessment of the overall toxicity of industrial and fresh water samples.[1] In recent years, whole-cell biosensors and microfluidic devices based on bacteria,[2] yeast[3] and microalgae[4] cells have been reported. One of the major challenges in the development of either electrochemical sensors or microfluidic chips based on living cells is to retain the viable cells on the surface of a transducer. The conventional immobilisation techniques utilise the covalent immobilisation of cells in a protein matrix cross-linked with glutaraldehyde[5] or the entrapment of cells in viscous membranes using the screen-printed technique,[5] in addition, the sol–gel technology[7] and the electrostatic adsorption of cells into conducting polymer matrices[8] have been used. Some of these methods are elaborate and time-consuming, whereas the others may potentially damage the viability of the cells. In addition, most of the existing immobilisation techniques irreversibly bind the cells to the surfaces, therefore it is not possible to replace or remove the immobilised cells to produce reusable biosensors. The straightforward replacement of the cells is especially important in the fabrication of biosensors for toxicity measurements which require substitution of the toxicant-exposed cells after each measurement. To overcome these drawbacks, the magnetic retention of yeast cells functionalised with magnetic nanoparticles (MNPs) in microfluidic devices and biosensors has been recently proposed.[9] In a similar way, microalgae cells were successfully functionalised with biocompatible polymer-stabilised MNPs,[10] suggesting the application of magnetic retention in algae biosensors.

Herein we report for the first time a simple biosensing methodology based on magnetically functionalised microalgae cells combined with an electrochemical screen-printed electrode. Green photosynthetic unicellular microalgae are currently used in biosensors and bioassays for aquatic risk assessment,[5a,6,11] cytotoxicity[1] and heavy metals[5b] screening, and detection of toxic gases[12] and herbicides.[13] The interest in rapid and inexpensive detection of toxicants (*i.e.* herbicides) using biosensors and biochips[14] is constantly increasing because the pollution of drinking water is a problem of paramount importance, especially in the developing countries. Microalgae are intrinsically sensitive to pollutants[5b,13,15] which affect the photosystem II (PSII).[16] This allows using either electrochemical[1] or fiber optic[4,7] biosensors with immobilised microalgae to detect the toxicant-induced inhibition of photosynthesis. Nevertheless, microalgae-based whole cell biosensors suffer from the inconvenient irreversible cell immobilisation techniques. Functionalisation of microbial cells with polyelectrolytes and nanomaterials has been reported previously.[17] Here we present a novel biosensor which takes advantage of the magnetic retention of the viable microalgae directly magnetized with polymer-stabilised biocompatible MNPs. MNP-functionalised cells were immobilised using a permanent mini-magnet positioned near the screen-printed electrode. As a proof-of-principle, we performed a quantitative assay of triazine herbicides to show that the biosensing methodology based on the magnetic retention of MNP-functionalised cells is effective in toxicity screening.

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Furthermore, we suppose that the methodology reported here may find a wider area of application in whole-cell biosensors.

Materials and methods

Reagents, cell culture and media

Poly(allylamine hydrochloride) (PAH, M_w *ca.* 15 000 Da), iron(II) and iron(III) chlorides (99%) were purchased from Sigma-Aldrich. Herbicides atrazine (2-chloro-4-(ethylamine)-6-(isopropylamine)-*s*-triazine) and propazine (6-chloro-*N*,*N*′-bis(1-methylethyl)-2,4-diamine)-*s*-triazine) were kindly provided by the regional Agriculture Commission (Kazan, Republic of Tatarstan). The stock solutions of herbicides in acetone (HPLC grade) were further diluted with water. All other reagents were of analytical grade and used without further purification. MilliQ water purified by reverse osmosis was used throughout. *Chlorella pyrenoidosa* microalgae cells (KFU culture collection) were harvested as described previously.[10]

Magnetic functionalisation of microalgae

MNPs were prepared as described previously.[9] After stabilising with PAH, the MNPs were separated from the excess of PAH by centrifugation, washed and redispersed in MilliQ water and sonicated. Finally, the MNPs dispersion was filtered with 0.22 μm Millipore syringe filter. The average diameter of MNPs was ~15 nm. The magnetic functionalisation of microalgae was performed as described elsewhere.[10] Briefly, 300 μL of *C. pyrenoidosa* cells (at 20 mg mL^{-1} wet weight concentration) were introduced into 1 mL of aqueous PAH-stabilised MNPs (0.4 mg mL^{-1}) and vortexed for 10 min. Next, the MNP-functionalised *C. pyrenoidosa* cells were separated from the unbound MNPs using a permanent neodymium–iron–boron (NdFeB) magnet and washed several times with MilliQ water. The viability of the magnetically functionalised cells was tested examining chlorophyll autofluorescence.[5b]

Characterisation techniques

Optical and epifluorescence microscopy images were obtained using a Carl Zeiss AxioScope microscope (objectives: 5×, NA 0.13; 40×, NA 0.65; 100×, NA 1.3) equipped with an AxioCam MRc5 CCD camera. The transmission electron microscopy (TEM) images of the thin-sectioned MNP-functionalised microalgae were obtained using a Jeol 1200 EX microscope (Japan) operating at 80 kV. The cells were fixed, dehydrated, embedded into resin and then thin sections were cut using a LKB ultramicrotome and mounted on copper grids. Finally, the samples were stained with 2% aqueous uranyl acetate and lead citrate. ζ-Potential of the MNPs and cells was determined using a Malvern Zetasizer Nano ZS instrument.

Fluorescence intensity measurements

Fluorescence microscopy images of MNP-functionalised microalgae were obtained before and after the 1 h treatment with 1.14×10^{-5} M aqueous herbicide solutions. For fluorescence intensity measurements the camera exposure time was set to 50 ms, and the images were recorded using AxioVision software. The cell greyscale intensities ($n > 15$) were analysed with ImageJ software (http://rsbweb.nih.gov/ij).

Amperometric measurements

Biosensor setup. Screen-printed electrodes 8 × 40 mm were fabricated using a DEK 248 printer on a polycarbonate support. Silver tracks were first printed and cured at 75 °C, followed by deposition of the curable graphite paste onto the silver tracks to form the counter and working electrodes. Then, Ag/AgCl ink was printed onto the area of the pseudo-reference electrode. The insulating resistive layer was deposited and dried at 90 °C. Graphite paste was purchased from Gwent Electronic Materials Ltd. and other printing materials from Delta-Pasta. After drying, the electrodes had a resistance of 140–180 Ω between the electrical contact point and working electrode area. The magnetic retention biosensor setup was fabricated by connecting the screen-printed electrode to a 4 mm thick tetrafluoroethylene plate. A 2 mm diameter NdFeB cylindrical magnet was inserted into the cavity below the working electrode area to produce the strong magnetic field in the vicinity of the electrode.

Immobilization of magnetically functionalised microalgae on screen-printed electrodes and electrochemical measurements. The detailed description of preliminary experiments performed in order to establish the most optimal experimental conditions is given in the ESI†. To immobilize the cells, 100 μL of magnetically functionalised algae were first mixed with 50 μL of 0.02 M $K_3Fe(CN)_6$ and 50 μL of 0.2 M Na_2SO_4. Then, 50 μL of the mixture were dropped onto the working area of the screen-printed electrode to overlap the insulating gaps between the electrodes. The drop was covered with a plastic opaque lid of an appropriate diameter. The electrode was polarized at −600 mV *vs.* pseudo-Ag/AgCl for 600 s. Then the lid was periodically removed to establish the equal periods of daylight illumination and darkness, 30 s each.

The difference in the extreme current values recorded in light and dark periods (as explained in more detail in the ESI†) was measured as the biosensor signal. To improve reproducibility, the temporal trend of the minimal currents was subtracted from the original current–time curve prior to signal calculation. The standard deviation of the signal recorded for the same volume of the microalgae suspension did not exceed 5%.

The influence of triazine herbicides on the signal was quantified as follows. First, the stable signal was obtained by alternation of light and dark periods as described above. Then, an aliquot of 1–2 μL of herbicide solution was introduced into the drop of microalgae onto the electrode surface and the alternation of light and dark periods was continued until stabilization of the reduced current value. The inhibition $I\%$ (1) caused by herbicides was calculated from the signals measured before (I_0) and after (I) the herbicide addition ($I\% = ((I_0 - I)/I_0) \times 100\%$. All the measurements were performed in six replications and mean ± standard deviation (P-value 0.10) was calculated.

Results and discussion

In this communication, we demonstrate the utilisation of magnetically functionalised *C. pyrenoidosa* cells in the simple electrochemical biosensor based on a screen-printed electrode as a signal transducer. To magnetise negatively charged algae cells (ζ potential around −18 mV), we used positively charged biocompatible PAH stabilised 15 nm MNPs (ζ potential around +48 mV) which provide a very fast direct deposition onto the cells (typically < 20 min). The magnet-facilitated

spatial manipulations can be easily performed with the functionalised cells. After the deposition of the MNPs onto the surface of the cells, we examined the TEM images and observed a thin (~90 nm) MNPs layer deposited on the cell walls (Fig. 1a and b). The viability of the MNP-coated cells was confirmed by fluorescence microscopy. Fig. 1c demonstrates a merged bright field and fluorescence microscopy image of the viable microalgae. We found that the viability of the cells was not affected at least during one week after functionalisation.

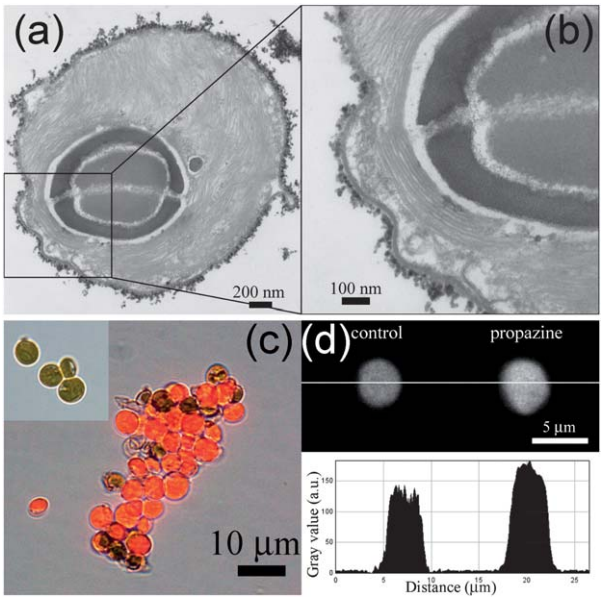

Fig. 1 (a) and (b) TEM images of *C. pyrenoidosa* cells coated with MNPs at lower and higher magnifications; (c) a merged white light and fluorescence microscopy image of viable magnetically functionalised *C. pyrenoidosa* cells (the inset shows a white light image of the cells); (d) a fluorescence microscopy image (top) and a corresponding plot profile taken along the line (bottom) of the control and 10–5 M propazine-treated magnetically functionalised *C. pyrenoidosa* cells demonstrating the autofluorescence induction in the herbicide-treated cell.

Further, autofluorescence induction was measured to confirm that the magnetic functionalisation did not affect the interaction of the algal PSII with triazine herbicides used as model toxicants in this study. Triazines disrupt the electron flow in PSII, therefore the excitation energy is re-emitted as autofluorescence.[18] The increase in autofluorescence induced by PSII herbicides is currently utilised in fiber optic biosensors based on microalgae[13] and isolated PSII.[19] The MNP-functionalised cells were incubated for 1 h in 1.14×10^{-5} propazine. Then the mean fluorescence intensity (MFI) values were quantified in single cells using 8 bit greyscale images. We found that the MFI values for intact and propazine-treated magnetised microalgae cells were 98 ± 11 a.u. and 151 ± 13 a.u., respectively. The typical autofluorescence greyscale images of the intact and the propazine-treated single cells along with the grey value plot are given in Fig. 1d. The similar results were obtained for atrazine (data not shown). The increase in MFI of propazine-treated magnetic microalgae demonstrates the autofluorescence induction due to the interaction of propazine with PSII, which suggests that the MNPs do not affect the uptake of the herbicide by the cells, this results are in good agreement with the previous studies.[9,10]

Since the MNP-functionalised *C. pyrenoidosa* cells are viable and herbicide-responsive, they were used to facilitate the assembly of the cells in a biosensor setup based on a screen-printed electrode and a permanent magnet. The sketch of the biosensor proposed is given in Fig. 2a. The MNP-coated cells were mixed with the electron mediator ($K_3Fe(CN)_6$) and electrolyte (Na_2SO_4), then the drop of the cell suspension was placed onto the electrode. The cells are immediately assembled above the magnet (Fig. 2b).

Amperometric measurements (Fig. 3) were conducted at −600 mV where the difference between the background current and the signal indicating the photosynthetic activity of microalgae was maximal. No signals associated with oxygen reduction were observed because of the kinetic limitation of this electrode reaction. The illumination of MNP-functionalised microalgae magnetically retained on the

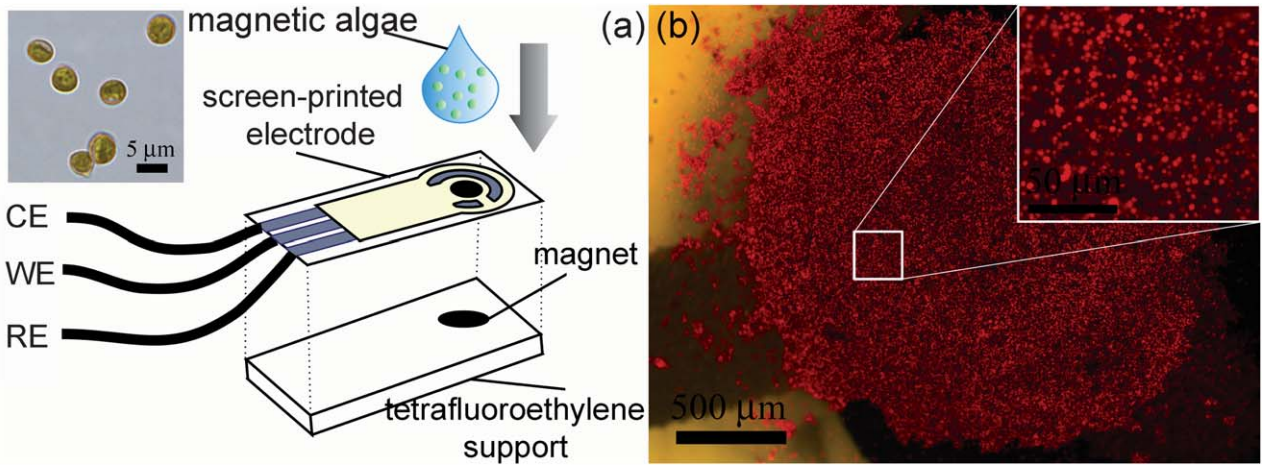

Fig. 2 (a) A sketch illustrating the biosensor setup based on a screen-printed electrode, a tetrafluoroethylene support with the embedded permanent magnet and magnetically functionalised microalgae cells. The connections legend: CE—counter electrode, WE—working electrode, and RE—reference electrode. The arrow indicates the order of assembly. The inset shows a typical optical microscopy image of the MNP-functionalised *C. pyrenoidosa* cells; (b) a typical epifluorescence microscopy image of the MNP-functionalised *C. pyrenoidosa* cells assembled above the permanent magnet positioned below the screen-printed electrode. The inset shows a higher magnification image of the cells.

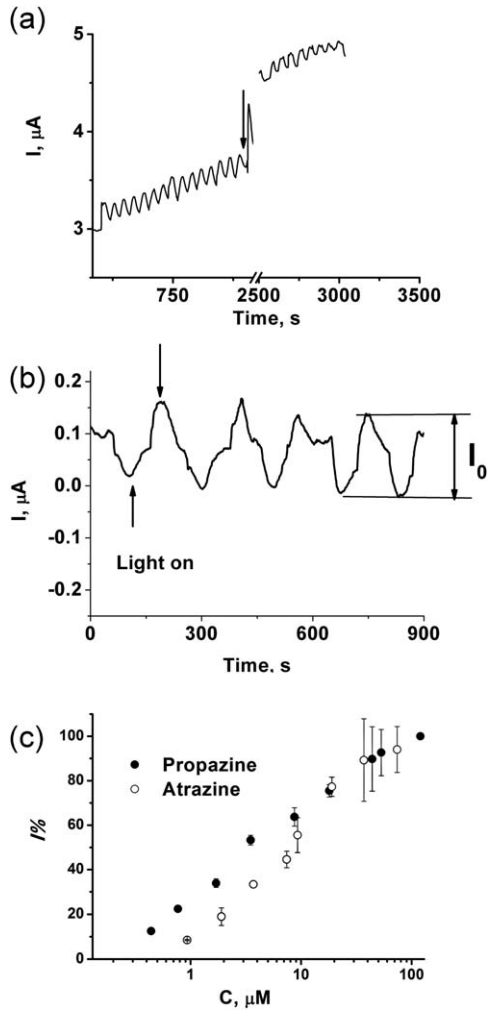

Fig. 3 (a) Cathodic current *vs.* time curve obtained with 50 μL drop of MNP-functionalised microalgae suspension magnetically retained on the screen-printed electrode (arrow indicates the addition of herbicide); (b) typical current curve after baseline subtraction; (c) calibration curves of propazine and atrazine, error bars correspond to the standard deviation of six replications.

screen-printed electrode resulted in a fast increase in the cathodic current due to the involvement of ferricyanide ions in the photosynthesis electron transduction chain. The reaction is fast enough and takes about 1–2 s of illumination for the remarkable changes in signal which became almost constant within further 5–15 min. For this reason, we decided to alter the light and dark periods. The stable oscillation of the cathodic current recorded at −600 mV was reached by 2–3 min and remained relatively constant for at least 30 min. The period of current stabilization within the cycle was about 15–20 s, *i.e.* somewhat longer than that reported for oxygen reduction by amperometric biosensor based on screen-printed electrode with LED illumination.[1] This can be due to a fairly faint daylight illumination of the electrode used here if compared to LED. The baseline subtraction (Fig. 3b) enables avoiding difficulties in the current shift calculation common for the regular drift of the current as shown in Fig. 3a. Among others, this approach suppresses interference related to non-regular distribution of herbicide after its addition and improves the reproducibility of the inhibition quantification results. To demonstrate the relationship between the photosynthetic activity and the signal observed, similar measurements with thermally inactivated algae cells modified with MNPs were performed. We found that just very small current oscillations were observed, not exceeding 2–3% of those obtained with living MNP-functionalised cells.

The microscopy observations suggest that all the cells are assembled on the electrode surface. However, we suppose that the electron transduction is most effective for the cells directly contacting the electrode. This conclusion is supported by a relatively smooth dependence of the signal on the cells concentration. Increasing the volume of the drop from 10 to 150 μL resulted in the signal increase 0.22 to 0.34 μA. Meanwhile, the standard deviation of the signal decreased from 30% for 5 μL portion to 7% for 30 μL and 6% for 150 μL. It is interesting that the absolute error was the same (0.02 μA) for the samples in volumes from 20 to 150 μL. The suppressed sensitivity of the response toward the volume of the drop (and, as a result, the concentration of the cells) placed on the electrode simplifies the measurement protocol and the estimation of the herbicide activity, especially in field conditions.

The inhibition of the biosensor signal increased with the concentration of the herbicide. The appropriate calibration graphs of atrazine and propazine are given in Fig. 3c. Triazine herbicides specifically fix to the quinone binding site of PSII which blocks the photosynthetic electron flow and the rate of current changes while illumination was switched off and on. Linear part of the curve corresponds to the concentration range from 0.9 to 74 μM for atrazine and from 0.6 to 120 μM for propazine. The appropriate limits of detection calculated from $s/n = 3$ ratio were found to be 0.7 and 0.4 μM, respectively. The increase in the standard deviation observed for rather high concentrations of the herbicides can be contributed to acetone, a herbicide stock solution solvent that could evaporate during the measurement or affect the solubility of the herbicide. No inhibitory effect of acetone itself was observed in the measurements without herbicides. The possibility to re-use the biosensor after its contact with the herbicide solution is limited by the drop volume. The more injections are made the less is the stability of the drop that tends to spread over the working area with partial losses of liquid and hence reducing reproducibility of the signal. Special experiments performed showed that the same electrode can be used for repeated deposition of several drops of the magnetically functionalised microalgae after the removal of the previous sample (data not shown). No changes in the current recorded and its sensitivity toward herbicide added for at least ten consecutive measurements have been observed.

Conclusions

To summarize, here we report a novel biosensor based on magnetic retention of MNP-functionalised microalgae on the surface of a screen-printed electrode. The sensitivity towards the triazine herbicides is close to that reported for the more complicated biosensor devices.[1,15] Meanwhile, the very simple design and measurement protocol provide the advantages of the approach proposed, together with the satisfactory reproducibility and no requirements to sample treatment (like thermostating or degassing). The removal of the magnetic field can be used to replace the biosensing elements. This might be very useful for the possible field application of the biosensor. We believe that the practical applications for the biosensors based on magnetized cells are not limited to

microalgae, but can be further extended to bacterial or fungal whole cell biosensors and microchips.

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