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Enzyme inhibition-based biosensor for the electrochemical detection of microcystins in natural blooms of cyanobacteria

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Abstract

An electrochemical biosensor for the detection of microcystin has been developed based on the inhibition of the protein phosphatase 2A (PP2A) by this cyanobacterial toxin. The enzyme has been immobilised by entrapment using a poly(vinyl alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP). Electrode supports and immobilisation conditions have been optimised by colorimetric assays, the highest immobilisation yields being obtained with screen-printed graphite electrodes and the 1:2 PP2A:PVA ratio. Catechyl monophosphate (CMP), α-naphthyl phosphate (α-NP) and 4-methylumbelliferyl phosphate (4-MUP) have been used as phosphorylated substrates to monitor the protein phosphatase activity by electrochemical methods, the former providing the highest chronoamperometric currents at appropriate working potentials (+450 mV *versus* Ag/AgCl). Incubation with standard microcystin solutions has demonstrated the inhibition of the immobilised enzyme, proportional to the toxin concentration. The standard inhibition curve has provided a 50% inhibition coefficient (IC_{50}) of 83 μg L^{-1}, a limit of detection (LOD; 35% inhibition) of 37 μg L^{-1}, and 100% inhibition at about 1000 μg L^{-1}. Real samples of cyanobacterial blooms from the Tarn River (Midi-Pyrénées, France) have been analysed using the developed amperometric biosensor and the toxin contents have been compared to those obtained by a conventional colorimetric protein phosphatase inhibition (PPI) assay and high-performance liquid chromatography (HPLC). The results clearly justify the use of the developed amperometric biosensor as screening method for microcystin detection.

Keywords: Microcystin (MC); Protein phosphatase 2A (PP2A); Poly(vinyl alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP) Entrapment; Inhibition; Catechyl monophosphate (CMP); Electrochemical detection

1. Introduction

Microcystins (MCs) are cyanobacterial toxins usually produced in freshwaters throughout the world. Cyanobacteria (blue-green algae) growth and toxin production are associated to several factors: (a) eutrophication, *i.e.* the presence of excessive nutrients coming from agricultural run-off, deforestation and urban pollution; (b) low flow regimes, with the corresponding long retention times; (c) high light intensity, necessary for the photosynthesis; (d) warm water temperatures, which explains why most blooms occur in summer; (e) the presence of trace metals [1,2]. MCs are cyclic heptapeptides, with five constant amino acids and two variable ones. More than 70 variants are known, showing variations in structure and toxicity [3,4]. Microcystin-LR (MC-LR) was the first MC chemically identified and it is known to be the most toxic and frequently found.

MCs are potent hepatotoxins. The peculiar hydrophobic Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) chain allows them to penetrate the hepatocytes, where MCs irreversibly bind to serine/threonine protein phosphatases type 2A (PP2A) and type 1 (PP1), inhibiting their enzymatic activity [5–8]. These enzymes are involved on the dephosphorylation of proteins. Consequently, their inhibition results in hyperphosphorylation and reorganisation of the microfilaments, promoting tumours and liver cancer [9]. Apart from the 60 patients of a renal dialysis unit who died due to an accidental intravenous uptake [10–12], no other human fatalities due to MCs have been documented. In fact, MCs are not likely to be ingested in lethal doses by humans due to the repulsive aspect of contaminated water. However, numerous cases of human illnesses and lethal poisoning of animals have been reported [13–22]. The main human exposure routes to MC are the oral

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consumption of contaminated drinking water and the dermal contact with poisoned recreational water. Although to a lower extend, other human exposure routes are the inhalation of aqueous aerosols [23,24] and the consumption of toxic cyanobacterial dietary supplements [25], vegetables irrigated by contaminated water [26], and poisoned animals [27–29]. The high chemical stability of MCs conferred by their cyclic structure, together with their water solubility, explain their environmental persistence and the difficulty to be degraded or removed. The potential risk for the public health has led the World Health Organisation (WHO) to adopt a provisional guideline value of 1 $\mu g\,L^{-1}$ for MC-LR in drinking water [30].

To minimise the potential risk to human health, it is necessary to develop fast, sensitive and reliable methods to detect MCs. Several biological screening methods and also more sophisticated analytical techniques are currently in use. The first screening method to detect MCs was the mouse bioassay [31,32]. This assay gives an indication of the toxicity of the sample, but suffers from low sensitivity, lack of reliability and ethical implications. Another screening method is the protein phosphatase inhibition (PPI) assay [7,33–38]. Like the mouse bioassay, this method informs about the toxicity of the sample (although it is necessary to mention that *in vivo* toxicities may not be directly correlated to *in vitro* PP inhibition), but is not specific to MCs and positive results may indicate the presence of other enzyme inhibitors. Nevertheless, this method is sensitive to subnanogram levels. Enzyme-linked immunosorbent assays (ELISAs) based on antibodies are also highly specific but they are not selective and usually have problems of cross-reactivity among MC and even nodularin congeners [39–41]. Apart from these screening methods, more sophisticated identification and quantification techniques have been applied to MC detection. The most widespread is high performance liquid chromatography (HPLC), usually coupled to a UV detector and preferably to a photodiode array (PDA) detector, due to its inherent high sensitivity and selective detection [42,43]. However, only a few standards are commercially available and it requires trained personnel, expensive equipment and sample pre-treatment, resulting in long analysis times. Liquid chromatography/mass spectrometry (LC/MS) enables the simultaneous separation and identification of MCs in a mixture [43,44]. This analysis technique has been coupled to several ionisation technologies, i.e. fast atom bombardment (FAB) [45], electrospray ionisation (ESI) [46] and matrix assisted laser desorption ionisation time of flight (MALDI-TOF) [47]. Alternative analytical methods are thin-layer chromatography (TLC) [48], suitable as screening method due to its simplicity and low cost; capillary electrophoresis (CE), which achieves appropriate detection limits when coupled with fluorescence detection [49] or ESI/MS [50]; the detection of 2-methyl-3-methoxy-4-phenyl-butyric acid (MMPB), an oxidation product of MCs, by gas chromatography/mass spectrometry (GC/MS) [51] or fluorescence [52]; and the electrochemical detection of the arginine and tyrosine aminoacids [53,54], only valid for the variants with electroactive residues. The choice of the detection method depends on the purpose. They can be used in combination to exploit their complementarities and obtain a complete identification.

Screening techniques for an initial evaluation of MC in cyanobacterial samples should be improved, the ideal method being simple, robust, reliable and sensitive. Biosensors offer themselves as bioanalytical tools potentially able to fulfil all these requirements. Most of the PPI-based assays are colorimetric and in order to develop a biosensor, other transduction techniques should be found. Electrochemistry has been widely used in biosensors because of the simplicity, ease of use, portability, disposability and cost-effectiveness of the electrochemical devices. For the first time, our group has developed an electrochemical approach for the detection of the PP inhibition by cyanobacterial hepatotoxins. This electrochemical biosensing strategy has led to the development of an amperometric biosensor for MC screening, with significant advantages over current analytical methods in respect to analysis time, cost and simplicity.

2. Materials and methods

2.1. Reagents and materials

MC-LR was purchased from Sigma (St. Quentin Fallavier, France). MC standard solutions were firstly prepared in 50:50 methanol:water and subsequently diluted in a buffer solution at pH 8.4 containing 30 mM Tris–HCl, 2 mM ethylene diamine tetraacetic acid (EDTA) and 20 mM $MgCl_2$. Protein phosphatase 2A (PP2A) was obtained from Upstate Biotechnology (New York, USA). PP2A is isolated as the heterodimer of 60 kDa (A) and 36 kDa (C) subunits from human red blood cells. The activity of the stock solution was 1100–2000 $U\,mL^{-1}$, 1 unit being defined as the amount of enzyme required to hydrolyse 1 nmol of *p*-NitroPhenyl Phosphate (*p*-NPP) in 1 min at 37 °C. *p*-NPP, α-Naphthyl Phosphate (α-NP), 4-MethylUmbelliferyl Phosphate (4-MUP) and components of buffers were purchased from Sigma. All solutions were prepared using Milli-Q water. Catechyl MonoPhosphate (CMP) was synthesised according to the method given by Kreuzer et al. [55]. Poly(vinyl alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP) (solid content 6 wt %, pH 6 – 6.5) was provided by Toyo Gosei Kogyo Co. (Chiba, Japan). Gold and platinum foils were purchased from Sigma (St. Quentin Fallavier, France). Copper chips were obtained from Biosentec (Toulouse, France). Silver (Electrodag PF 410), graphite (Electrodag 423 SS) and silver/silver chloride (Electrodag 418 SS) inks were obtained from Acheson (Erstein, France). The insulating layer was a Dulux Valentine paint (Asnières, France). PVC sheets (200 mm × 100 mm × 0.5 mm), supplied by SKK (Denzlingen, Germany), were used as support for the screen-printed electrodes. Microtiter plates were obtained from Nunc (Roskilde, Denmark). Real samples of cyanobacteria from the Tarn River (Midi-Pyrénées, France) were kindly provided by CRITT Bio-Industries (Toulouse, France).

2.2. Apparatus

Colorimetric measurements were performed with a Beckman DU520 UV/Vis spectrophotometer (Beckman Coulter France, S. A., Roissy CDG, France) or a Labsystems Multiskan EX

microtiter plate reader (Thermo Life Sciences, Cergy-Pontoise, France). Cyclic voltammetries and chronoamperometric measurements were performed with an AUTOLAB PGSTAT12 potentiostat interfaced to a PC. Screen-printed 3-electrode systems, with graphite as working and counter electrodes and Ag/AgCl as reference electrode (on Ag conductive tracks), were fabricated using a DEK 248 screen-printing system (Weymouth, UK) as previously reported for screen-printed 2-electrode systems [56]. MC separation was carried out using an HPLC system (Merck, Whitehouse Station, USA), which consisted of an pump (type L-7100), an autosampler (L-7250) with a 20-μL loop and a UV detector (L-7400). Data were collected and evaluated by HSM 4.1 chromatographic software from Merck.

2.3. Extraction of MCs from cyanobacterial samples

Forty milligrams of lyophilised cyanobacterial cells were extracted three times with 1.5 mL of 75:25 methanol:water by sonication for 5 min and centrifugation for 10 min. The three supernatants were mixed together and passed through a 0.2-μm cut-off Acrodisc® syringe filter (Pall Corporation, Saint-Germain-en-Laye, France). After evaporation of the solvent in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at room temperature, the residue was resuspended in 500 μL of 30 mM Tris–HCl, 2 mM EDTA and 20 mM $MgCl_2$, pH 8.4, containing 10% (v/v) methanol.

2.4. Enzyme immobilisation and colorimetric optimisation

PP2A was immobilised by entrapment with poly(vinyl alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP), following the same protocol than with poly(vinyl alcohol) bearing styrylpyridinium groups, also named Poly(vinyl alcohol)-stilbazolium quaternary (PVA-SbQ) [57–63]. The stock enzyme solution was mixed with the polymer in a 2:1, 1:2 or 1:3 PP2A:PVA ratio on a Vortex mixer. Controls without enzyme were also performed. After homogenisation, 3 μL of this solution were dropped on screen-printed graphite electrodes, gold or platinum foil, and copper chips. The supports were then exposed to neon light (two 15-W lamps) for 3 h at 4 °C to allow entrapment of the enzyme by polymerisation. Finally, the supports were dried for 22 h at 4 °C and rinsed with water prior use. The amount of entrapped enzyme was calculated to be 3.8 U when using stock PP2A solution in a 2:1 ratio, 1.9 U when using stock PP2A solution in a 1:2 ratio, and 1.4 U when using PP2A solution in a 1:3 ratio.

Activity assays were performed in order to study the possible enzyme leakage during the toxin incubation. To this end, the electrode supports with immobilised PP2A were introduced into microtiter wells containing 270 μL of buffer solution at pH 8.4 with 30 mM Tris–HCl, 2 mM DTT, 2 mM EDTA, 0.2 mg mL^{-1} BSA and 20 mM $MgCl_2$, and incubated for 30 min (the time subsequently used for the MC incubation) at room temperature. The supports were then rinsed with water and transferred to new microtiter wells containing fresh buffer. Thirty microliters of the colourless 100 mM *p*-NPP solution were added and after 1 h at room temperature, 200 μL of solution were transferred to fresh microtiter wells to measure the absorbance of the yellow *p*-NP produced with the microtiter plate reader. The enzymatic activity arising from the incubation buffer was also measured. Controls without enzyme were always performed. Assays were performed in triplicate. Results were compared to those obtained with supports that had not been incubated in the buffer for 30 min.

2.5. Electrochemical enzymatic assays

The enzymatic activity of PP2A towards phosphorylated compounds was investigated by electrochemical methods with the purpose to develop an amperometric biosensor for MC detection. Electrochemical experiments were performed at room temperature using a single-drop configuration on a horizontally supported screen-printed 3-electrode system. The enzymatic substrates Catechyl MonoPhosphate (CMP), α-Naphthyl Phosphate (α-NP) and 4-MethylUmbelliferyl Phosphate (4-MUP) were dissolved in a buffer solution at pH 8.4 containing 30 mM Tris–HCl, 2 mM EDTA, 20 mM $MgCl_2$ and 100 mM KCl. Preliminary experiments were performed with alkaline phosphatase in solution, used as model enzyme (results not shown).

(a) Cyclic voltammetry: 80 μL of buffer were placed on the 3-electrode system with PP2A immobilised on the working electrode. Three scans were performed in the quiescent solution between +100 and +700 (for CMP), 0 and +600 (for α-NP), and +300 and +1000 mV (*versus* Ag/AgCl) (for 4-MUP) at 50 mV s^{-1} and one at 5 mV s^{-1}. Afterwards, 10 μL of 50 mM phosphorylated substrate were injected and mixed, and after 20 min one scan was performed again at 5 mV s^{-1}. Controls without enzyme were always performed. Assays were performed in triplicate.
(b) Steady-state chronoamperometry: 90 μL of buffer and 10 μL of 50 mM phosphorylated substrate were placed on the 3-electrode system with PP2A immobilised on the working electrode. After 20-min incubation, the corresponding working potential (+450, +300 and +700 mV *versus* Ag/AgCl when using, respectively, CMP, α-NP and 4-MUP, according to the oxidation peaks obtained by cyclic voltammetry) was applied for 1 min in the quiescent solution and the current was measured at the end point. Controls without enzyme were always performed. Assays were performed in triplicate.

2.6. MC detection in real cyanobacterial samples

After extraction of MC from cyanobacterial cells, samples were analysed by a colorimetric PPI assay and with the developed amperometric biosensor. In both techniques, the standard curves for the enzyme inhibition were firstly plotted. Results were also compared to those obtained by HPLC, the most widespread method for MC analysis.

(a) Colorimetry (*enzyme in solution*): the colorimetric assays for the inhibition of the enzyme in solution were performed by the addition of 20 μL of pure, 10-, 100- and 1000-fold

diluted real samples or MC standard solutions at different concentrations into microtiter wells containing 160 μL of enzyme solution (0.3 U mL^{-1} final concentration in the well after the addition of all reagents) in 30 mM Tris–HCl, 2 mM EDTA, 20 mM $MgCl_2$ and 100 mM KCl buffer, pH 8.4. After 30-min pre-incubation at room temperature, 20 μL of 100 mM *p*-NPP solution were added and after 1 h, the absorbance was measured using the microtiter plate reader. Controls without enzyme and without MC were always performed. Assays were performed in triplicate.

(b) Electrochemistry–steady-state chronoamperometry (*immobilised enzyme*): 80 μL of 30 mM Tris–HCl, 2 mM EDTA, 20 mM $MgCl_2$ and 100 mM KCl buffer, pH 8.4, and 10 μL of pure and 100-fold diluted real samples or MC standard solutions at different concentrations were placed on the 3-electrode system with PP2A immobilised on the working electrode. After 30-min incubation, 10 μL of 50 mM CMP were added and incubated for 20 min. Enzyme inhibition was then detected by steady-state chronoamperometry, by applying a potential of +450 mV *versus* Ag/AgCl for 1 min in the quiescent solution and measuring the current value at the end point. Controls without enzyme and without MC were always performed. Assays were performed in duplicate.

(c) HPLC: Separation of cyanobacterial samples (prepared in 50:50 methanol:water) was performed on a Phenomenex C18(2) Luna 3-μm analytical column (150 mm × 4.6 mm i.d.) using a 20 μL injection volume at a flow rate of 0.75 mL min^{-1} with a mixture of acetonitrile and water both containing 0.05% trifluoroacetic acid, using the following linear gradient: 30% of acetonitrile at 0 min, 35% of acetonitrile at 10 min, 70% of acetonitrile at 40 min, 100% of acetonitrile at 42 min and 30% of acetonitrile at 46 min. UV detection was performed at 238 nm. All the assays were performed in triplicate.

3. Results and discussion

3.1. Choice of the appropriate enzyme:polymer ratio and the electrode support

As demonstrated in our previous work [57], the immobilisation of PP2A using the entrapment method improves the stability of the enzymatic activity, when compared to the enzyme in solution. In this work, a water-soluble polymer with pendant photosensitive azide units has been used as cross-linked matrix. Its biocompatibility characteristics make it appropriate for enzyme immobilisation and biosensor construction.

Different electrode materials (gold, platinum, copper and graphite) were tested in order to choose the best support for PP immobilisation, *i.e.* the support that provides higher immobilisation yields. The 1:2 PP2A:PVA ratio was chosen to carry out this study. When gold and platinum foils were used, the activity assays of the incubated electrodes did not result in any colour development. In fact, it was noticed that the polymer was not adsorbed on the surface and that it was removed by rinsing. When copper chips were used, the polymer was also desorbed. These results do not demonstrate the lack of retention of the enzyme into the polymer network, but the poor adsorption of the polymer on those materials. It is interesting to note that if the supports were not rinsed prior the colorimetric assay (consequently, the totality of the deposited enzyme was in the microtiter well, either immobilised or free in solution), the activity assays showed colour development for gold and platinum foils, but no response from copper chips. This result may be explained by the inhibitory effect of the copper metal on the PP enzymatic activity, already demonstrated when using 0.2 mM Cu^{2+} salt with PP from bacteriophage lambda [64]. When graphite electrodes were used, the activity assays of the incubated electrodes showed 62% (relative standard deviation (R.S.D.) = 17%, $n = 3$) of the response of non-incubated electrode controls. The missing 38% could be due to the leakage of the enzyme from the network during the 30-min incubation step required for the subsequent MC incubation and/or to its partial inactivation during that period. In fact, the "leaked" enzymatic activity (arising from the incubation buffer) was not 38%, but 8% (R.S.D. = 8%, $n = 3$). The fact that the balance does not close can be explained by the partial enzyme inactivation, higher for the enzyme in solution. Although this result is far from ideal, the response was high enough to continue with the development of the electrochemical biosensor.

After the electrode material optimisation, several PP2A:PVA ratios were investigated for the immobilisation on graphite electrodes. A high polymer proportion may provide a high enzyme immobilisation yield, however, it may also restrict the enzyme freedom and flexibility, decreasing or totally inhibiting the enzymatic activity. Therefore, a compromise needs to be found between a high immobilisation yield and a measurable signal. Similarly, a high enzyme proportion may provide higher responses, however the sensitivity of the inhibition assay decreases as the enzyme activity increases. Hence another compromise needs to be found between a significant response and a low limit of detection. The absorbance values of the activity assays of the incubated electrodes were 0.041 (R.S.D. = 9%, $n = 3$), 0.259 (R.S.D. = 17%, $n = 3$) and 0.189 AU (R.S.D. = 15%, $n = 3$) for, respectively, the 2:1, 1:2 and 1:3 PP2A:PVA ratios. Although the enzyme amount used in the 1:2 ratio (1.9 U) was lower than that in the 2:1 ratio (3.8 U), the colorimetric response was higher, indicating that the enzyme was better immobilised and/or retained in that matrix. The 2:1 ratio was discarded, as the maximum absorbance (0.041 AU) was too low to perform an inhibition experiment. Compared to the 1:3 ratio (1.4 U), the 1:2 ratio provides only slightly higher absorbance values. Like the 0.259 AU value, the 0.189 AU value could also be appropriate for inhibition experiments. Looking at the immobilisation and/or retention yields, the activity assays of the incubated electrodes for the 2:1, 1:2 and 1:3 PP2A:PVA ratios, respectively, showed 16 (R.S.D. = 9%, $n = 3$), 62 (R.S.D. = 17%, $n = 3$) and 41% (R.S.D. = 15%, $n = 3$) of the response of non-incubated electrode controls. The "leaked" enzymatic activity was 16 (R.S.D. = 6%, $n = 3$), 8 (R.S.D. = 8%, $n = 3$) and 3% (R.S.D. = 5%, $n = 3$). Once again, the balances do not close due to the partial enzyme inactivation. The results demonstrate that the 1:2 PP2A:PVA ratio provides the higher immobilisation and/or

retention (during MC incubation) yield. Although looking at the absorbance values, both the 1:2 and 1:3 PP2A:PVA ratios could be used, the 62% of retention and/or immobilisation in front of the 41% induced us to chose the 1:2 ratio for the subsequent electrochemical enzyme inhibition experiments.

3.2. Choice of the appropriate substrate for PP2A activity electrochemical detection

Most of the enzymes participating in electrochemical biosensors are redox enzymes and, consequently, the electron transfer is guaranteed by the enzyme itself (direct electron transfer) or by the inclusion of redox mediators that react with the enzyme and with the electrode surface (mediated electron transfer). In this work, the enzyme used for the MC detection is not electrochemically active and other electrochemical transduction pathways should be found. The use of an enzymatic substrate electrochemically active only after dephosphorylation by the enzyme would be the solution. In order to choose the optimum phosphorylated substrate to monitor the PP2A activity by electrochemistry, CMP, α-NP and 4-MUP were tested. Preliminary experiments using alkaline phosphatase in solution as model enzyme demonstrated the possibility to detect the dephosphorylated products both by cyclic voltammetry and steady-state chronoamperometry.

Cyclic voltammetry experiments with PP2A in solution demonstrated the ability of the PP2A to dephosphorylate the three substrates. Fig. 1A shows an oxidation peak at approximately +450 mV (*versus* Ag/AgCl), corresponding to the catechol coming from the reaction between CMP and PP2A. The reaction between α-NP and PP2A resulted in an oxidation peak at approximately +300 mV (*versus* Ag/AgCl) (Fig. 1B). Although in the development of electrochemical biosensors, low oxidation peaks are preferred, as they lower the working potential and decrease the interference level, CMP was preferred to α-NP due to the less pronounced electrode fouling [57]. The reaction between 4-MUP and PP2A resulted in an oxidation peak at approximately +700 mV (*versus* Ag/AgCl) (Fig. 1C). Despite the high currents obtained with this substrate, the oxidation potential was too high to be used as working potential in an amperometric biosensor. In the chronoamperometric experiments, the background-subtracted steady-state oxidation currents were 637 (R.S.D. = 18%, $n = 3$), 98 (R.S.D. = 20%, $n = 3$) and 429 nA (R.S.D. = 25%, $n = 3$) for, respectively, CMP, α-NP and 4-MUP. The CMP provides not only the highest oxidation currents but also the highest reproducibility. However, this reproducibility should be improved, as it compromises the reliability of the assay. As the rough graphite surface may be responsible for the high R.S.D. values, other types of electrodes are currently under study in our group. In conclusion, both voltammetric and amperometric results led us to choose CMP as phosphorylated substrate for the subsequent electrochemical enzyme inhibition experiments.

3.3. MC detection in real cyanobacterial samples

Real samples of cyanobacterial blooms from the Tarn River (Midi-Pyrénées, France) were tested using the developed amperometric biosensor. Electrochemical results were compared to those obtained by a colorimetric PPI assay with the enzyme in solution and by HPLC. Firstly, calibration curves with MC standard solutions were plotted for both the colorimetric (Fig. 2A) and the electrochemical (Fig. 2B) approaches. Whereas the R.S.D. values for the colorimetric assay were always less than 7%, the biosensor showed much higher values (R.S.D. = 35%), due to the less stepper slope of the curve (which induces a higher uncertainty on the concentration values), but also to reproducibility problems associated to the electrode construction and to the fouling phenomena. The regression equations obtained for the linear regions ($y = 88.462 \ln(x) - 47.312$, $R^2 = 0.9962$, and $y = 18.344 \ln(x) - 31.077$, $R^2 = 0.9968$, for the colorimetric and the electrochemical strategies, respectively), subsequently used for quantification of MC concentrations in the real samples, indicate 50% inhibition coefficients (IC_{50}) of 3 and 83 $\mu g\,L^{-1}$. The limit of detection (LOD) in the colorimetric approach, considered as 10% inhibition, was 2 $\mu g\,L^{-1}$. In the electrochemical approach, the LOD was considered to be 35% inhibition (due to the lower reproducibility values, which compromise the reliability of the MC determination) and it was 37 $\mu g\,L^{-1}$. The curves show 100% inhibition at about 10 and 1000 $\mu g\,L^{-1}$, for the colorimetric and the electrochemical strategies, respectively. Thus the colorimetric method is almost 20 times more sensitive, but the electrochemical strategy provides a larger working range. This larger working range is very useful for screening purposes, since when unknown samples are analysed, subsequent dilutions have to be performed to meet the linear section of the calibration

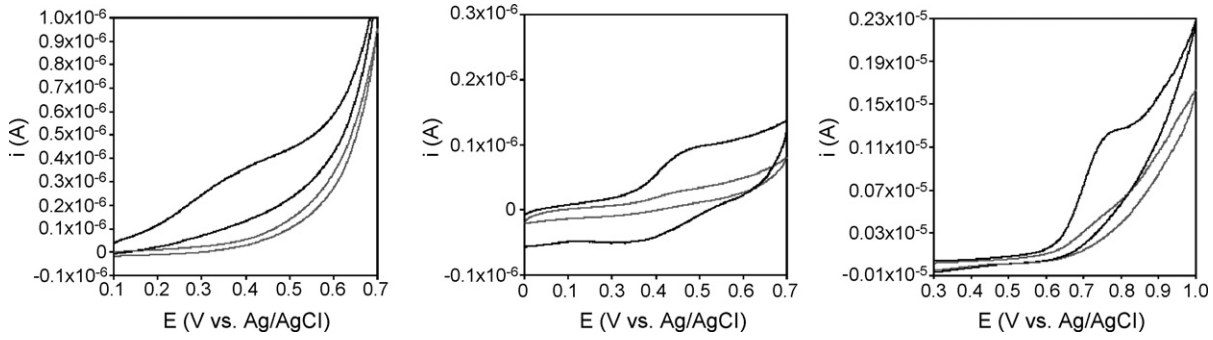

Fig. 1. Cyclic voltammogram of 1.1 U PP2A (grey line) and 1.1 U PP2A + 5 mM substrate (CMP for 1A, α-NP for 1B and 4-MUP for 1C; black line) at 5 mV s^{-1} using a single-drop configuration on a horizontally supported screen-printed 3-electrode system.

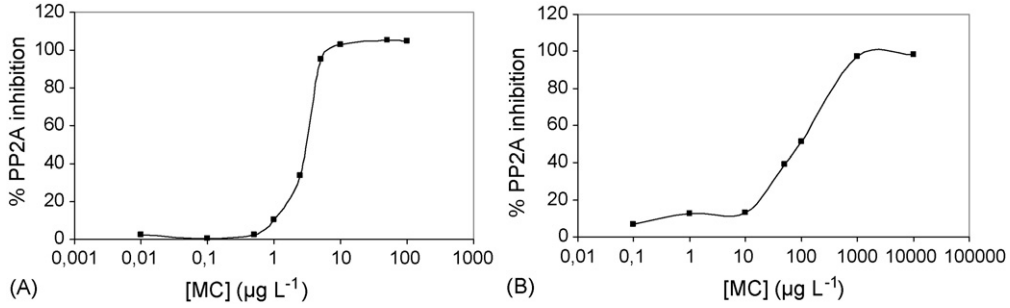

Fig. 2. Colorimetric (enzyme in solution) (A) and electrochemical (immobilised enzyme); (B) standard curves for the inhibition of PP2A by MC-LR. Inhibition is expressed as percentage of the control (no MC). Concentrations refer to the initial MC standard solutions. R.S.D. values are not shown for clarity (for more details, see in the text).

curve. Although electrochemical techniques are usually more sensitive than colorimetric assays, in this case problems associated to the reproducibility between electrodes derived from the screen-printing process and the partial electrode fouling have restricted the sensitivity of the biosensor. As previously mentioned, work is in progress to improve both the reproducibility and the limits of detection by the use of new types of electrodes. At this moment, since the determination level of the biosensor ranges from 37 to 188 $\mu g\,L^{-1}$ (from 35 to 65% inhibition), the biosensor is not directly applicable to natural water samples and a prior concentration step is necessary. However, in the analysis of microcystin content in algae, the residue resuspension step may include this concentration step.

MC-LR equivalent contents in natural blooms of cyanobacteria obtained by the colorimetric PPI assay, with the amperometric PPI-based biosensor and by HPLC are summarised in Table 1. From the 7 real samples tested, all of them contained MC at levels detectable by the amperometric biosensor, 6 by the colorimetric assay and 5 by HPLC. The correlation between the two enzymatic methods is very good (Fig. 3A), but always with higher toxin levels when analysed with the biosensor. Taking into account that when MC standard solutions were used, the colorimetry assay showed lower limits of detection, it seems that the electrochemical biosensor gives an overestimation of the toxin content compared to the colorimetric assay. This effect could be due to the fouling of the electrode by some of the cell extract components, which would decrease the steady-state oxidation currents, simulating an enzymatic inhibition. HPLC correlations with the colorimetric assay (Fig. 3B) and the electrochemical biosensor (Fig. 3C) are poorer. HPLC indicates slightly higher toxin contents for samples 1, 2, 4 and 6 when compared with both PPI-based techniques. Since all the real samples had MC-LR as main or only component, this slight underestimation of the enzymatic techniques is not certainly due to the presence of a MC variant less active than MC-LR (although only three MC variants – MC-LR, MC-LF and MC-RR – were used as standards). Neither to the presence of intracellular PPs, first because cells were lyophilised and second because the methanol used in the extraction process would have neutralise their activity. The explanation to the underestimation should be found in the potential matrix effects. Work is in progress to evaluate them. Sample 3 presented slightly higher toxin contents than those

Table 1
MC-LR equivalent concentrations ($ng\,mg^{-1}$ dry weight) in cyanobacterial blooms derived by the colorimetric PPI assay, the amperometric PPI-based biosensor and HPLC (MC variant composition obtained by HPLC is provided)

Sample	Colorimetric	Electrochemical	HPLC
1	4.0 (7.4)	10.2 (1.4)	28.7 (0.6) 100% MC-LR
2	6.1 (8.3)	14.3 (21.8)	15.7 (1.1) 100% MC-LR
3	32.4 (1.9)	149.7 (18.3)	83.3 (0.9) 99% MC-LR 1% MC-LF
4	–	0.5 (9.6)	4.1 (1.4) 100% MC-LR
5	3.5 (2.5)	9.2 (25.3)	–
6	0.1 (6.3)	0.3 (16.5)	5.9 (3.5) 100% MC-LR
7	0.4 (2.9)	2.5 (19.6)	–

In parentheses, the R.S.D. (%) values are given.

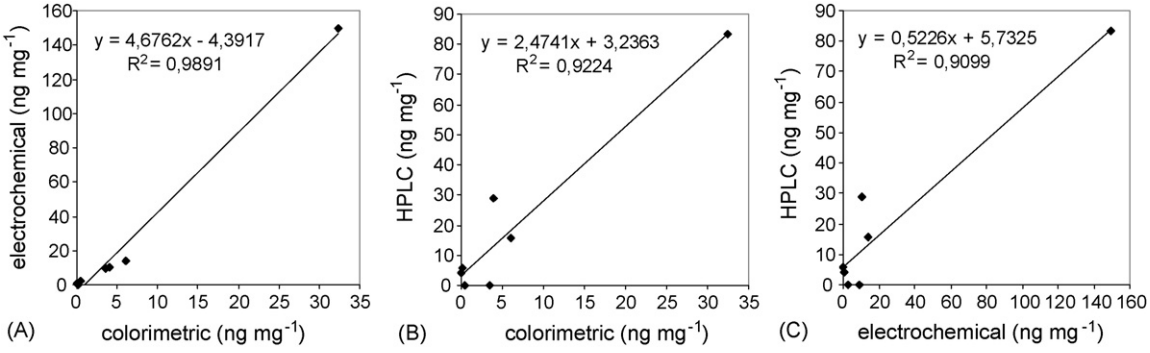

Fig. 3. Correlations of MC-LR equivalent concentrations ($ng\,mg^{-1}$ dry weight) in cyanobacterial blooms derived by the colorimetric PPI assay, the amperometric PPI-based biosensor and HPLC. The regression lines and equations are shown.

determined by colorimetry but lower than those determined with the biosensor. This result clearly demonstrates the biosensor overestimation, more evident at high toxin concentrations. Samples 5 and 7 were indicated to contain MC by PPI at levels that should have been detected by HPLC. The non-detectable contents by HPLC could be due to the fact that only MC-LR, MC-LF and MC-RR were used as standards and other variants, whose standards were not tested or are not commercially available, could be present in those samples. In fact, because the PPI-based techniques detect all inhibitory MC variants at the same time, its sensitivity compared to HPLC is further enhanced. One can not ignore the fact that the MC presence detected in samples 5 and 7 by the enzymatic methods could be a false positive due to the presence of PP2A inhibitors others than microcystins, such as nodularins, okadaic acid and its derivatives, cantharidin, tautomycin, calyculin A; however, only nodularins can be present in cyanobacterial cells, which, in any case, are less spread in the world than microcystins.

Although there was a poor correlation between HPLC and the electrochemical device, the high sensitivity, the simplicity and the broad working range of the approach justify the use of the amperometric biosensor as screening and monitoring tool to roughly estimate the microcystin content in sample. In positive samples, the use in parallel of other analytical techniques, such as LC/UV or LC/MS, would eliminate the possibility of having other inhibiting compounds, would provide accurate MC determination and would enable the identification of the variant/s.

4. Conclusions

The enzymatic methods for MC detection present the advantage over HPLC of being more sensitive and less expensive and do not require trained personnel. Although enzymatic assays are not specific to MCs, they give the microcystin content expressed into a MC-LR equivalent concentration. In this work, a PPI-based amperometric biosensor for MC detection has been developed for the first time, combining the advantages of the PPI assays and the electrochemical transducers. The low working potential required, provided by the lab-made CMP enzyme substrate, avoids the oxidation of interferences that might be present in real samples. Despite the overestimation of the toxin content, potentially due to the electrode fouling by some cell extracts components, the applicability of the biosensor to rapidly assess the environmental and health risk due to MCs is demonstrated. To minimise the risk of false positive results or if accurate toxin determination and quantification are desired, the amperometric biosensor should be used in parallel with other analytical techniques. The simplicity of both the biosensor construction and the electrochemical measurement, together with the electrode disposability and the possibility to be used *in situ*, make the amperometric biosensor attractive as screening tool for rapid and early MC detection.

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