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Sensitive biosensor based on recombinant PP1α for microcystin detection

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ABSTRACT

A novel electrochemical microcystin-LR (MC-LR) biosensor based on the inhibition of recombinant protein phosphate type 1 (PP1α) is reported in this work. The use of innovative recombinant enzyme led to investigate new commercially available substrate, electrochemically active after their dephosphorylation by PP1α. Only two of selected substrates, 1-naphylphosphate and phosphoparacetamol, showed a good affinity toward PP1α. Kinetic parameters were performed by classical colorimetric assays and revealed that phosphoparacetamol is an excellent synthetic substrate with a K_m value of 1.2 mM. The reported biosensor is constructed by entrapment of the enzyme in Polyvinyl Alcohol (azid unit) on Cobalt-Phtalocyanine (CoPC) modified screen printed electrode. Electrocatalytic mediator demonstrated a significant improvement in the electrochemical detection of dephosphorylated substrate. The standard inhibition curve has provided a limit of detection at 0.93 μg/L and a broad dynamic range from 0.93 to 40.32 μg/L for MC-LR, demonstrating the improved analytical performance.

1. Introduction

Cyanobacterias also known as "blue-green algae" are found over the fresh, brackish and marine waters all over the world, and play a preponderant role in the functioning of aquatic ecosystems (Kehr et al., 2011). However, some species of cyanobacteria represent a serious problem to human health due to their associated toxicity. Climate changes and anthropogenic eutrophication are important factors responsible for an abnormal increase of harmful cyanobacteria blooms (HABs) (Pearson et al., 2010; Paerl and Paul, 2012; O'Neil et al., 2012). HABs contain different types of cyanotoxins that are classified according to their mode of action into hepatotoxins (e.g. microcystins), neurotoxins (e.g. anatoxins), skin irritants, and other pathogens (Carmichael, 1997; Codd et al., 2005). The most commonly encountered cyanotoxins are microcystins (MCs) produced in freshwater by various species of the genera *Microcystis*, *Planktothrix*, and *Anabaena*. MCs constitute a group of hepatotoxic cyclic heptapeptide, that are produced none ribosomally in the cytoplasm. They consist of two variable and five constant amino acids including the unusual C20 amino acid responsible to hepatotoxicity. More than 80 MCs congeners have been identified which exhibit structure variations, and vary in their mode of toxicity (Pearson et al., 2010). Microcystin-LR (MC-LR) is one of the most prevalent and potent toxin among cyanotoxins (Krishnamurthy et al., 1986). Recent studies have witnessed an increase of incidents of toxicity involving MCs (Carmichael, 1996, 2001; Codd et al., 1999; Hitzfeld et al., 2000; Funari and Testai, 2008). This potential risk for the public health led the World Health Organization (WHO) to establish a provisional guideline value of 1 μg/L for MC-LR in drinking water (WHO, 1998). The strong toxicity and ubiquity of MCs make necessary the development of fast, sensitive, reliable and portable monitoring system to detect low level of MCs, and to manage the risk associated with the health.

Numerous analytical techniques are currently available for identifying MCs including bioassays, chromatographic techniques, chemical assays and immunoassay techniques (Falconer et al., 1994; Campbell et al., 1994). Among them, the most commonly used methodology is High Pressure Liquid Chromatography (HPLC) in combination with different detectors (Chorus and Bartram, 1999). However, these techniques are expensive, time consuming and they require highly trained operators. Additionally, they are not suitable for rapid processing of multiple samples. Alternative screening methods have been developed based on antigen and antibody affinity interaction. Although, antibody-based ELISA assays are highly specific but they still have shortcomings such as poor selectivity and cross-reactivity with other MCs congeners (Nagata et al., 1995). MCs are specific inhibitors of serine/threonine protein phosphatases (PPs), making PPs assays suitable for their

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detection and screening (Carmichael and An, 1999; Bouaïcha et al., 2002). Enzymatic methods based on the PPs inhibition provides insight on toxicology, and are highly sensitive compare to immunoassays. Our group has developed sensitive colorimetric inhibition assays with the use of commercial and recombinant PPs for the MCs detection (Sassolas et al., 2011a, 2011b; Covaci et al., 2012). However, all these methods do not address properly the need for field studies.

Herein, we propose an electrochemical biosensor that combines the significant selectivity of the innovative recombinant protein phosphatase type 1 (PP1α) towards MC-LR (Sassolas et al., 2011a, 2011b) and the sensitivity and relative simplicity of an electroanalytical measurement. The first part of this study aimed to select appropriate substrates for the recombinant PP1α. It was observed that majority of selected substrates are phosphorylated phenol having an important fouling effect after hydrolysis, while other suffers from instability and also require high potentials for the detection of corresponding dephosphorylated products. To circumvent theses drawbacks, characterization of substrates and their corresponding products was performed with bare and Cobalt-PhtaloCyanine (CoPC) modified screen printed electrode. CoPC mediator is known to decrease electrode passivation, and to facilitate the detection of phenol compounds at lower potential (Yin et al., 2009). Afterwards, PP1α assays with selected substrates were performed by cyclic voltammetry, and enzymatic product detection was carried out according to the graphical abstract. Kinetic characteristics of appropriate substrates were determined by colorimetric assay based on the quantification of release inorganic phosphate. Finally, the inhibition of immobilized and free PP1α with different amount of MC-LR was conducted with differential pulse voltammetry (DPV) detection in order to evaluate the suitability of the newly designed biosensors.

2. Materials and methods

2.1. Reagents and solutions

The recombinant protein phosphatase type 1α (PP1α) was produced by CRITT (Toulouse, France). Synthetic gene coding for rabbit PP1α (emb XO7798) was constructed according to Denis-Quanquin et al. (2007). Three mutations were introduced to increase the production and solubility of the protein (C127S, C38G, C105V), and a histidine tail was added at the N-terminus to facilitate their purification. A green fluorescent-protein (GFP) tag was fused to the C-terminal extremity to simultaneously select synthetic genes devoid of frame-shift mutations and with mutations providing satisfactory expression and folding of the protein. The construction was introduced into the pETG vector downstream of the T7 Promoter and the lac operator. *Escherichia coli* BL21 (DE3) strain transformed harboring the plasmid including this construction, was grown in the presence of 1 mM $MnCl_2$ until an optical density (OD) of 1 was reached. T7 RNA polymerase-mediated transcription was induced with 1 mM of isopropyl-*β*-thiogalactopyranoside (IPTG), and the culture temperature was decreased to 25 °C. The cells were harvested by centrifugation and lysed by sonication. Protein was purified by affinity chromatography using nickel-modified resin. Purified extracts were found to be devoid of any contaminant, as assessed by overloaded SDS-PAGE.

The enzyme solution was prepared by dissolving 140 mg of lyophilized enzyme in 200 μL of enzyme diluents containing 1% glycerol, 50 mM HEPES pH 7.0, 200 mM NaCl, 1 mM $MnCl_2$, 1 mM dithiothrietol (DTT), 0.1 mM Ethylene Glycol Tetraacetic Acid (EGTA), 0.025% Tween 20, 0.1 mg/L Bovine Serum Albumin (BSA) and stored at −20 °C.

Further, the stock enzyme solution was diluted in reaction buffer 30 mM Tris-HCl at pH=8.4 containing, 2 mM Ethylene Diamine Tetraacetic Acid (EDTA), 20 mM $MgCl_2$, 200 mM KCl (Campas et al., 2005) and daily supplemented with 0.5 mM DTT, 0.02% BSA for assays.

Malachite green, ammonium molybdate tetrahydrate, *p*-nitrophenyl phosphate (*p*-NPP), sulphuric acid reaction buffer were used for colorimetric assays, and all components were provided by Sigma-Aldrich (Saint Quentin en Fallavier, France).

Enzyme was immobilized with a photo-crosslinkable polyvinyl alcohol azide-unit pendant water soluble photopolymer (PVA-AWP) (solid content 6 wt% pH 66.5) for electrochemical assays, which were provided by Toyo Gosei Kogyo Co. (Chiba, Japan).

The selected enzyme substrates as *p*-Aminophenyl Phosphate (*p*-APP) was purchased from DiagnoSwiss (Monthey, Switzerland), Phosphorylated PARacetamol (PPAR) and HydroQuinone DiPhosphate (HQDP) from dropsens (Llanera, Spain). The remaining of substrates and their respective dephosphorylated forms as Ascorbic Acid-2-Phosphate (AAP), *1*-Naphthyl PhosPhate (*1*-NPP), Phenyl-Phosphate (PP), Phospho-Tyrosine (PTyr), Ascorbic Acid (AsAc), *p*-AminoPhenol (*p*-AP), 1-NaPhtol (1-NP), PARacetamol (PAR), phenol (PH), Tyrosine (Tyr) and HydroQuinone (HQ) and buffer components to design biosensors were purchased from Sigma (Saint Quentin en Fallavier, France).

MC-LR purchased from Alexis (San Diego, USA), was firstly dissolved in absolute ethanol (1 g/L) and subsequently diluted in distilled water.

2.2. Apparatus

Colorimetric measurements were performed with a lab system Multiskan EX microtiter plate reader (Thermo Life Sciences, France). Maxisorp microtiter plates were obtained from Nunc (Roskilde, Denmark).

Electrochemical measurements were performed with an AUTOLAB PGSTAT100 potentiostat/galvanostat equipped with general purpose electrochemical system (4.9) for cyclic voltammetry (CV) and DPV (Eco Chimie, The Netherlands).

Screen-printed electrode system consists of conventional three electrode configuration with a disk-shaped graphite working electrode (0.4 cm diameter, 0.125 cm^2) geometrical, a graphite counter and *Ag/AgCl* as pseudo-reference electrode, were fabricated using a DEK 248 screen-printing system (Weymouth, UK) as previously reported for screen-printed two-electrode (Istamboulie et al., 2007). Based on the same principle CoPC screen-printed electrode were fabricated using carbon inks containing CoPC as mediator material for print working electrode.

2.3. Colorimetric assays

The immobilized enzyme is a key element in the development of the biosensor. It is crucial to control the amount of enzyme immobilized on electrode surface.

2.3.1. Assay for PP1α activity by colorimetric method

PP1α activity was assayed in solution at 37 °C by monitoring the conversion of *p*-nitrophenyl phosphate (*p*-NPP) to *p*-nitrophenol (*p*-NP) as described (Sassolas et al., 2011a, 2011b). Briefly, 50 μL of diluted enzyme solution, 20 μL of 100 mM *p*-NPP and 130 μL of reaction buffer was mixed in microtiter wells at 37 °C. After 1 h, the absorbance was measured at λ=405 nm using the microtiter plate reader. Control tests without enzyme were always performed. The activity is expressed as the mean μmol phosphate released per min per ml of enzyme (U/mL).

2.3.2. Kinetics parameters

This method was applied only for selected substrates to design the biosensor: PPAR and *1*-NPP. The procedure used to quantify inorganic phosphate released from dephosphorylation of the substrate by the PP1α is an adjusted protocol as described by Wheldrake et al. (1996).The assay was carried out in two stages. The first part involves the enzymatic hydrolysis of the phosphorylated substrates, followed by the measurement of liberated inorganic phosphate (Pi). The enzyme activity towards different substrates concentration 0.625, 1.25, 2.5, 5 and 10 mM was determined by incubating 80 μl of PP1α at 2.5 mU/mL, 25 μl of *1*-NPP or PPAR (phosphoralated subtrates) in 100 μL of reaction buffer. The mixture was incubated at 37 °C, after which the reaction was stopped at different times by the addition of 10 μl H_2SO_4 (3 M) and frozen. Each sample was centrifuged at 10 000 rpm for 5 min. The supernatant (150 μl) was combined with 25 μl of freshly prepared molybdate ammonium solution at 12.5 mM in 3 M sulfuric acid, and incubated at room temperature in microtiter plate. Finally, 25 μL of malachite green solution at 0.9 mM prepared in 0.1% PVA, H_2SO_4 3 M solution was added and incubated for 10 min. The resulting absorbance of the malachite green-phosphomolybdate complex was measured at 620 nm in triplicate. The kinetic parameters V_{max} and K_m were calculated from the double reciprocal plot of velocity versus substrates concentration

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\right) * \left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

2.4. Electrochemical procedures

The enzymatic activity of PP1α towards several substrates was investigated using CV, in order to select the most appropriate substrate to develop an electrochemical biosensor for MC-LR detection. Then the MC-LR effect was determined in presence of selected substrates by DPV. Each electrochemical assay was conducted in a single-drop configuration.

2.4.1. Selection of electrochemical substrates

First of all, the behavior of selected enzyme substrates was characterized by CV. Briefly, 180 μL of reaction buffer, 20 μl of 50 mM phosphorylated substrate were placed on the 3-electrode system at 37 °C for 30 min. The incubation time was reduced to 30 min compared to initial colorimetric parameters. In fact, the enzymatic reaction takes place on electrode surface allowing fast electrochemical detection of the catalytic product. Then, three scans were performed between −0.400 and +0.800 V (versus *Ag/AgCl*) to avoid the interference of CoIII/CoII transition occur at −0.600 V at 50 mV/s (Blank).

CV was carried out with 0.5 mM of hydrolysis product to confirm the oxidation or reduction peak position and characterize electrochemical behavior.

Afterwards, 20 μL of 50 mM phosphorylated substrate were mixed with 180 μl pure desalting PP1α and after 30 min of incubation, three scans were performed at 50 mV/s. Controls without enzyme were always performed to verify natural hydrolysis of substrate (Blank). All assays were carried out in triplicate.

2.4.2. Electrochemical detection of MCs

The effect of MC-LR on the enzyme activity was determined by incubating free or immobilized PP1α (1.0 mU) with various concentrations of MC-LR in reaction buffer; selected phophorylated substrates (5 or 1 mM final concentration) was added, at 37 °C for 30 min. The substrate hydrolysis rate was determined by DPV. DPV was carried out in reaction buffer under following conditions; (modulation time=0.04 s, interval time=0.5 s, initial potential=0.2 V, end potential=0.5 V, step potential=0.01 V, modulation amplitude=0.1 V, stand by potential=0 V.

The maximum ip values recorded correspond to the oxidative waveform height peak in the presence of an enzyme substrate and without MC called control signal.Then, calibration curves were obtained by plotting the degree of PP1α inhibition express as percentage of control signal (*y*-axis) against MC concentrations (*x*-axis). Assays were performed in triplicate.

3. Results and discussion

3.1. Selection of substrate for electrochemical measurement PP1α activity

An innovative recombinant enzyme (PP1α) was produced as recognition element to develop the biosensor. It has been reported that proteins phosphatase 1 and 2A are the prime targets affected by MCs. Genetic modifications (Section 2.1) were performed in order to improve the MCs sensitivity of wild PPs. Previous investigations have demonstrated that genetically alteration led to increased PPs sensitivity towards MCs (Covaci et al., 2012; Sassolas et al., 2011a, 2011b). The commonly used enzyme used for the MCs detection is not electrochemically active, therefore other electrochemical transduction pathways need to be explored. The solution may be the use of an enzymatic substrate that is electrochemically active only after dephosphorylation by the PP1α. Previous studies have shown the ability of PP2A to dephosphorylated a number of substrates (Campas and Marty, 2007), but this activity can be degraded by the PP1α modifications. Consequently, a wide range of substrate was selected including those of PP2A and alkaline phosphate (Massón et al., 2004; Preechaworapun et al., 2008), to choose the appropriate substrate.

CV was used to examine the electrochemical properties of substrates, and products of the enzymatic reaction. Firstly, seven commercial substrates including AAP, PP, *p*-APP, PPAR, *1*-NPP, PTyr, HQDP were tested.

The substrates displayed similar electrochemical behavior and none of them was electrochemically active in the potential range from −0.400 to +0.800 V (versus SCPE and versus CoPc modified SCPE) with the exception of *p*-APP. The anodic peak current decreased, while the peak cathodic current increased for *p*-APP. Therefore, this substrate was excluded for further study, because background signal, due to its chemical instability, significantly affects the sensibility of respective product detection.

Afterwards, electrochemical measurements of products were investigated on bare and CoPc modified SCPEs to characterize electrochemical behavior and to determine the optimized parameters for the detection. To achieve this, three successive cyclic voltammetry were performed under in similar inhibition condition. CoPc was employed in the electrocatalytic detection of dephosphorylated substrates, because the majority of selected substrates led to production of mono-substituted phenolics compounds. However, most of the phenolic compounds are oxidized at high positive potentials posing problem for their electrochemical detection. Their electro-oxidation is known to form derivatives of phenols radicals which adsorb onto the electrode surface and induce the passivation of electrode (Ferreira et al., 2006). The modification of SCPEs with mediators as prussian blue (Lete et al., 2010) carbon nanotubes (Wang et al., 2003) and CoPC (Yin et al., 2009) have been successfully used to reduce the relatively high working potential, and to increase the sensitivity.

Fig. 1 shows the relative response of the anodic peak current for dephosphorylated substrates oxidation during successive cyclic voltammetric scans with carbon and CoPC modified electrode. Subsequent scanning led to significant decrease in the anodic

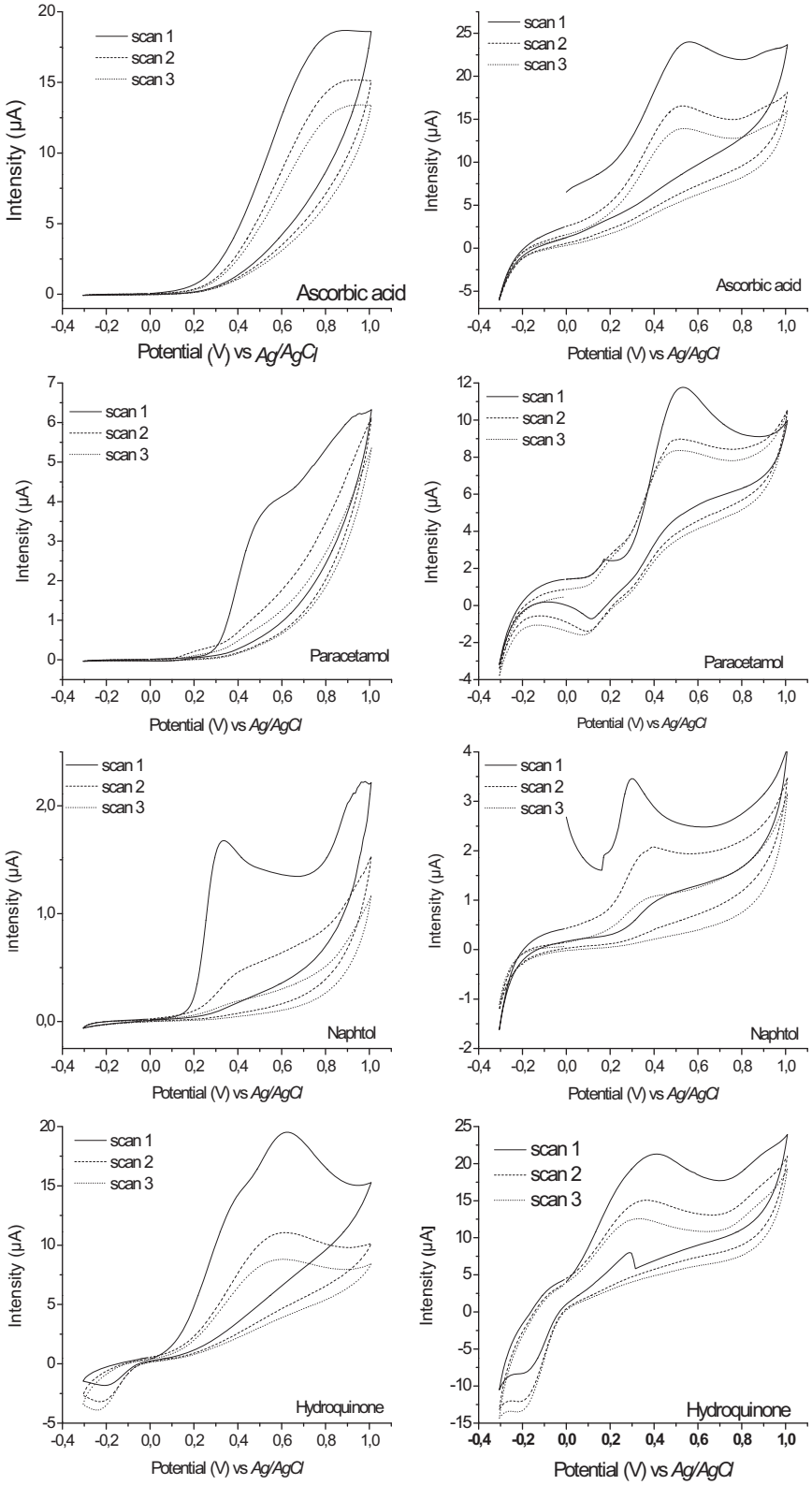


Fig. 1. Three successive cyclic voltammograms obtained for 5 mM substrate (*1*-NPP/PPAR/HQDP/AAP/PP/PTyr) in reaction buffer pH=8.6 at 37 °C on bare (on left side) and Co-PC SPCEs (on the right side) with a similar scan rate=50 mV/s.,

current except for tyrosine and ascorbic acid due to the use of CoPC mediator considerably minimizing the passivation of electrode.

In the presence of mediator, an interesting effect in the form increased anodic current was observed, and was attributed to the electrocatalytic activity of CoPC (Zagal, 1992), Furthermore, this resulted in increase electron transfer rate with a shift of anodic potential to less negative values. The electrochemical parameters to demonstrate the performance of modified electrode compared to bare electrode are summarized in Supplementary Table 1.

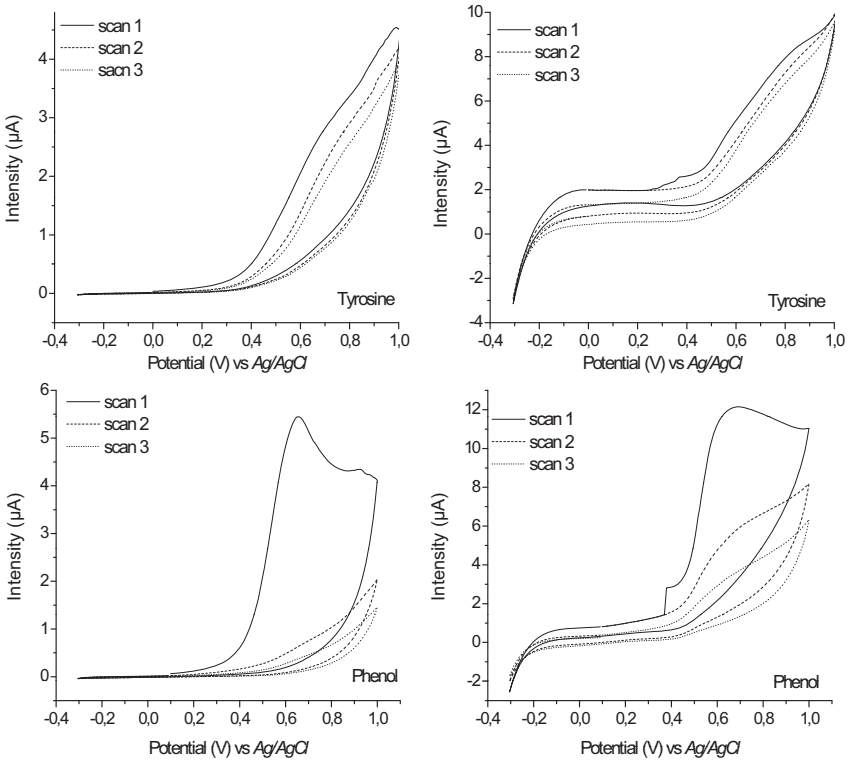


Fig. 1. (*continued*)

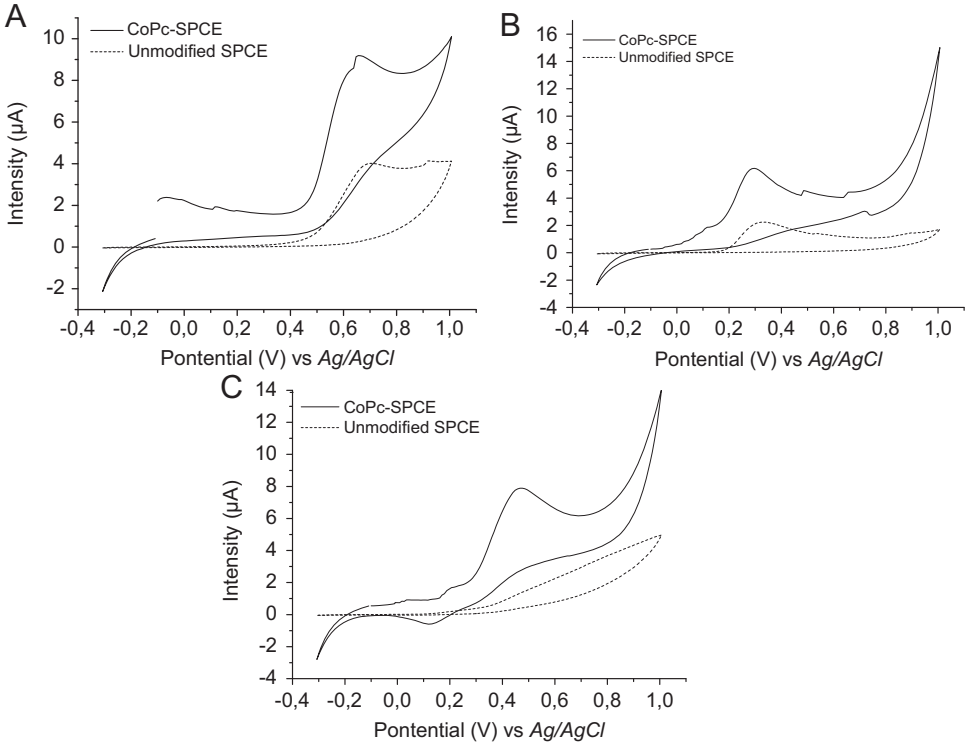


Fig. 2. Cyclic voltammograms of nondiluted enzyme + 5 mM substrate (PP (A)/*1*-NPP (B)/PPAR(C)) at 50 mV/s using single-drop configuration on a horizontally supported bare and CoPc-SPCEs.

Finally, assays were performed with pure desalting PP1α in solution to demonstrate its ability for dephosphorylatation of the selected substrates. CV experiments showed that only three selected substrates (PP, *1*-NPP, PPAR) were hydrolyzed by the enzyme with the appearance of an oxidation peak indicating that PP1α converted the non-electrochemical substrates into electrochemical products. Despite similar chemical structure, dephosphorylated products have different electrochemical properties mainly due to the nature of substituent (Enache and Oliveira–Brett, 2011). Fig. 2 presents the redox behavior of product resulting

from the reaction between PP1α and PP, *1*-NPP, PPAR on bare and CoPc-SPCE.

Overall, the oxidation potential and the current response of dephosphorylated product were dependent on the degree of substitution and the type of substituent on phenol ring. The current response was found to be lower for products substituted with mesomeric electron donating group compared to the phenol. Indeed, in the absence of mediator, the anodic current of phenol is 1.5 and 1.2 fold higher than recorded for 1-NP and PAR. Similarly, the oxidation potential decreases from +0.700 V for phenol to +0.575 V and +0.330 V for PAR and 1-NP, respectively. The addition of mediator not only changed the oxidation potential pattern but also oxidative peak potentials were negatively shifted 50 mV for PP and 1-NP and 115 mV for PAR, which indicates that CoPC electrode has better electrocatalytic effect towards oxidation of enzymatic products.

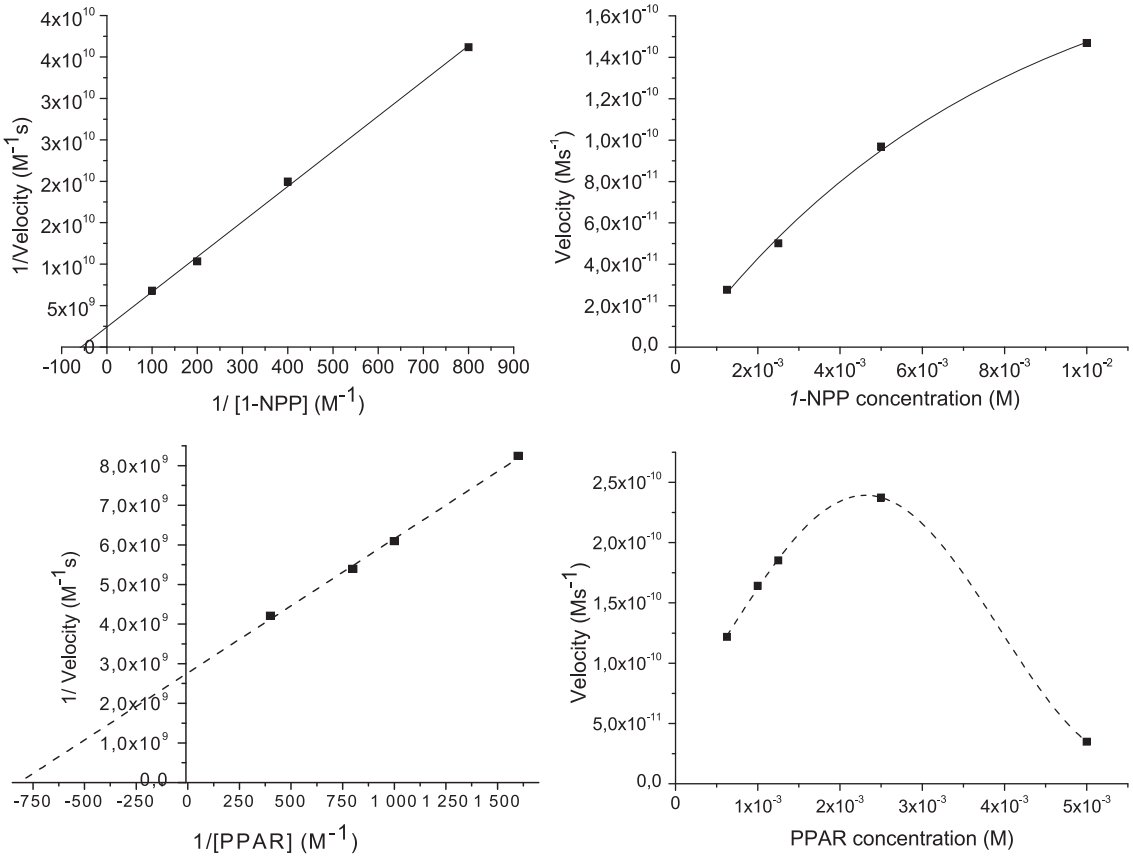
1-NPP and PP after hydrolysis by PP1α have generated electrochemically active 1-NP and phenol (Pemberton et al., 1999). On bare and modified *SPCEs*, their latter presented irreversible oxidation peaks, and this corresponds to naphtoquinone (NPQ) and quinone (Fig. 2A and B).The electrochemical oxidation led to N-acetyl-p-benzoquinone-imine (NAPQI) for PAR (Fig. 2C) (Nematollahi et al., 2009) confirmed by an irreversible behavior with not well defined anodic peak at +575 mV on bare electrode. However, PAR electroxidation on CoPc-SPCEs showed a well defined anodic pick +0.460 V and a small cathodic peak at −150 mV versus *Ag/AgCl*, which confirmed CoPC displayed better electrocatalytic property for PAR.

Nevertheless, PP was dephosphorylated in the presence of PP1α, CV data shown important electrode fouling and higher oxidation potential than other substrates. However, in the development of electrochemical biosensors, low oxidation potential is preferred to decrease the interference level. In conclusion, PPAR and *1*-NPP as phosphorylated substrates for the subsequence electrochemical enzyme inhibition experiments and CoPC-SPCE as transduction platform were used due to improved electrochemical detections toward selected substrates including low oxidation potential, high current responses, and good anti-fouling performance.

3.2. Kinetics parameters of PPAR and 1-NPP hydrolysis

For further assessment regarding substrate specificity, the kinetics parameters K_m and V_{max} of substrate hydrolysis were investigated. Classical Michaelis−Menten kinetics analyses were performed at various substrate concentrations ([S]).

As demonstrated in Fig. 3, the velocity of the reactions is increased as the PPAR and *1*-NPP substrates concentration is increased up to 2.5 mM in the presence of 0.2 mU/mL of desalting PP1α. However, up to 5 mM of PPAR, a decrease of velocity was



Substrates			
PPAR		1-NPP	
Km	Vmax	Km	Vmax
1.2 ± 1mM	1.7 nmol s^{-1}	22 ± 2mM	0.5 nmol s^{-1}

Fig. 3. Michaelis–Menten plots for the serial dilution of substrate with PP1α (0.2 mU) in reaction buffer (pH 8.6) versus velocity of product formation measured by the absorbance of 620 nm and kinetic parameters extract to double reciprocal plot of velocity versus substrate concentration.

observed due to an inhibition by substrate excess, this observation should be taken into consideration for the choice of best substrate concentration in the development of biosensors.

An estimation of V_{max} and K_m was determined using Lineweaver – Burk transformation plots.

A high K_m value indicates weaker affinity, whereas a low value suggests a higher affinity. According to Fig. 3, the K_m value of PP1α toward *1*-NPP is around 18 times greater than that of PPAR, indicating weaker binding affinity for *1*-NPP. Further, K_m value of PPAR is 16 times lower is compared to Km of colorimetric substrate (*p*-NPP) (Covaci et al., 2012). The catalytic activity of PP1α is enhanced when it operates at a high rate in the presence of sufficient amount of substrates. The selected substrates concentrations were 1 mM for PPAR and 5 mM in case of *1*-NPP.

3.3. Biosensors design and microcystin-LR detection: Comparison of calibration curves

3.3.1. Biosensor construction

The electrochemical assays were performed with nondiluted enzyme solution at 1.5 U/mL; the number of enzyme units deposited on the electrode surface is a key point in the construction of the biosensor. High enzyme activity may provide higher electrochemical response and low enzyme activity may provide lower detection limits. Consequently, there is a compromise between a significant response and a high sensitivity. Further, the major problem of PP1α is poor enzymatic stability. To circumvent this problem, enzyme immobilization was used to stabilize the PP1α activity (Campas et al., 2008).

Several amount of free enzyme were tested by DPV with 1 mM of PPAR and 5 mM *1*-NPP as enzyme substrate on unmodified and CoPC-SPCEs. For 0.4 mM of free PP1α, on bare electrode, the observed current was 600 nA (R.S.D.=7.1%). When using 0.2 mU, the obtained current values was 300 nA (R.S.D.=1.9%) while 0.1 mU of enzyme decreased current to 150 nA (R.S.D.=3.7%). The current obtained with 0.1 mU was considered as enough to perform calibration plots (the lowest measurable current should be 15 nA in presence of toxin and was chosen for the construction of the corresponding biosensors.

3.3.2. Microcystin detection

In order to validate the feasibility of this method for the analysis of MC-LR, the quantitative determination of dephophoryladed substrates was achieved by measuring the oxidation peak current after background subtraction with different concentration of MC-LR by DPV. Calibration curves using 0.1 mU of free and immobilized PP1α in the presence of 1 mM PPAR or 5 mM *1*-NPP were performed (Fig. 4).

In both cases, the standard curves were well described by the sigmoidal logistic four-parameter equation.

$$y = A2 + \frac{A1 - A2}{1 + (x/x_0)^p}$$

where *A*1 and *A*2 are the asymptomatic minimum and maximum values, respectively, x_0 is the *x* value at the inflection point and *p* is the slope at the inflection point (IC_{50}). Table 1 summarizes the curve parameters and correlation coefficients (*R*) from the regression curves. Electrochemical detection limits has been defined as the 10% inhibition coefficient (concentration that indicates toxin occurrence, even though this value is out of dynamic range). The regression equations obtained from the linear regions are subsequently used for quantified the MC-LR concentration which led to 50% (IC_{50}) of PP1α inhibition and the dynamic range of developed method.

Comparing the inhibition assays based on the free PP1α (Fig. 4 and Table 1), the results showed that enzyme had similar sensitivity toward MC-LR in presence of two selected substrates. The LOD was 2 times lower for *1*-NPP and the dynamic range is slightly larger 0.39–3.25 μ/L with the linear equation, $y=34.30\ln(x)-48.29$, $r^2=0.992$) compared to as those of PPAR, 0.52–2.51 μg/L ($y=44.118\ln(x)-43.061$, $r^2=0.992$). Moreover, it was observed that enzyme immobilization induced a decrease in limit of detection from 0.52 to 0.93 μg/L, for PPAR ($y=17.15\ln(x)-26.29$, $r^2=0.996$) and 0.28 to 0.42 μg/L for *1*-NPP.The dynamic range, on the other hand, was enlarged by more than 2 orders of magnitude. Additionally, the reproducibility is greatly improved with the enzyme entrapment strategy. These results could be explained by diffusion barrier created by the polymeric matrix which stabilizes enzyme activity and avoids the residual fouling effect.

As expected, the electrochemical detection led to better limit detection and IC_{50} compared to those obtained by PP1α colorimetric tests (Sassolas et al., 2011a, 2011b). The electrochemical biosensor described in this work resulted in a 1.8-fold lower IC_{50} than the IC_{50} value (12.2 μg/L) of previously reported colorimetric assay. Moreover, the sensitivity of the biosensor has been enhanced by decreasing the fouling effect with the used of CoPc mediator.

Preliminary assays were carried out on real samples using developed biosensor. Our method based on the evaluation of MC-LR toxicity allows a screening of water bodies with a sensibility better than detection limit adopted by the WHO. Two samples considered as positive according to electrochemical results containing respectively 1.12 and 1.34 μgL^{-1} were analyzed by the reference method HPLC–MS–MS. The MC-LR amount provided by conventional method for each sample were 1.01 and 1.07 μgL^{-1}, these concentrations are 10% to 20% lesser than obtained with developed

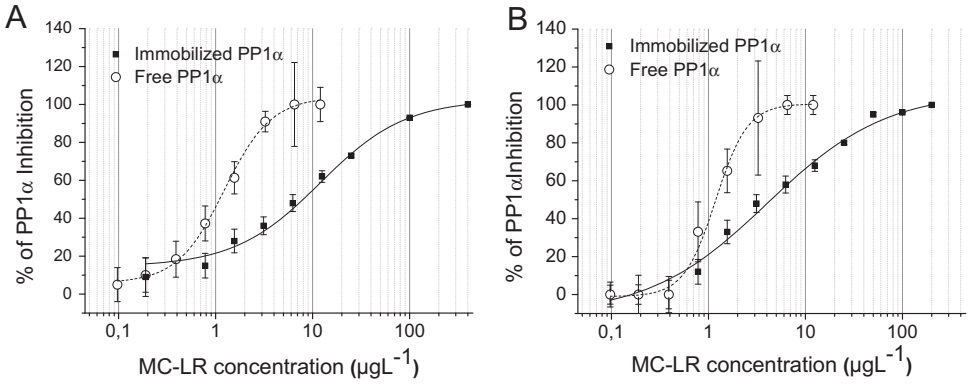


Fig. 4. Electrochemical standard curves for free and immobilized PP1 inhibition by different concentration of MC-LR. With curve (A) *1*-NPP and curve (B) PPAR as enzyme substrate. Inhibition is expressed as percentage of the control (no MC-LR).And *x* values refer to final MC concentration on SPCEs.

Table 1
Curve parameters derived from the sigmoidal logistic four-parameters fitting for the PP1α inhibition by MC-LR.

	CoPC-SPCE	Detection limits (μg/L)	IC_{50} (μg/L)	Dynamic range (μg/L)	Sigmoïd equation	r^2
Free PP1α	PPAR	0.52	1.31	0.52–2.51	$y = 102.05 + \frac{-91.00}{1+(x/1.62)^{1.37}}$	0.998
	1-NPP	0.28	1.10	0.39–3.25	$y = 102.43 + \frac{-101.04}{1+(x/5.93)^{0.78}}$	0.997
Immobilized PP1α	PPAR	0.93	4.42	0.93–40.32	$y = 100.59 + \frac{-1.04}{1+(x/2.64)^{1.18}}$	0.997
	1-NPP	0.42	7.88	0.78–100	$y = 104.99 + \frac{-91.00}{1+(x/1.23)^{1.79}}$	0.997

biosensor. The difference is due to the simultaneous presence of other variants at minor's concentrations. The method comparison underlines the pertinence of our approach which estimates the global toxicity of the sample. The analysis of bloom from contaminates sites could be also envisaged. The intracellular concentration of MCs present in reservoir may give us an indication of potential water toxicity. Concerning interference effects, the two samples were tested with biosensor built with denatured enzyme to take into account the eventual interference of matrix. These was no electrochemical response, in consequence developed method cannot provide false negative tests. However, in case of inhibition assay, some compound as detergent present in the medium can inhibit enzyme activity and lead to the overestimation of toxin concentration and conclude to false positive tests.

4. Conclusions

This paper describes the development of sensitive biosensors for the detection of MC-LR based on inhibition recombinant PP1α. In this work, we demonstrated the excellent sensitivity of our recombinant PP1α. We also demonstrated the possibility to use a new and inexpensive substrate PPAR. The simplicity and the sensitivity of our biosensor gave us the possibility to use it "*in situ*" for a rapid control of MCs. An indirect detection of dephosphorylated substrate with is bienzymatic system could be implemented to avoid the residual fouling effect and to improve the sensitivity. The next step of this work will be to systematically test real samples from contaminated sites to validate the developed device. A correlation between biosensor responses and the concentration of MCs determined by classical method based on HPLC-SM-SM will be demonstrated.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.10.030.

References

Bouaïcha, N., Maatouk, I., Vincent, G., Levi, Y., 2002. Food Chem. Toxicol. 40, 1677–1683.
Campas, M., Szydlowska, D., Trojanowicz, M., Marty, J.L., 2005. Biosens. Bioelectron. 20, 1520–1530.
Campas, M., Marty, J.L., 2007. Biosens. Bioelectron. 22, 1034–1040.
Campas, M., Olteanu, M.G., Marty, J.L., 2008. Sens. Actuators B 129, 263–267.
Campbell, D.L., Lawton, L.A., Beattie, K.A., Codd, G.A., 1994. Environ. Toxicol. Water Qual 9, 71–77.
Carmichael, W.W., 1996. Liver failure and human deaths at a haemodialysis center in Brazil: microcystins as a major contributing factor. Harmful Algae News. IOC-UNESCO, pp.11 – 15.
Carmichael, W.W., 1997. The cyanotoxins. In: Callow, J.A. (Ed.), Advances in Botanical Research, vol. 27. Academic Press Inc., San Diego, pp. 211–256.
Carmichael, W.W., An, J., 1999. Nat. Toxins 7, 377–385.
Carmichael, W.W., Azevedo, S.M.F.O., An, J.S., Molica, R.J.R., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., 2001. Environ. Health Perspect. 9, 663–668.
Chorus I., Bartram J., 1999. A guide to their public health consequences, monitoring and management. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water. London and New York, p. 362.
Codd, G.A., Metcalf, J.S., Beattie, K.A., 1999. Toxicon 37, 1181–1185.
Codd, G.A., Lindsay, J., Young, F.M., Morrison, Metcalf, J.S., 2005. Harmful Cyanobacteria: from mass mortalities to management measures. In: Huisman, J., Matthijs, Visser, P.M. (Eds.), Harmful Cyanobacteria. Springer, pp. 1–23.
Covaci, O.I., Sassolas, A., Alonso, G.A., Muñoz, R., Radu, G.L., Bucur, B., Marty, J.-L., 2012. Anal. Bioanal. Chem. 404, 711–720.
Denis-Quanquin, S., Lamouroux, L., Lougarre, A., Maheo, S., Saves, I., Paquereau, L., Demange, P., Fournier, D., 2007. J. Biotechnol. 131, 223–230.
Enache, T.A., Oliveira-Brett, A.M., 2011. J. Electroanal. Chem. 655, 9–16.
Falconer, I.R., Smith, J.V., Jackson, A.R.B., Jones, A., Runnegar, M.T.C., 1994. J. Toxicol. Environ. Health 24, 291–305.
Ferreira, M., Varela, H., Torresi, R.M., Tremiliosi-Filho, G., 2006. Electrochim. Acta 52, 434–442.
Funari, E., Testai, E., 2008. Crit. Rev. Toxicol. 38, 97–125.
Hitzfeld, B.C., Hoeger, S.J., Dietrich, D.R., 2000. Environ. Health Perspect. Suppl. 108, 113–122.
Istamboulie, G., Andreescu, S., Marty, J.-L., Noguer, T., 2007. Biosens. Bioelectron. 23, 506–512.
Kehr, J.C., Picchi, D.G., Dittmann, E., 2011. Beilstein J. Org. Chem. 7, 1622–1635.
Krishnamurthy, T., Carmichael, W.W., Sarver, E.W., 1986. Toxicon 24, 865–873.
Lete, C., Lupu, S., Marin, M., Badea, M., 2010. Rev. Roum. Chim. 55, 335–340.
Massón, M., Rúnarsson, ÖV., Jóhannson, F., Aizawa, M., 2004. Talanta 64, 174–180.
Nagata, S., Soutome, H., Tsutsumi, T., Hasegawa, A., Sekijima, M., Sugamata, M., Harada, K., Suganuma, M., Ueno, Y., 1995. Nat. Toxins 3, 78–86.
Nematollahi, D., Shayani-Jam, H., Alimoradi, M., Niroomand, S., 2009. Electrochim. Acta 54, 7407–7415.
O'Neil, M., Davis, T.W., Burford, M.A., Gobler, C.J., 2012. J. Harmful Algae 14, 313–334.
Paerl, H.W., Paul, V.J., 2012. Water Res. 46, 1349–1963.
Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B., 2010. Mar. Drugs 8, 1650–1680.
Pemberton, R.M., Hart, J.P., Stoddard, P., Foulkes, J.A., 1999. Biosens. Bioelectron. 14, 495–503.
Preechaworapun, A., Dai, Z., Xiang, Y., Chailapakul, O., Wang, J., 2008. Talanta 76, 424–431.
Sassolas, A., Catanante, G., Fournier, D., Marty, J.-L., 2011a. Talanta 85, 2498–2503.
Sassolas, A., Catanante, G., Hayat, A., Marty, J.-L., 2011b. Anal. Chim. Acta 702, 262–268.
Wheldrake, J.F., Bilney, A., Rosenberg, L., Murray, A.W., Bond, P.M., Steffensen, D.A., Nicholson, B.C., 1996. UWRAAR, Melbourne, Report No. 105.
WHO, 1998. Guidelines for Drinking-water Quality. Addendum to Vol. 2, Health Criteria and Other Supporting Information, second ed. World Health Organisation, Geneva.
Wang, J., Deo, R.P., Musameh, M., 2003. Electroanalysis 15, 1830–1834.
Yin, H.S., Zhou, Y., Ai, S.-Y., 2009. J. Electroanal. Chem. 62, 80–88.
Zagal, H., 1992. Coord. Chem. Rev. 119, 89–136.