

An SPR biosensor for the detection of microcystins in drinking water

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Abstract A surface plasmon resonance (SPR) biosensor for the detection of microcystins (MCs) in drinking water has been developed. Several assay formats have been evaluated. The selected format is based on a competitive inhibition assay, in which microcystin-LR (MCLR) has been covalently immobilized onto the surface of an SPR chip functionalized with a self-assembled monolayer. The influence of several factors affecting sensor performance, such as the nature and concentration of the antibody, the composition of the carrier buffer, and the blocking and regeneration solutions, has been evaluated. The optimized SPR biosensor provides an IC_{50} $0.67 \pm 0.09 \mu\text{g L}^{-1}$, a detection limit of $73 \pm 8 \text{ ng L}^{-1}$, and a dynamic range from 0.2 to $2.0 \mu\text{g L}^{-1}$ for MCLR. Cross-reactivity to other related MCs, such as microcystin-RR (88%) and microcystin-YR (94%), has also been measured. The SPR biosensor can perform four simultaneous determinations in 60 min, and each SPR chip can be reused for at least 40 assay–regeneration cycles without significant binding capacity loss. The biosensor has been successfully applied to the direct analysis of MCLR in drinking water samples,

below the provisional guideline value of $1 \mu\text{g L}^{-1}$ established by the World Health Organization for drinking water.

Keywords Microcystin-LR · Label-free biosensor · Self-assembled monolayer · Surface plasmon resonance

Introduction

The concern about the effects of cyanobacteria (blue-green algae) on human health has grown in recent years, due to the increasing eutrophication of surface waters, resulting in proliferation of cyanobacterial water blooms [1, 2]. Outbreaks of human poisoning attributed to toxic cyanobacteria have been reported worldwide, following consumption of contaminated drinking water or after recreational exposure [3]. The worst incident associated with the cyanotoxins to date occurred in 1996 in Caruaru (Brazil), where a fatal intoxication at a hemodialysis clinic led to the death of over 50 renal patients [4]. The use of water from a reservoir suffering a cyanobacterial bloom, along with an insufficient water treatment to eliminate the MCs, was identified as the main cause. In addition to water, the population can be exposed to dangerous cyanotoxins through the consumption of freshwater fish or seafood and micro-algae dietary supplements [5, 6].

Among the cyanotoxins, perhaps the most studied are the so-called microcystins (MCs) that are cyclic heptapeptides, produced by several bloom-forming cyanobacteria genera (e.g., *Microcystis*, *Planktothrix*, and *Anabaena*). Among over 80 known toxic variants of MCs, microcystin-LR (MCLR), where L and R represent the variable amino acids leucine and arginine, respectively, is the most frequently present and toxic MC. Although the primary target is the liver (e.g., it promotes the formation of hepatic

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tumors by chronic ingestion through food or drinking water), MCLR can also affect the kidney and the gastrointestinal tract. Less acute MCs toxicosis symptoms generally include headache, blurred vision, abdominal pain, nausea, and vomiting. Their toxicity is related to the inhibition of the protein phosphatases, thus disrupting the cellular processes.

MCs are most widely found in freshwater (e.g., rivers, lakes, and reservoirs) where they are released in substantial amounts after bacterial cell lysis or bacterial death. Moreover, their high chemical stability and water solubility lead to a long MCs persistence in the surface water bodies. Therefore, the removal of cyanotoxins and toxic cells during the water treatment processes has become a high priority for researchers and drinking water supply companies [7, 8].

Due to the significant impact of MCs on human health, the World Health Organization (WHO) has adopted a provisional guideline value of $1 \mu\text{g L}^{-1}$ for MCLR in drinking water (as it is the most toxic and frequent MC), and many countries have followed this guideline for drinking water and food as well [9]. However, recent toxicity studies suggest that even a low concentration of MCLR in water could significantly interrupt cellular processes, and thus more care should be taken in determining the criterion for MCLR content in drinking water [10].

The most widespread analytical methods for the determination of MCs in raw or drinking water are based on reversed-phase liquid chromatography (LC) combined either with ultraviolet (UV) [11, 12] or mass spectrometry detection [13–16]. These methods often require lengthy and time-consuming sample preparation procedures that usually involve the pre-concentration of considerable water volumes prior to LC analysis to reach the required sensitivity. Furthermore, the ability of these techniques to identify unknown MCs in environmental samples has been hindered by the lack of analytical standards for many MCs variants [17].

Biochemical screening methods, such as enzyme-linked immunosorbent assays [18–20], competitive enzyme immunoassays [21, 22], or protein phosphatase inhibition assays (PPIA) [20, 23], have also been described. Immunoassays and PPIA are sensitive methods with low equipment requirements that report the total MCs content, but they do not distinguish between MCs and other unrelated protein phosphatase inhibitors [24]. Recently, a commercial lateral flow dipstick (MCs ImmunoStrip®) [25] and dipsticks based on colloidal gold particles [26] have become available, and they can be used equally in the field and in the laboratory with minimum sample processing or technical expertise.

Alternatively, immunosensors based on different transduction schemes, such as optical [27–29], electrochemical

[30], and nuclear magnetic resonance [31] measurements, have been also applied to MCs determination.

Surface plasmon resonance (SPR) spectroscopy has become a widely used analytical technique due to its advantages, such as the possibility to perform direct, real-time, and label-free detection of molecules in complex matrices, without the need of previous cleanup.

In this work, we describe the development of an SPR-based immunosensor for the determination of MCLR. Due to the small size of MCLR, several assay formats have been tested by immobilization of either an anti-MCLR antibody, an MCLR conjugate, or the MCLR itself. The resulting SPR immunosensor is based on a competitive inhibition assay, where MCLR is covalently attached to the SPR chip. Several parameters affecting the immunosensor performance have been optimized (antibody nature and concentration and carrier buffer composition). The immunosensor has been successfully applied to the determination of MCLR in tap water samples below the WHO recommended levels for drinking water.

Experimental

Reagents and materials

Microcystin-LR (MCLR), biotin-MCLR conjugate, ovalbumin-MCLR (OVA-MCLR) conjugate, and monoclonal anti-MCLR antibodies were obtained from AbKem Iberia S.L. (Vigo, Spain). Microcystin-RR (MCRR), microcystin-YR (MCYR), and monoclonal MC10E7 antibodies that rose against MCs were provided by Alexis (Läufelfingen, Switzerland). C64C12 and C64A1 monoclonal antibodies against MCLR were purchased from HyTest Ltd. (Turku, Finland). Alkanethiol $\text{HS}(\text{CH}_2)_{11}\text{OH}$, amine-alkaneithiol $\text{HS}(\text{CH}_2)_{11}\text{NH}_2$, oligo ethylene glycol alkaneithiol $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_4\text{OH}$ (EG4OH), carboxylic acid-terminated oligo ethylene glycol alkaneithiol $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OCH}_2\text{COOH}$ (EG6COOH), and alkaneithiol $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{-biotin}$ (BAT) were purchased by ProChimia (Gdansk, Poland).

Ethanolamine hydrochloride (EA), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were all included in the amine coupling kit from Biacore (Uppsala, Sweden). Casein and gelatin-blocking buffers, bovine serum albumin (BSA), glutaraldehyde solution, triethylamine, and dextran sulfate sodium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). SuperBlock® T20 blocking solution was provided by Thermo-Scientific (Rockford, IL, USA).

MCs stock solutions were prepared in dimethyl sulfoxide (1 mg mL^{-1}) and stored at -20°C . MCs standard solutions for calibration purposes were prepared daily upon dilution

of the stock solutions in phosphate-buffered saline (PBS; 10 mM, pH 7.4). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). All solutions were filtered through 0.22- μm nylon membranes (Whatman, Maidstone, UK). All other chemicals used were of analytical reagent grade.

SPR sensor

A four-channel SPR sensor platform developed at the Institute of Photonics and Electronics (Prague) [32, 33] was used. The SPR sensor is based on the Kretschmann geometry of the attenuated total reflection method and spectral modulation. In this configuration, broadband light from a halogen lamp is collimated and reflected from the base of the prism to which an SPR chip is attached. The chip consists of a thin gold layer (50-nm thickness), deposited on a glass substrate. Upon the incidence of light on the chip, the excitation of the surface plasmons occurs. The light reflected from four areas (sensing channels) on the gold layer is collected via GRIN lenses into four optical fibers, which are connected to a four-channel spectrograph. A flow-cell with four separate flow chambers is interfaced with the chip to confine the sample during experiments. Each flow chamber is aligned to cover one sensing channel. A multichannel peristaltic pump is used to deliver liquid samples over each sensing channel. The acquired spectra are analyzed in real-time by a special software package that allows determination of the resonant wavelength in each sensing channel. In the reported experiments, the flow rate of 30 $\mu\text{L min}^{-1}$ was used, unless otherwise stated. Temperature was kept at 25 °C.

Assay formats

Several competitive inhibition assay formats, based on the immobilization of the anti-MC antibody, MCLR, or MCLR conjugates, have been evaluated for immunosensor development (Fig. 1).

Prior to functionalization, the gold SPR chip was cleaned by washing with absolute ethanol, drying with nitrogen stream, and by cleaning in UV ozone cleaner for 10 min to remove organic contaminants. The chip surface was washed with deionized water followed with absolute ethanol and dried with a stream of pure nitrogen.

Immunoassays based on the immobilization of anti-MC antibodies

The gold layer on the SPR chip (the sensor surface) was coated with the anti-MC antibody following two different approaches: (a) covalent immobilization on a self-assembled monolayer (SAM) via the terminal carboxylic groups and (b)

immobilization as a covalently cross-linked double layer. Due to the small size of MCLR, the detection was carried out by competition between the MCLR and an OVA-MCLR conjugate (1 $\mu\text{g mL}^{-1}$ in PBS), flowed along the sensor surface for 30 min. The amount of the OVA-MCLR conjugate bound to the immobilized antibody was measured both in the presence and in the absence of MCLR.

Covalent immobilization via carboxylic-SAM (surface I)

The anti-MCs antibody was covalently attached by amino coupling to the sensor chip functionalized with a terminal carboxylic-SAM. The chips were incubated with a 200- μM solution of thiols EG4OH and EG6COOH (7:3, v/v) in degassed absolute ethanol [34] for 30 min at 40 °C and left overnight at room temperature in a dark place. Afterwards, they were cleaned with ethanol, dried with N_2 , and mounted in the SPR device. The carboxylic groups on the sensor surface were activated with 200 mM EDC and 50 mM NHS (20 $\mu\text{L min}^{-1}$, 5 min) followed by incubation (30 $\mu\text{L min}^{-1}$, 30 min) with the antibody solution (1 $\mu\text{g mL}^{-1}$ in 10 mM sodium acetate, pH 5.0). Non-covalently bound antibody was removed by washing with high ionic strength phosphate buffer (HIS-PB; 10 mM, pH 7.4, 0.75 M NaCl, 5 min), followed by EA (1 M, pH 8.5, 5 min) to deactivate residual carboxylic groups.

Covalently cross-linked double layer (surface II) The antibodies were immobilized onto the chip surface following the procedure described by Koubová et al. [35]. Briefly, the sensors were sequentially treated with an anti-MC antibody solution (1 $\mu\text{g mL}^{-1}$ in 60 mM citrate buffer, pH 4.0, 15 min), a solution of dextran sulfate polyanions (1 mg mL^{-1} in citrate buffer, 20 min), and again with the anti-MC antibody solution. The resulting multilayer was cross-linked with a solution of 0.5% glutaraldehyde in citrate buffer. Finally, dextran sulfate was removed from the cross-linked antibody assembly by replacing citrate buffer by PBS.

Immunoassays based on the immobilization of MCLR or MCLR conjugates

The following assay formats have been tested based on the immobilization of (a) a biotin-MCLR conjugate, (b) an OVA-MCLR conjugate, or (c) MCLR. Detection was carried out by real-time monitoring of the interaction between the anti-MCLR antibodies and the immobilized MCLR or MCLR conjugates, respectively.

Immobilization of a biotin-MCLR conjugate (surface III)

The biotin-MCLR conjugate was immobilized on neutravidin-coated chips via a non-covalent, high-affinity,

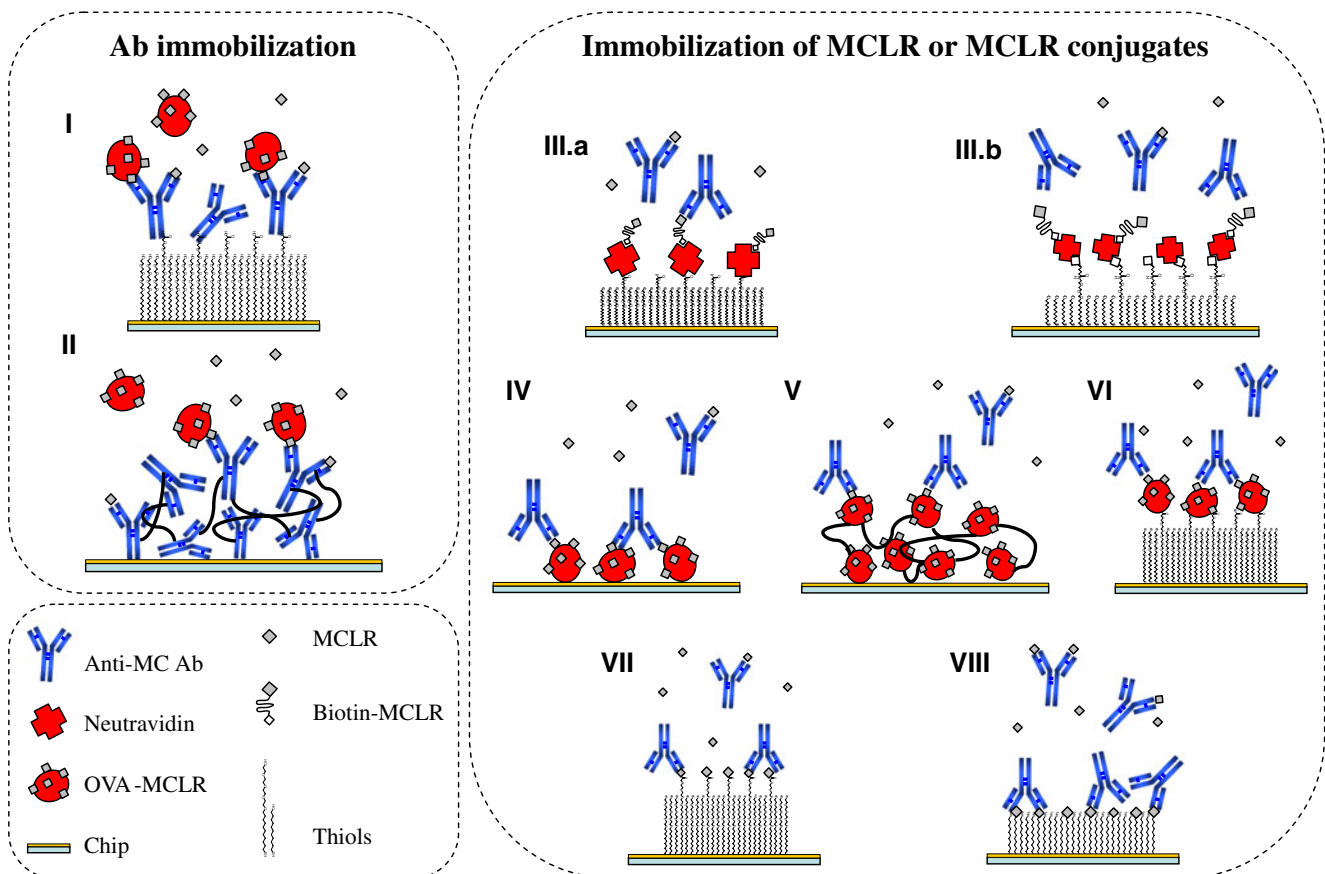


Fig. 1 Assay formats. (I) Covalent immobilization of the antibody via carboxylic-SAM; (II) covalently cross-linked antibody double layer; (III) immobilization of a biotin-MCLR conjugate on a neutravidin layer (III.a, covalent immobilization of the neutravidin; III.b, neutravidin immobilization via biotin-avidin bond); (IV) physical

adsorption of an OVA-MCLR conjugate; (V) covalently cross-linked OVA-MCLR double layer; (VI) covalent immobilization of an OVA-MCLR conjugate; (VII) covalent immobilization of MCLR via amino groups, and (VIII) covalent immobilization of MCLR via carboxylic groups

and stable neutravidin–biotin bond. Two different approaches were investigated for neutravidin coupling:

- Covalent attachment of neutravidin to a carboxylic-SAM (surface III.a): Neutravidin was covalently attached to the chip surface functionalized with a terminal carboxylic-SAM, following the procedure described in “Covalent immobilization via carboxylic-SAM (surface I).” The activated carboxylic groups were reacted with a solution of neutravidin ($50 \mu\text{g mL}^{-1}$ in sodium acetate) for 5 min and then with the biotin-MCLR conjugate ($10 \mu\text{g mL}^{-1}$ in PBS) for 30 min.
- Non-covalent binding of neutravidin on a biotin-SAM (surface III.b): The chip was functionalized with a mixture of alkanethiol $\text{HS}(\text{CH}_2)_{11}\text{OH}$ and biotinylated alkanethiol (BAT; 9:1, total concentration of $100 \mu\text{M}$) in ethanol, following the procedure described in “Covalent immobilization via carboxylic-SAM (surface I),” mounted in the SPR instrument and treated with a solution of neutravidin ($50 \mu\text{g mL}^{-1}$ in sodium acetate) for 10 min.

Immobilization of an OVA-MCLR conjugate

- Physical adsorption (surface IV): The chips were mounted in the SPR instrument, and a solution of OVA-MCLR ($25 \mu\text{g mL}^{-1}$ in sodium acetate) was allowed to flow over the gold surface for 5–10 min.
- Covalently cross-linked double layer (surface V): The OVA-MCLR conjugate ($25 \mu\text{g mL}^{-1}$) was immobilized on the chip according to the protocol described in “Covalently cross-linked double layer (surface II).”
- Covalent immobilization (surface VI): The OVA-MCLR conjugate ($25 \mu\text{g mL}^{-1}$) was covalently immobilized by amino coupling onto the chip surface functionalized with a carboxylic-SAM, following the procedure described in “Covalent immobilization via carboxylic-SAM (surface I).”

Immobilization of MCLR

- Covalent binding via amino groups (surface VII): MCLR ($10 \mu\text{g mL}^{-1}$) was covalently immobilized by

amino coupling onto the sensor surface functionalized with a carboxylic-SAM (see “Covalent immobilization via carboxylic-SAM (surface I)”).

- (b) Covalent binding via carboxylic groups (surface VIII): The chips were immersed in an amine-alkanethiol HS $(\text{CH}_2)_{11}\text{NH}_2$ ethanolic solution (0.2 mM), containing 2% of triethylamine and kept in the dark at room temperature for at least 1 day in order to obtain the amine-SAM. Afterwards, the chips were sequentially rinsed with ethanol, a solution of 10% acetic acid in ethanol (v/v), ethanol, and were dried with N_2 . A mixture of EDC/NHS and MCLR in PBS (final concentration in solution, 80 mM EDC, 20 mM NHS, and 10 μM MCLR) was then pumped for 30 min, and finally, the non-covalently bound MCLR was removed with HIS-PB.

Assay protocol

The measuring principle was based on a competitive inhibition assay between MCLR, immobilized onto the surface of the SPR chip, functionalized with an amine-alkanethiol-SAM as described previously (“Immobilization of MCLR,” surface VIII), and MCLR present in the sample for a limited number of antibody binding sites.

All solutions were prepared in PBS (carrier solution). The MCLR standard solutions (950 μL) were mixed with 50 μL of the antibody solution (4 $\mu\text{g mL}^{-1}$). After pre-incubation at room temperature for 10 min, the mixture was pumped over the sensor surface for 30 min. During this step, the free antibody interacts with the immobilized MCLR, resulting in a signal increase that depends on the concentration of MCLR in the sample. Figure 2 shows a typical sensorgram for real-time detection of MCLR. Finally, the sensor surface was regenerated by flowing of a 50-mM NaOH solution (15 min). A complete cycle, including chip regeneration, can be accomplished in 60 min.

The SPR sensor response was normalized according to the following expression (Eq. 1):

$$\text{Normalized response} = (B - B_{\infty}) / (B_0 - B_{\infty}) \quad (1)$$

where B is the signal measured in the presence of increasing MCLR concentrations, B_{∞} is the background obtained in the presence of an excess of MCLR, and B_0 is the signal in absence of MCLR. The normalized response was plotted against the concentration of MCLR (in logarithmic scale), and the experimental data were fitted to a four-parameter logistic equation (sigmoidal; Eq. 2):

$$\text{Normalized signal} = \frac{A_{\max} - A_{\min}}{1 + \left(\frac{[\text{Analyte}]}{\text{IC}_{50}} \right)^b} + A_{\min} \quad (2)$$

where $A_{\max}$ is the asymptotic maximum (maximum signal in the absence of MCLR), b represents the curve slope at the inflection point, IC_{50} is the concentration of analyte at the inflection point (concentration giving 50% inhibition of $A_{\max}$), and $A_{\min}$ is the asymptotic minimum. The detection limit (LOD) was calculated as the MCLR concentration for which the antibody binding to the immobilized MCLR was inhibited by 10%, and the dynamic range (DR) of the method was evaluated as the MCLR concentrations that produced a normalized signal between 20% and 80% of B_0 .

Selectivity studies

Cross-reactivity (CR) studies were carried out by measuring the competitive inhibition curves for other MCs variants under the optimized conditions. CR was calculated according to the following equation (Eq. 3):

$$\% \text{CR} = \frac{\text{IC}_{50}(\text{MCLR})}{\text{IC}_{50}(\text{cross-reacting compound})} \times 100 \quad (3)$$

Analysis of water samples

Tap water samples were collected in plastic bottles previously rinsed with ultrapure water. The pH and ionic strength of the water samples was adjusted by addition of PBS, and they were stored at 4 °C until analysis. The samples were filtered through a 0.22- μm nylon membrane and finally spiked with MCLR. The analyses were performed in triplicate.

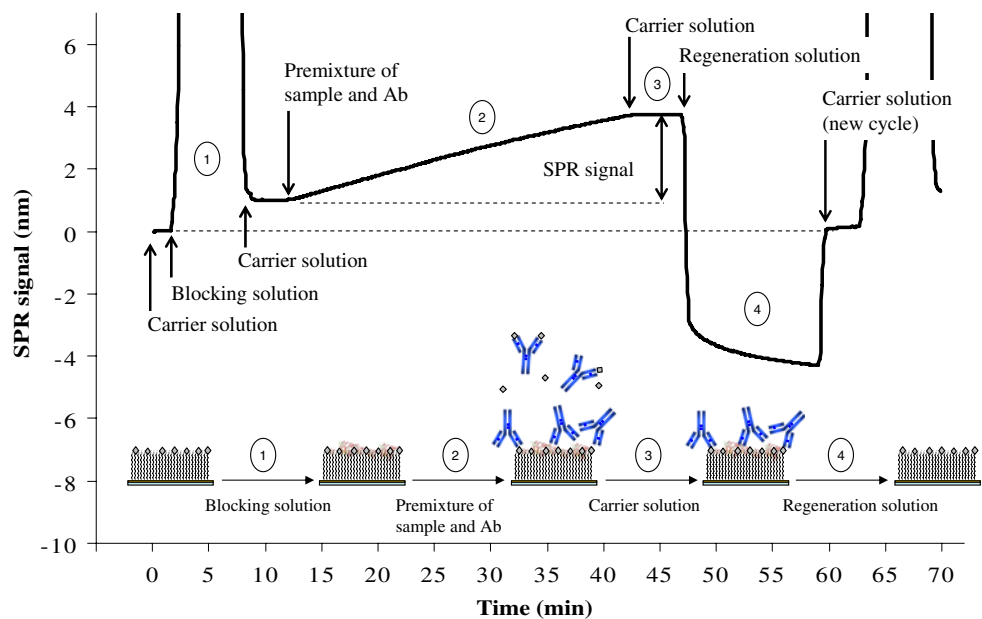
Results and discussion

Comparison of the different assay formats

In order to select the most appropriate format and to achieve a defined and sufficiently high density of immobilized receptors without loss of their biological activity, two assays based on anti-MCLR antibody immobilization and seven based on MCLR or MCLR conjugate (OVA-MCLR or biotin-MCLR) immobilization were evaluated. The results are shown in Fig. 3.

When MCLR was immobilized onto the chip functionalized with a carboxylic-SAM by covalent binding through the amino groups (surface VII), a high SPR signal was obtained, but it was mainly due to the non-specific binding of the antibody to the sensor surface. As follows from Fig. 3, this high non-specific adsorption of the antibody causes a minimal reduction of the signal in the presence of a high amount of MCLR in the sample. Therefore, this assay format was not sensitive enough to MCs. To improve

Fig. 2 Selected biosensor assay format and typical sensorgram for the detection of MCLR



the sensitivity of the assay, several parameters were optimized, such as the nature of the carrier buffer and blocking solutions before the detection step. However, no improvement in sensitivity was observed.

A poor SPR signal and a high degree of non-specific binding was also observed when a biotin-MCLR conjugate was immobilized onto a neutravidin carboxylic-SAM-modified chip surface (surface III.a). In this case, in the reference channels, the biotin-MCLR conjugate was replaced by biotin during the chip functionalization step. To improve the sensitivity, the covalent immobilization of neutravidin was replaced by a non-covalent immobilization via biotin-neutravidin interaction (surface III.b). Moreover, the use of streptavidin instead of neutravidin, or of different carrier buffers and/or blocking reagents, did not increase the assay sensitivity.

The best results in terms of sensitivity (Fig. 3) were obtained for the assay formats V (covalently cross-linked double layer of OVA-MCLR conjugate), VI (covalent immobilization of the OVA-MCLR conjugate), and VIII (immobilization of MCLR onto an amine-SAM-functionalized chip). However, the assay format VIII resulted in a higher SPR response, showing the maximal signal in the absence of MCLR (B_0). The effect of the concentration of the OVA-MCLR conjugate in both V and VI approaches was evaluated. Thus, the chips were functionalized with concentrations of 25, 50, and 75 $\mu\text{g mL}^{-1}$ of OVA-MCLR, but similar results in terms of response intensity were observed. The low SPR response when using OVA-MCLR may originate from the low ratio of MCLR per protein molecule. Considering the results obtained, we selected the assay format VIII, based on the immobilization of MCLR onto an amine-SAM-functionalized SPR chip, as the most appropriate for further biosensor development and optimization.

Biosensor optimization and characterization

Several parameters affecting the performance of the SPR biosensor have been evaluated: the concentration of MCLR in the immobilization solution, the nature of the carrier

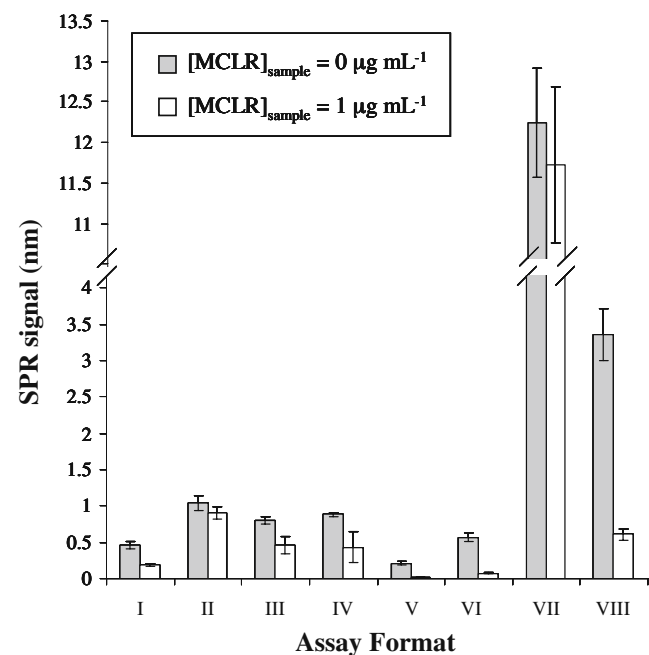


Fig. 3 Comparison of the different assay formats. (I) Covalent immobilization of the antibody via carboxylic-SAM; (II) covalently cross-linked antibody double layer; (III) immobilization of a biotin-MCLR conjugate on a neutravidin layer; (IV) physical adsorption of an OVA-MCLR conjugate; (V) covalently cross-linked OVA-MCLR double layer; (VI) covalent immobilization of an OVA-MCLR conjugate; (VII) covalent immobilization of MCLR via amino groups, and (VIII) covalent immobilization of MCLR via carboxylic groups ($n=3$)

buffer and regeneration solutions, concentration of the antibody, etc.

Optimization of the MCLR immobilization step

Two different experimental approaches have been applied for the covalent immobilization of MCLR onto the surface of the SPR chip, functionalized with an amine-thiols SAM: (1) immobilization in situ, within the SPR instrument, by flowing a solution of MCLR and EDC/NHS across the chip surface (see “Immobilization of MCLR,” b) and (2) a static immobilization by covering the chip-sensing area with the MCLR and EDC/NHS mixture and left incubate overnight at 4 °C.

Afterwards, calibration was performed using standard MCLR solutions, in the range from 0 to 1 $\mu\text{g mL}^{-1}$ and 0.2 $\mu\text{g mL}^{-1}$ of antibody. Although similar results, in terms of detection limit, IC_{50} and dynamic range were obtained, an important difference in chip stability was observed depending on MCLR immobilization approach. The chip resulting from the static immobilization was stable (retaining 98% of the initial response) after at least 15 detection–regeneration cycles. By contrast, the chip obtained by in situ immobilization showed a decrease of 40% in the response after ten assay–regeneration cycles. These results suggest that the static immobilization yield a higher amount of immobilized MCLR than the in situ immobilization. On the other hand, it is also possible that the MCLR immobilized by the in situ procedure was partly adsorbed and not covalently bound to the surface. Therefore, the adsorbed MCLR could be easily washed out after several regenerations steps, which would explain the stabilization of the SPR signal in a 60% of the initial response after ten assay–regeneration cycles. Unfortunately, the small size of the MCLR does not allow real-time monitoring of the amount of immobilized analyte. Therefore, the static immobilization was selected for further studies.

No signal was detected when a solution of MCLR in the absence of EDC/NHS was injected in the reference channels.

The concentration of the MCLR solutions used for chip functionalization was also evaluated. An increase of the MCLR concentration led to a lower LOD and a wider dynamic range (see Fig. 4). Therefore, an MCLR concentration of 160 $\mu\text{g mL}^{-1}$ was chosen for chip functionalization. Concentrations higher than 160 $\mu\text{g mL}^{-1}$ were not tested to balance the obtained improvement of the analytical characteristics of the biosensor and consumption of costly and toxic MCLR.

Immunoassay optimization

Influence of the carrier solution We have tested several buffer composition, such as HEPES, Tris, PB, PBS,

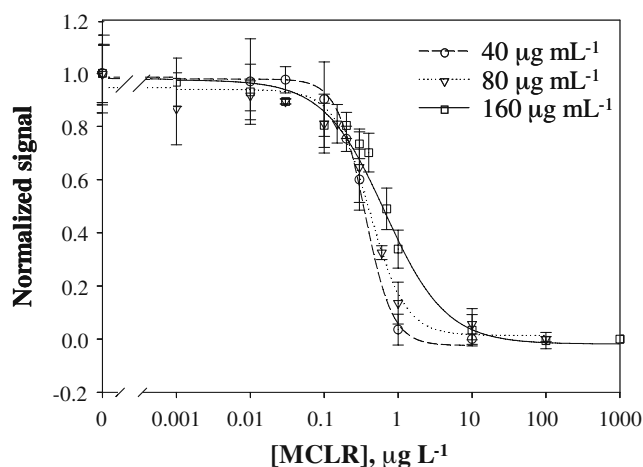


Fig. 4 Competitive inhibition curves as a function of the concentration of the MCLR solution used for chip functionalization ($n=3$)

glycine-NaOH (Gly-NaOH), and bicarbonate (HCO_3^-), at a concentration of 10 mM and pH 7.4.

The SPR signal intensity was measured, both in the absence (B_0) and in the presence (B) of a 1- $\mu\text{g L}^{-1}$ MCLR in solution. The results depicted in Fig. 5 show that PBS provided the highest sensitivity (in terms of lowest B/B_0 ratio) and the highest B_0 of all studied buffers. This finding can be attributed to the presence of small cations, such as Na^+ or K^+ , which favor a closer interaction between the epitope and the paratope and increase the stability of the immunocomplex [36]. The effect of pH within the range from 5.0 to 9.0 was also studied, but no significant differences were observed. Therefore, PBS at pH 7.4 was selected as the carrier solution for further experiments.

Influence of the nature and concentration of the antibody Four different anti-MCLR monoclonal antibodies (from Abkem Iberia, Alexis, and HyTest Ltd.) were tested. C64C12 and C64A1 monoclonal antibodies from Hytest showed the

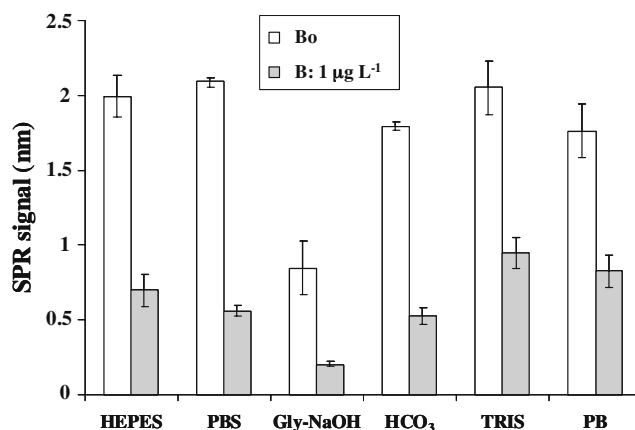


Fig. 5 Influence of the carrier solution on the B/B_0 ratio ($n=3$)

lowest B_0 signal (<0.2 nm), whereas the highest B_0 signal was measured when Abkem antibodies were used (>5 nm). Nevertheless, the latter was mainly due to the non-specific binding of the antibody to the chip surface, which was also observed in the control channels (chip surface functionalized with the amine-SAM, but in the absence of immobilized MCLR). The best results, in terms of sensitivity (lowest B/B_0 ratio), were obtained when using MC10E7 monoclonal antibodies from Alexis that provided a sufficient B_0 signal (between 2.5 and 3 nm) with a low non-specific binding to the chip surface ($\sim 20\%$ of the measured signal) and were selected for further experiments.

In order to reduce the non-specific binding and avoid false negative results, several blocking solutions have been assayed including, SuperBlock® T20, casein, gelatin, and BSA solutions, all prepared in PBS at a flow rate of $30 \mu\text{L min}^{-1}$. A 5-min treatment with the SuperBlock® T20 solution provided a slightly higher SPR sensor response compared to the other blocking agents.

The effect of the antibody concentration (0.1 to $0.5 \mu\text{g mL}^{-1}$) was evaluated in the presence of different MCLR solutions (0 to $1 \mu\text{g mL}^{-1}$; Fig. S1 electronic supplementary material). A decrease of the antibody concentration from 0.5 to $0.2 \mu\text{g mL}^{-1}$ yields to lower IC_{50} , detection limits, and wider dynamic ranges; however, concentrations below $0.2 \mu\text{g mL}^{-1}$ led to the weakest response and lower precision. Thus, a concentration of $0.2 \mu\text{g mL}^{-1}$ was selected for further experiments.

Regeneration solution Four different media were tested for sensor regeneration, namely, NaCl 1 M, glycine-HCl buffer (0.1 M, pH 2.0), HCl, and NaOH solutions.

The efficiency of the regeneration step was determined by measuring the amount of antibody that remains bound to the chip surface after a 5-min regeneration step. The use of NaCl yielded the highest percentage of residual antibody bound to the surface: over 85% against 34% and 26% for 30 mM HCl and 30 mM NaOH, respectively. The glycine-HCl buffer did not improve these results. Increasing the NaOH concentration from 30 to 60 mM led to a more effective antibody removal. Finally, efficient regeneration ($99 \pm 5\%$) of the sensor surface was achieved using 50 mM NaOH ($30 \mu\text{L min}^{-1}$, 15 min).

The optimized assay conditions are summarized in Table 1.

Analytical characteristics

The SPR instrument used in this work incorporates a four-channel chamber, thus allowing the measurement of four samples simultaneously. The reproducibility of biosensor was evaluated both, among channels and cycles. The

Table 1 Summary of the optimized immunoassay conditions

Parameter	Studied range	Optimized conditions
[MCLR] for immobilization, $\mu\text{g L}^{-1}$	40–160	160
Anti-MCs antibody brand	Alexis AbKem Iberia Hytest	Alexis
Concentration of anti-MCs antibody, $\mu\text{g mL}^{-1}$	0.1–0.5	0.2
Pre-incubation time, min	10–60	10
Carrier solution	PB PBS HEPES Tris Glycine Bicarbonate	PBS
Carrier solution pH	5.0–9.0	7.4
Blocking solution	Superblock T20 Casein BSA Gelatin	Superblock T20
Regeneration solution	NaCl HCl glycine-HCl NaOH	NaOH (50 mM)
Chip stability	–	≥ 40 cycles

reproducibility of the biochips was investigated under the optimized conditions by monitoring the SPR signal in the absence of MCLR (B_0 ; nine to ten cycles per day) over 6 days. The biochips were stored at 4°C protected from light when not used.

The change in biosensor response was negligible after 40 assay–regeneration cycles (in total, 160 measurements), maintaining about 95% (RSD, $<13\%$) of the original B_0 value. The reproducibility among channels was excellent with RSDs values below 7%. The reproducibility among biochips ($n=4$) was found to be in the range from 5% to 15% (depending on the MCLR concentration).

Figure 6 shows a typical competition inhibition curve measured under the optimized conditions using MCLR standard solutions in the range from 0 to $1,000 \mu\text{g L}^{-1}$. The IC_{50} value and the LOD were $0.67 \pm 0.09 \mu\text{g L}^{-1}$ and $73 \pm 8 \text{ ng L}^{-1}$, respectively. The dynamic range (normalized signal in the range from 20% to 80%) was from 0.2 to $2.0 \mu\text{g L}^{-1}$.

The sensitivity of the developed SPR biosensor was superior to that previously reported for other SPR-based devices [29], and the LOD is comparable, or even lower, than those reported for other immunosensors [26–28, 31, 37]. Moreover, it presents other advantages, such as the

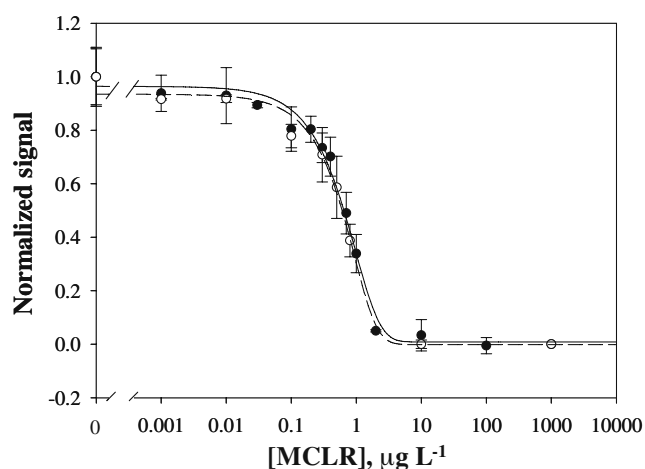


Fig. 6 Competitive calibration curves obtained with the SPR immunosensor for (closed circles) MCLR standard solutions in PBS (10 mM, pH 7.4) and (open circles) MCLR standard solutions prepared in tap water ($n=3$)

possibility of real-time and label-free monitoring up to four samples simultaneously.

Cross-reactivity

Among the 80 MC variants identified up to date, MCLR, MCRR, and MCYR are the most common [1]. Therefore, the cross-reactivity for MCRR and MCYR was also tested, and the results are summarized in Table 2.

The selected MC10E7 monoclonal antibodies show group specificity for [4-arginine]-MCs, as it has been already reported [38]. Therefore, the high cross-reactivity exhibited for MCRR and MCYR can be explained considering the presence of the 4-arginine residue and the Adda group, the common moiety of MCs that seems to play an important role for antibody recognition. Since the antibody recognizes MCLR, MCRR, and MCYR with similar sensitivities, the biosensor allows the quantification of the total concentration of the three most common MCs in water samples.

Analysis of tap water samples

The SPR biosensor has been successfully applied to the determination of MCLR in drinking water samples. Tap

water samples were spiked with MCLR, in the concentration range from 0 to 1,000 $\mu\text{g L}^{-1}$, and further analyzed under the optimized conditions. The samples were checked previously to contain undetectable levels of the toxin. A comparison of the dose–calibration curves obtained both in tap water and ultrapure Milli-Q water (Fig. 6) show that there is no matrix effect, and therefore, the developed immunosensor allows direct determination of MCLR in these samples.

Table 3 shows the results obtained with SPR biosensor. Mean recoveries of analyzed samples were $105 \pm 17\%$. These results demonstrate that the developed biosensor is suitable for the analysis of MCLR at levels below the provisional guideline value for drinking water proposed by WHO (1 $\mu\text{g L}^{-1}$ for MCLR) without the need of previous sample cleanup and pre-concentration.

Conclusions

An SPR biosensor has been developed that allows the determination of MCLR in drinking water samples in a fast and sensitive way. The biosensor working principle is based on a competitive inhibition assay, in which the analyte (MCLR) has been covalently immobilized onto the SPR chip surface functionalized with an amine-SAM. The new immunosensor allows detection of MCLR at levels below the provisional guideline value of 1 $\mu\text{g L}^{-1}$ proposed by WHO. Moreover, it shows high stability, reusability (at least 40 assay–regeneration cycles with the same chip), and excellent precision. The configuration of the SPR instrument used for this work incorporates a four-channel chamber, thus allowing the measurement of four samples simultaneously with short analysis times (one complete measurement–regeneration cycle can be accomplished in 60 min). The IC_{50} and the LOD were of 0.67 $\mu\text{g L}^{-1}$ and 73 ng L^{-1} , respectively, clearly lower than those reported for other SPR-based devices. Besides MCLR, the biosensor has shown excellent cross-reactivity for other MCs variants, such as MCRR and MCYR, which are together with MCLR, the most abundant in the environment.

Finally, the MCs biosensor has been successfully applied to the direct analysis of tap water samples without any cleanup or pre-concentration steps.

Table 2 Cross-reactivity study ($n=3$)

Analyte	LOD, $\mu\text{g L}^{-1}$	IC_{50} , $\mu\text{g L}^{-1}$	DR, $\mu\text{g L}^{-1}$	CR (%)
MCLR	0.073 ± 0.008	0.67 ± 0.09	0.2–2.0	100
MCRR	0.24 ± 0.06	0.76 ± 0.02	0.4–1.4	88 ± 3
MCYR	0.20 ± 0.04	0.72 ± 0.04	0.3–1.5	94 ± 5

Table 3 Analysis of tap water samples ($n=3$)

MCLR spiked level, $\mu\text{g L}^{-1}$	Detected concentration, $\mu\text{g L}^{-1}$	Recovery (%)
0.3	0.37 ± 0.07	123
0.5	0.44 ± 0.12	88
0.8	0.83 ± 0.03	103

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