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Portable sensing system based on electrochemical impedance spectroscopy for the simultaneous quantification of free and total microcystin-LR in freshwaters

Marília Barreiros dos Santos[a,1], Raquel B. Queirós[a,1], Álvaro Geraldes[a], Carlos Marques[a], Vânia Vilas-Boas[a,2], Lorena Dieguez[a], Elvira Paz[a], Ricardo Ferreira[a], João Morais[b], Vitor Vasconcelos[b,c], João Piteira[a], Paulo P. Freitas[a], Begoña Espiña[a,*]

[a] INL, International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-330, Braga, Portugal
[b] CIIMAR, Interdisciplinary Centre of Marine and Environmental Research of the University of Porto, 4450-208, Matosinhos, Portugal
[c] FCUP, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4069-007, Porto, Portugal

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ABSTRACT

Microcystins are the most worldwide extended and common toxins produced by cyanobacteria in freshwater. Microcystin-leucine arginine (MC-LR), associated with the most toxic incidents involving microcystins, are within the cyanobacteria (intracellular) until released into the surrounding waters (extracellular) during cell lysis. Therefore, the relationship between intracellular and extracellular cyanotoxins will allow a comprehensive risk of cyanobacteria-containing waters, preventing disease and improving human safety. In this work, we present the development of a novel portable microfluidic sensing platform for the simultaneous detection of free (extracellular) and total MC-LR (intracellular and extracellular). The integrated system contains the sample processing and detection modules capable of performing the chemical lysis, filtration, sample mixing with antibodies, and electrochemical detection of MC-LR based on an indirect strategy. The performance of the immunosensors was evaluated by electrochemical impedance spectroscopy, showing a linear dynamic range between 3.3×10^{-4} and 10^{-7} g L^{-1} and a limit of detection of 5.7×10^{-10} g L^{-1}. The results demonstrate the potential of the developed portable biosensor platform and its suitable application for the analysis of MC-LR at regulated levels for drinking water. Finally, the integrated system was able to simultaneously detect the free and total MC-LR on a *Microcystis aeruginosa* culture. To the best of our knowledge this is the first described system that can differentiate between intracellular and extracellular concentration of MC-LR. This novel electrochemical sensing platform avoids the multiple processing steps typically needed for standard MC-LR analysis in the laboratory and provides an early warning system for MC-LR remote monitoring in water.

1. Introduction

Harmful algal blooms (HABs) and harmful cyanobacterial blooms (cyanoHABs) compose a major threat to aquatic resources. They occur under a wide range of environmental conditions and have been increasing their intensity, frequency and geographic distribution due to global climate change, rising agricultural activities, and introduction of invasive species (Bykova et al., 2006; Carey et al., 2011; Paerl and Huisman, 2009; Preece et al., 2017; Visser et al., 2016). The most frequent and widespread cyanobacterial toxins are microcystins (MCs). This group of hepatotoxins (liver damaging) can promote the formation of hepatic tumors upon the prolonged ingestion of contaminated water. They can also affect kidneys and the gastrointestinal tract as well as provoke headache, nausea, vomiting, and abdominal pain (Bell and Codd, 1994). Due to the toxicity of MCs, in particular Microcystin-leucine arginine (MC-LR), the World Health Organization (WHO) set a guideline value of 10^{-6} g L^{-1} for total MC-LR in drinking water, since this is the major route to human exposure (WHO, 2003).

With the proliferation of toxic cyanoHABs and the increase of their severity (Preece et al., 2017), the awareness of this hazard has

* Corresponding author.
E-mail address: begona.espina@inl.int (B. Espiña).
[1] both authors contributed equally to the work.
[2] Present address: Department of In Vitro Toxicology and Dermato-Cosmetology, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium.

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increased (Wang et al., 2009). A broad diversity of methods have been recently reported for their detection (Gan et al., 2016; Moore et al., 2016; Tan et al., 2015; Weller, 2013; Zhang et al., 2018). For a more detailed information we recommend the consultation of the following reviews (Kaushik and Balasubramanian, 2013; Reverte et al., 2016; Vogiazi et al., 2019). Although all these methods are very sensitive and suitable for the detection and quantification of MC-LR, they do not satisfy the demanding requirements for the routine monitoring of HABs in terms of lack of portability, inexpensive instrumentation, user-friendliness, little or no sample pre-treatment, and low-cost (Wang et al., 2009). Importantly, there is no suitable method yet for the detection of cyanotoxins in freshwaters with the required frequency and accuracy needed to guarantee water quality and minimize the potential risk to human health (Kaushik and Balasubramanian, 2013; Wang et al., 2009). In the past years, several works have been focusing on automated systems for MC-LR detection. Herranz et al. (2012) described an automated portable array biosensor for microcystin analysis, a commercial system for the detection of the contaminants. A quite similar device proposed by Lui et al. shows an integrated channel waveguide-based fluorescent immunosensor with the ability to detect a maximum of 32 contaminants simultaneously. Both those works required manual sampling and processing and only evaluate the free toxin content. Zhang et al. (2011) presented an immuno-enzyme assay integrated into a microfluidic chip for automatic analysis of algal toxins that performs all the chip operations to complete the immunoassay. However, the system does not allow the direct analysis of water samples and is also not portable, requiring a fluorescence microscope for the detection of the algal toxins. Lebogang et al. (2017) present a sequential micro flow-ELISA based on amperometric detection using a fully automated and computer-controlled system. This amperometric immunoanalysis system was developed for the quantification of MC-LR but it also only analyses the free toxin content and does not allow the direct analysis of water samples as it uses a commercial bench-top potentiostat for the electrochemical quantification. The information regarding the total content is of upmost importance since the recommendation level of MC-LR in drinking water. Bickman and co-workers (Bickman et al., 2018) developed an innovative portable biosensor system for the total content analysis of MC-LR including a portable lysis module based on mechanical disruption of the cells. Although the mechanical method is very effective the lysed sample should be added manually to the detection system, limiting its applicability in unassisted monitoring.

Monitoring MCs levels in environmental waters is quite difficult and challenging (Wang et al., 2009). These toxins are secondary metabolites, which are formed at all stages of cyanobacterial growth. In most cases, they are within the cells (intracellular) until released into the surrounding waters (extracellular) during cell lysis due to stress or age (EPA, 2014; Kaushik and Balasubramanian, 2013; Wang et al., 2009). The relation between intracellular and extracellular presence of cyanotoxins is important for a comprehensive risk assessment of cyanobacteria-containing waters, whether for drinking water supply or recreational activities. To overcome these difficulties, it is necessary to develop new methods combining high sensitivity, portability and easiness of sample processing to be able to analyse the full amount of toxin present in the water column (extracellular and intracellular content).

The present work describes a portable system to detect (i) the total MC-LR content (intracellular and extracellular) and (ii) the free MC-LR toxin (extracellular) in water, see Fig. 1. The portable system comprises a sample processing module (a) and a detection module (b) using electrochemical immunosensor. The sample processing module consists on a set of sample preparation operations, namely lysing, filtering and mixing. The microfluidic mixers coupled with micro-reservoirs (μFM-μR) containing lyophilised molecules allow the sample and reagents mixing prior to the measurement. The sensing principle is based on electrochemical impedance spectroscopy (EIS) using electrochemical-cell-chips (ECCs), which allow multiple and independent measurements. The detection strategy is based on an indirect approach, in which MC-LR is covalent immobilised onto the gold electrode. The toxin present in the sample competitively inhibits the binding of an anti-MC-LR antibody to the MC-LR modified surface. The surface functionalisation of the ECCs was characterised and the analytical performance tested by increasing concentrations of MC-LR. The integrated system was used to the simultaneous analysis of the free and the total MC-LR toxin in *Microcystis aeruginosa* culture and the results were validated by enzyme-linked immunosorbent assay (ELISA). The results demonstrate the potentiality of the developed sensing platform and its suitable application for the analysis of MC-LR at regulated levels for drinking water. To the best of our knowledge, this is the first system able to perform the simultaneous quantification of intracellular and extracellular MC-LR in freshwater.

2. Methods

2.1. Reagents and solutions

Sodium phosphate monobasic monohydrate (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), phosphate buffered saline (PBS), spermine, cysteamine, fluorescent antibody (Anti Mouse IgG-Atto 655), lysozyme, pluronic acid and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sulfo-NHS (N-hydroxysulfosuccinimide) and N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide hydrochloride (EDC) were purchased from Thermofisher (Massachusetts, USA). Anti-MC-LR monoclonal antibody, MC-LR, Microcystin-YR (MC-YR) and Microcystin-RR (MC-RR) were purchased from Enzo Life Sciences (NY, USA). Isopropanol (IPA) and acetone were purchased from Honeywell. Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) was purchased from Ellsworth adhesives Iberica (Madrid, Spain), while PMMA (Poly (methyl methacrylate)) was purchased from Plexicril (Braga, Portugal). *Microcystis aeruginosa* cells (LEGE 91094), microalgae that produce MC-LR, were provided by CIIMAR (University of Porto, Portugal) and were cultured in Z8 medium.

Phosphate buffer (PB, 0.01 M, pH 7.4) was used for all the experiments and it was prepared by mixing solutions of NaH_2PO_4 (Sigma Aldrich) and Na_2HPO_4 in Milli-Q water. The electrolyte solution used was 5 mM $[Fe(CN)_6]^{3-/4-}$ in 10 mM PBS solution (pH 7.4).

2.2. Sample processing module

The sample processing module contains several components, including micro-reservoirs (μR), microfluidic mixers (μFM) and ultrafilters, used in sample pretreatment (Fig. 1(a)). For the total MC-LR content (i), a lysis buffer (LB) is used to disrupt the cyanobacteria and release their intracellular content. The LB is lyophilised in a μR and mixed with the sample by means of a μFM. Since simultaneous analyses are performed, a μFM-μR is used as a control device for the free toxin (ii) in order to obtain the same experimental conditions than in part (i). Ultrafilters (pore size: 0.2 μm) were used for both total and free MC-LR pathways to remove the bigger sized particles that could affect the measurement. The filtered sample flows through the μR for the resuspension of the lyophilised antibody solution. Next, it flows through the serpentine mixer (Yoon and Kim, 2012) to ensure a complete mixing of the solution. The scheme of the mixing system is shown in Fig. 2(a).

The components of the sample processing module were fabricated applying standard microfabrication techniques and replica moulding using PDMS (Fig. 2(b)). The μFM-μRs were assembled by O_2 plasma bounding of the PDMS mixers to a glass substrate, and subsequently bounding the μR over the mixers. Two μFM-μR connected by tubbing and incorporating an ultrafilter were used per measurement (see Fig. 2(c) and A.1.2 for details in the fabrication and assembly).

The μFM-μRs were passivated with a solution of 1% pluronic acid in PBS, washed and dried, before the lyophilisation to prevent the

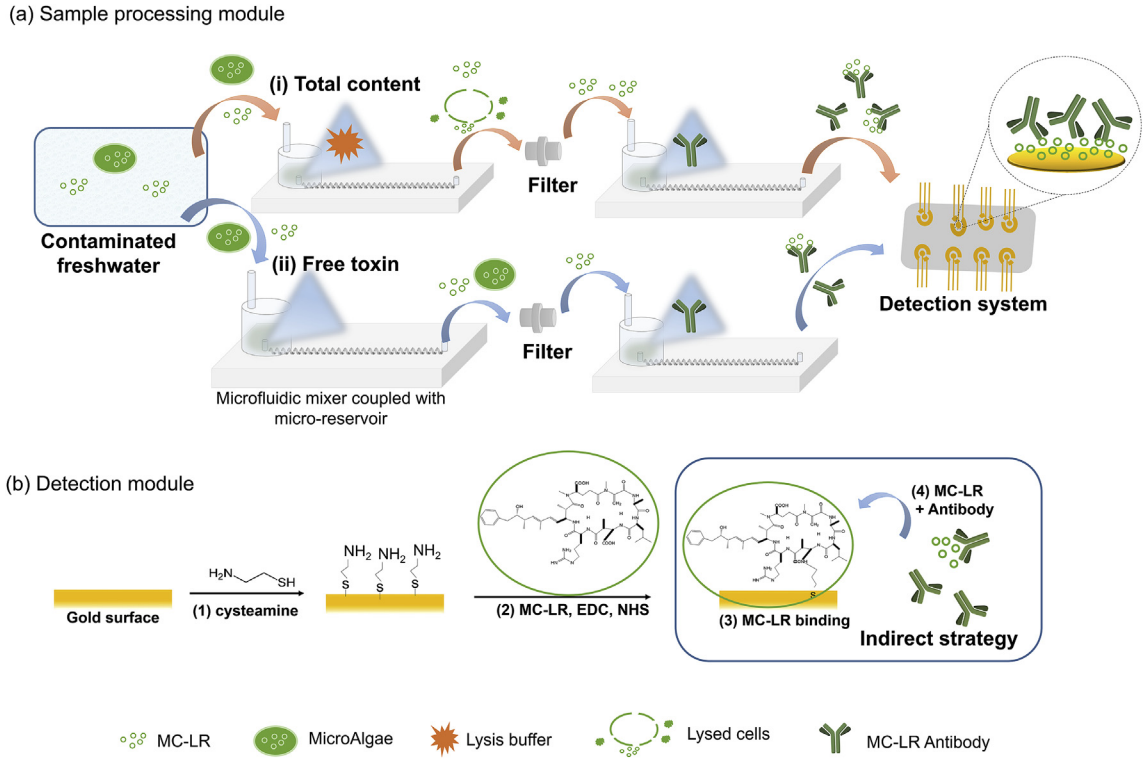

Fig. 1. Schematic representation of the complete system for the detection of MC-LR integrating the sample processing (a) and detection (b) modules. The sample processing system consist on two microfluidic mixers coupled with reservoirs (μFM-μR) used for the freshwater cyanobacteria sample processing with (i) and without (ii) lysis buffer. The sample was filtered and incubated with the antibodies lyophilised on a second μFM-μR before it reached to the detection module. (b) Diagram for the immunosensor fabrication: (1) SAM with cysteamine, (2) MC-LR activation and (3) MC-LR binding to the SAM-modified surface. An indirect strategy was used to detect MC-LR by adding different concentrations of MC-LR pre-incubated with antibody.

adsorption of the molecules on the PDMS. The solutions of LB (5 g L^{-1} lysozyme + 20 g L^{-1} spermine) and anti-MC-LR antibody in BSA 0.001% were lyophilised on each corresponding μR using a freeze dryer (Telstar–LyoQuest). For that, 150 μL of the solution were incorporated on the respective reservoir, frozen for 1 h at −80 °C, and then placed on the freeze dryer for 12 h. The cell disruption created by the LB was verified using flow cytometry S3™ (see A.1.3). Its efficacy was studied by the quantification of the initial value of MC-LR on the sample (free toxin) and the total amount of toxin after lysis (total content) using ELISA (see A.1.4). The amount and functionality of antibody during the lyophilisation process was also evaluated by ELISA.

2.3. Detection module

2.3.1. Design and fabrication of the electrochemical-cell-chips

The MC-LR detection module is based on an indirect strategy using antibodies (Fig. 1(b)) by an EIS transduction system. The ECCs consist on an array of 8 independent electrochemical cells with 1000 μm diameter working electrodes (WEs), using a 3-electrodes configuration, allowing multiplex and independent detection with statistical significance (Fig. 3(a)). ECCs were produced using standard microfabrication technologies, onto 200 mm diameter silicon wafers (crystalline silicon coated with 100 nm thermal oxide from Si-Mat, Germany). The devices were fabricated on a thin layer of gold (150 nm) deposited by physical-vapor deposition using a multi-target confocal

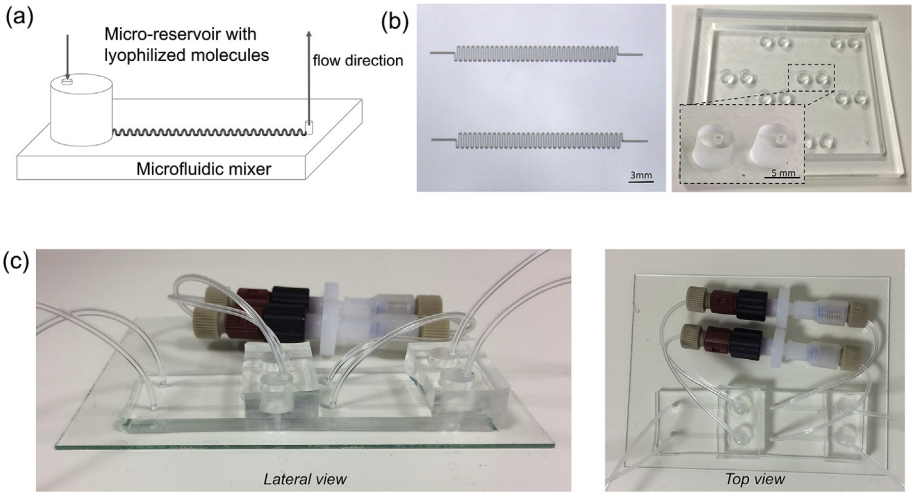

Fig. 2. (a) Scheme of the design for the microfluidic mixers and micro-reservoirs (μFM-μR) used to mix the lyophilised molecules with the sample. (b) Silicon master used for mixers fabrication (*left*) and PMMA mould fabricated to produce the micro-reservoirs in PDMS (*right*). (c) Complete sample processing system containing the μFM-μRs interconnected by an ultrafilter of 0.2 μm.

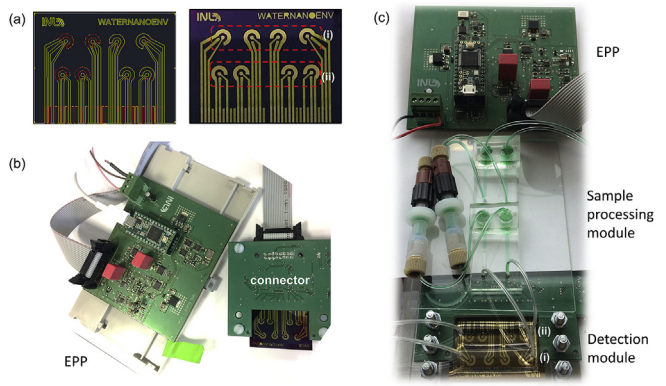

Fig. 3. (a) Electrochemical-cell-chips development: design (*left*) and fabricated chip (*right*); (b) EIS measurements were performed by a custom-made electrochemical impedance portable platform (EPP) (*left*) and ECCs connectors (*right*). The EPP has one controller/measurement PCB board build around the impedance analyzer integrated circuit by Analog Devices (AD5933) and a 32 bit ARM processor (excitation voltage 5 mV, potential applied 0 V, frequency range: 0.5–100000 Hz, 53 data points); The sensors were connected to the potentiostat through a connector support and electronics system composed by a PC board with dedicated analog front end for the sensor chip. (c) Complete portable system with the sample processing module, the detection module (chip and microfluidic reaction chambers (i and ii) defined by PDMS O-rings) and the EPP.

sputtering tool (Kenosystec, Italy). The lithography was performed by direct write laser lithography (Heidelberg DWL 2000, Germany) followed by ion milling etching process (Nordiko 7500, UK). An insulator Al_2O_3 layer was deposited by magnetron sputtering (Singulus Timaris FTM, Germany) on top of the devices. A second step of photolithography and etching define the active area and the contacts.

2.3.2. Electrochemical-cell-chips surface functionalisation and characterisation

Before the functionalisation, ECCs were cleaned with acetone in an ultrasounds bath for 90 min and dried with N_2. Afterwards, the ECCs were cleaned with air plasma during 2 min to remove the organic contaminants from the gold surface. Finally, the ECCs were rinsed with IPA and Milli-Q water and dried with N_2. For the functionalisation, the WE was covered with 1 µL of 18 mM cysteamine in Milli-Q water for 4 h in dark and humid atmosphere conditions. The excess solution was rinsed with Milli-Q water. The MC-LR was immobilised onto the self–assembled monolayer (SAM)-modified WE using an activated solution of MC-LR (10^{-2} g L^{-1}) overnight at 4 °C in a humid atmosphere. The activation of the MC-LR was performed by using sulfo-NHS (20 mM) and EDC (80 mM) at room temperature for 30 min at 350 rpm (thermomixer, Eppendorf). Finally, the sensors were rinsed with Milli-Q water and dried with N_2. The surface functionalisation was characterised by quartz crystal microbalance (QCM), atomic force microscopy (AFM), scanning electron microscopy (SEM), cyclic voltammetry (CV) and EIS (see A.1.5).

2.3.3. Microcystin-LR detection and quantification by electrochemical impedance spectroscopy

The EIS measurements for MC-LR quantification were performed by using a custom-made electrochemical impedance portable platform (EPP) (Fig. 3(b)). The EIS measurements were performed applying a sinusoidal perturbation of 0.005 V within the frequency range of 0.5–100,000 Hz. Data acquisition on the host computer was performed with a serial terminal (Termite, CompuPhase). The R_{ct} values were obtained from a fit module on the EC-Lab software (Bio-Logic SAS) to adapt the data to the Randles equivalent circuit [R (CPE[RW])]. The ECCs analytical performance was tested by EIS against known concentrations of MC-LR and antibody. First, the antibody (10^{-2} g L^{-1}) was incubated with different concentrations of MC-LR ($3.3. \times 10^{-4}$–10^{-7} g L^{-1}) in PB solution at RT, shaking at 350 rpm for 30 min. Then, the MC-LR functionalised sensors were exposed for 1 h to different concentrations of the mixture of MC-LR and anti-MC-LR at RT. Before the measurement, the sensors were rinsed with PB and incubated with the redox probe solution (0.005 M $[Fe(CN)_6]^{3-/4-}$ in PBS). The same procedure was performed with MC-YR, MC-RR and okadaic acid (OA) (10^{-4} g L^{-1}) to evaluate the biosensor selectivity.

2.4. Portable system for sample processing and detection of Microcystin-LR

The final integrated system consists of the assembling of the sample processing module, the detection module and the EPP (see Fig. 3(c)). Two independent chambers (i and ii), containing four electrochemical cells each, were used for the EIS measurements. The chambers were delimitated by PDMS O-rings and PMMA holders, and their volume was ~125 µL. PDMS O-rings were fabricated by replica moulding as described on A.1.2, the O-ring mould and PMMA pieces were designed in ArtCAM and fabricated in PMMA using a Computer Numerical Control (CNC) - High Speed Milling System (FlexiCam Viper, Germany). The top part of the PMMA piece was connected to the inlet tubbing (1.5 mm O.D., Tygon tube) for each chamber. Finally, the sample processing module was connected to each chamber by the individual tubbing creating the complete portable system.

A sample of a *Microcystis aeruginosa* culture (3.8×10^6 cells mL^{-1}) was used for the validation of the portable system. The diluted 10× sample was injected by using a syringe pump and incubated over the sensors for 1 h. Then, the detection chambers were washed with PB and injected with a redox probe solution for EIS measurement using the EPP according to the conditions described in section 2.3.3. The concentration of the free and total content MC-LR presence on the *Microcystis aeruginosa* sample was also quantified by ELISA according to A.1.4.

3. Results and discussion

3.1. Evaluation of the sample processing module

The sample processing module consists on a set of µFM-µRs and filters that allows the lysis of cyanobacteria, filtering and sample mixing with antibodies (see Fig. 1). The sample processing module can perform rapid and parallel pretreatment of the samples in order to obtain the total and free MC-LR toxin content. The total content is determined by the free content plus the toxin content released by the cyanobacteria lysate. A chemical lysis method was chosen due to its simplicity and ability to chemically process the sample in one-step that can easily be integrated in our system. Lysozyme and spermine were the enzymes and chemicals chosen for the LB (Mehta et al., 2015). The efficacy of the LB (5 g L^{-1} lysozyme + 20 g L^{-1} spermine) was evaluated on *Microcystis aeruginosa* cells by using flow cytometry to study the disrupted cells and by ELISA to quantify the amount of MC-LR on the samples (see A.2.1.1). The cytometer results showed an increase of 50% of dead cells after the disruption of the viable cells using the LB method. ELISA results showed that the concentration of the MC-LR increased up to 0.012 g L^{-1} using the LB chemical method. The efficiency of the LB compared with the ultrasounds method in MC release correspond to 59% of the MC-LR concentration present in the *Microcystis aeruginosa* culture. The results demonstrated that this simple lysis method can release great part of the intracellular MC-LR and thus its suitability to be integrated in our sample processing module.

The effect of the lyophilisation on the antibody and the efficiency of antibody re-suspension during the µFM-µR was also studied by ELISA using the same antibodies and functionalisation strategy applied to the sensor. The recovery obtained for the antibody solution lyophilised in the µFM-µR compared to the initial solution was 105% (A.2.1.2.). These results demonstrated that the lyophilisation processes do not affect the antibody functionality and there are no significant losses during the re-

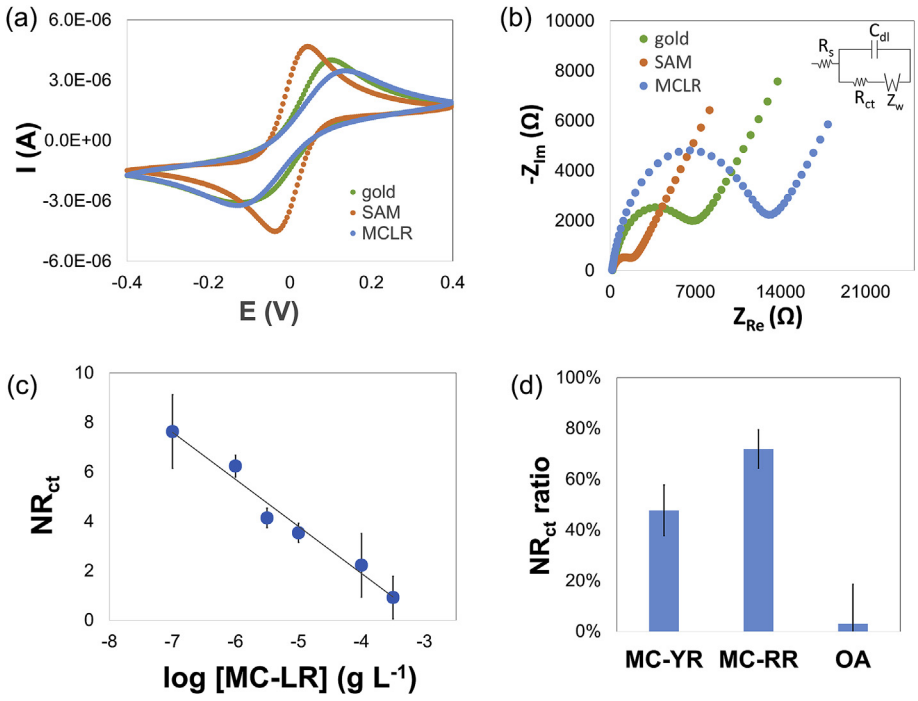

Fig. 4. (a) Typical cyclic voltammogram and (b) Nyquist plot obtained for the surface functionalisation steps, (inset) Randles equivalent circuit model used for EIS fitting, where R_{ct} is the charge transfer resistance, Z_w is the Warburg impedance, R_s is the resistance of the solution and for a better fitting the constant phase element (CPE) replaces a double layer capacity (C_{dl}). (c) Normalised signal of R_{ct} versus MC-LR concentrations (concentration of antibody 10^{-2} g L^{-1}). (d) Percentage of the NR_{ct} signal obtained with other toxins (MC-YR, MC-RR, OA) for the same concentration (10^{-4} g L^{-1}). The 100% correspond to the inhibition of the signal obtained with MC-LR. Data are shown as mean ± SD, n = 3.

suspension on μFM-μR. Furthermore, this process will ensure long-term stability of the reagents, ideal for portable detection system.

3.2. Functionalisation process characterisation

The functionalisation process of the gold electrode is shown in Fig. 1 (b). First, a monolayer of cysteamine was self-assembled on the clean gold surface, making use of the strong gold-thiolate interaction (1). Afterwards, the carboxylic acid functionalities of the MC-LR were activated by the reaction with a mixture of EDC and sulfo-NHS (2), which react with the amine groups of the self-assembled monolayer (SAM) (3). The surface functionalisation was firstly monitored using QCM (A.2.2.1), AFM and SEM (A.2.2.2.), and afterwards characterised by CV and EIS. The typical cyclic voltammogram obtained for the surface functionalisation steps is shown in Fig. 4 (a). The formation of the cysteamine monolayer onto the gold electrode led to an increase in the faradaic currents of CV due to an electrostatic attraction between the positively charged cysteamine monolayer (surface pK_a = 6.7) and negatively charged probe solution (Shervedani et al., 2007). The binding of MC-LR reduced the penetration of the redox probe and decreases the current response, suggesting the binding of the toxin to the surface. The Nyquist plot shown in Fig. 4(b) was fitted with the Randles equivalent circuit model (inset). The eight bare electrodes showed an average R_{ct} value of 6295 ± 940 Ω, with a coefficient of variation of 15%, indicating a good electrochemical signal reproducibility between the electrodes. The formation of the cysteamine SAM on the gold electrode led to a decrease on the R_{ct} (1825 ± 306 Ω) influenced by a high electrostatic attraction and a small increase in the thickness as also shown by Shervedani et al. (2007). A higher R_{ct} signal (17007 ± 4161 Ω) was obtained for MC-LR compared with the SAM, which demonstrates the successful binding of MC-LR onto the modified-SAM gold surface, as previously demonstrated by AFM, QCM and CV.

3.3. MC-LR detection and quantification by EIS

The EIS detection of MC-LR is based on an inhibition type-immunoassay: the binding of the MC-LR immobilised onto the sensor surface to a limited number of antibodies is inhibited by the MC-LR present in the sample that was allowed to react with the antibody before the exposure to the sensors.

In Fig. 4 (c) is shown the normalised signal of R_{ct} against varying concentrations of MC-LR. The R_{ct} of the redox probe on the faradaic impedance measurement reflects the amount of antibodies bound on the surface in a concentration-dependent manner. The correlation between R_{ct} and MC-LR concentrations were determined by measuring standard serial dilutions of MC-LR mixed with a constant antibody concentration. The sensor response was normalised (NR_{ct}) according to the following Equation (1):

$$NR_{ct} = (B - B_0)/B_0 \quad (1)$$

where B is the R_{ct} signal measured in presence of antibody and increasing MC-LR concentrations, and B_0 is the signal obtained in absence of MC-LR or antibody. The NR_{ct} signal decreased with the increasing concentration of MC-LR in the sample and it is inversely proportional to the logarithm of MC-LR concentration in the range between 3.3×10^{-4}–10^{-7} g L^{-1}, being the linear regression equation $NR_{ct} = -1.902 \times \log([\text{MC-LR}]) - 5.718$ (R^2 = 0.986). The limit of detection, LOD, was calculated accordingly to IUPAC definition (IUPAC, 1997) as $\chi_L = \chi_{bi} + 3xS_{bi}$, where S_{bi} is the standard deviation of blank measurement, and χ_{bi} mean of the blank and 3 is the numerical factor chosen according to the confidence level desired (95%). The developed biosensors obtained a LOD of 5.7×10^{-10} g L^{-1}, accomplishing the detection of a much lower concentration than the limit for MC-LR in drinking water (10^{-6} g L^{-1}) required by WHO provisional guideline. Previously reported sensors attained ultra-sensitive detection of up to 10^{-14} g L^{-1} (Ruiyi et al., 2013; Tang et al., 2018), however, their performance was usually not tested in environmental waters or toxic cyanobacteria cultures, always referring to free MC-LR concentrations.

The reproducibility of the MC-LR detection with the electrochemical sensors was also investigated for three concentrations of MC-LR. The relative standard deviations (RSDs) obtained in the intra-assays for 10^{-5}, 10^{-6} and 10^{-7} g L^{-1} ranging from 7% to 19% (n = 3), while the values for the inter-assays were between 14% and 36% (n = 3). These results indicated an acceptable reproducibility and feasibility of the sensors. The stability of the sensor, when stored at 4 °C, was as well evaluated comparing the mean of the higher expected NR_{ct} value (antibody) for 0, 1 and 2 days after the functionalisation. One-way ANOVA

results showed that the sample means did not differ significantly. The same conclusion was reached comparing the two means of the results obtained for the sensors tested on the same day of the functionalisation and after 12 weeks. These results indicate that the sensors presented a good stability and functionality over time.

3.4. Sensor selectivity

Finally, the detection system selectivity was addressed by exposing the sensor to other common toxic MCs (MC-YR and MC-RR) and to OA, belonging to another family of toxins which present the same toxic mechanism of action (cellular protein phosphatases inhibition). The percentage of the NR_{ct} signal obtained for the other toxins (10^{-4} g L^{-1}) is shown in Fig. 4 (d). The signal inhibition produced by other microcystins, MC-YR and MC-RR, was 47% and 73%, respectively. On the contrary, the signal inhibition produced by OA is much lower compared to the MC-LR (3%), demonstrating no specific interaction of the antibody with OA. The antibody used recognizes all the [4-arginine]-microcystins and, consequently, it recognizes MC-LR, MC-RR and MC-YR, as also demonstrated by Herranz et al., (2010). In fact, this could lead to an over-estimation of MC-LR concentration, depending on the congener present in the drinking water. MC-LR, MC-RR and MC-YR are among the most common analogues. MC-LR is the most toxic and common congener, and consequently, the most studied and currently the only one regulated. However, MC-RR and MC-YR are among the most frequently occurring analogues and relatively toxic as well (Díez-Quijada et al., 2019). This means that our system for monitoring of MC-LR allows for a better human safety protection. It is important to point out that although the main advantage of our system is the possibility of installing an unassisted device to predict the occurrence of microcystins blooms before reaching the regulated limit, once the alarm is triggered, the confirmation and proper identification and quantification of the analogues composition should be finally performed by the regulatory methods; HPLC-UV or LC/MS.

3.5. Validation of the integrated system for simultaneous detection of free and total MC-LR toxin

The validation of the portable system was performed using a *Microcystis aeruginosa* culture (initial concentration 3.8×10^6 cell mL^{-1}) in order to evaluate the system capability to simultaneously detect the free and the total toxin content in the sample. The sample was injected in the system, passed through both pathways (i) and (ii) in the sample processing module, and then through the detection module (see Fig. 1). The concentration of MC-LR, measured by the portable EIS sensing system, was 19.0×10^{-3} g L^{-1} for the total content and 9.0×10^{-3} g L^{-1} for the free toxin as shown in Table 1. For the ELISA method, the obtained concentration of MC-LR was 18.3×10^{-3} g L^{-1} for the total content, and 11.5×10^{-3} g L^{-1} for the free toxin. Comparing both methods, a recovery between 78 and 104% was obtained for the total and free toxin was obtained. The results for both methods were compared using a statistical *t*-test. The experimental value obtained was 0.04, a much lower value than the critical value ($t_{crit\ [5,\ 0.05\%]} = 2.57$), indicating that both methods are in good agreement for the determination of MC-LR concentration (total and free) in microalgae samples. The results showed that the free amount was about 2 times lower than the total content, corroborating the idea that *Microcystis aeruginosa* cells contain MC-LR inside the cell. This emphasizes the necessity of a novel method able to predict the release of MC-LR into freshwater when the cell dies, or the cell membrane ruptures.

Table 1
Validation of the integrated system: initial concentration values for the free and total MC-LR content in the *Microcystis aeruginosa* culture (3.8×10^6 cells mL^{-1}) using ELISA and EIS.

Detection method	$[MC\text{-}LR]_i$ (10^{-3} g L^{-1})	
	Total content	*Free toxin*
EIS	19.0	9.0
ELISA	18.3	11.5
Recovery (EIS/ELISA)	104%	78%

Finally, the information regarding the total content is of upmost importance since the recommendation level of MC-LR in drinking water, established by WHO, refers to the total amount of MC-LR (WHO, 2003). This presents a huge advantage of our system comparing to other devices described in the literature (Lebogang et al., 2017; Liu et al., 2017; Herranz et al., 2012; Zhang et al., 2011), which are only able to quantify the free toxin content. On the other hand, the previously reported system by Bickman et al. (2018) able to perform *in situ* total content analysis of MC-LR attained a 700-fold higher LOD.

4. Conclusions

This work shows the development of a portable device focused on the simultaneous detection of intracellular and extracellular content on MC-LR, one of the most common and toxic compounds produced by cyanobacteria in freshwater. The information of both the intra and extracellular content is very relevant because it could be used to take preventive measures to minimize the impact of the blooms. Considering that most part of the toxin remains inside the cells and only when the bloom is in senescence a higher quantity of toxin can be found free in the water, the knowledge about variations in both fractions provides very relevant information about the blooms' status. This analysis would enable a more comprehensive risk assessment of cyanobacteria-containing waters, predicting the amount of toxin that could be released to the water and detecting at the same time the total content of MC-LR as defined by the regulated limits.

To the best of our knowledge our system is the first described completely integrated system (sample processing and detection module) that can differentiate between intracellular and extracellular concentration of MC-LR. The efficiency of this system was good in terms of MCs recovery. The sensors' performance was stable, reproducible, presented a large linear range (3.3×10^{-4} to 10^{-7} g L^{-1}), a good specificity and a low LOD of 5.7×10^{-10} g L^{-1}. Consequently, this system could be used to monitor *in situ* cyanobacterial blooms and predict the release of toxin to the water at very low MC-LR concentrations. Additionally, this system is able to efficiently detect MC-RR and MC-YR as well, two of the most common toxic analogues, providing a broader safety protection in water quality monitoring.

Future work is envisioned for the adaptation of this system for unassisted monitoring with wireless communication of data to be included in decision support systems for water management bodies. Additionally, we will work on including multiplexing capabilities for the quantification of other relevant analogues such as MC-LA and other co-occurring freshwater toxins such as anatoxin-a, cylindrospermopsin or nodularin.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Marília Barreiros dos Santos: Conceptualization, Investigation, Validation, Formal analysis, Methodology, Writing - original draft. **Raquel B. Queirós:** Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing - review & editing. **Álvaro Geraldes:** Validation. **Carlos Marques:** Validation. **Vânia Vilas-Boas:** Methodology. **Lorena Dieguez:** Conceptualization, Methodology.

Elvira Paz: Methodology, Writing - review & editing. **Ricardo Ferreira:** Methodology, Writing - review & editing. **João Morais:** Resources, Writing - review & editing. **Vitor Vasconcelos:** Resources, Writing - review & editing. **João Piteira:** Methodology, Supervision. **Paulo P. Freitas:** Methodology, Supervision. **Begoña Espiña:** Conceptualization, Project administration, Resources, Funding acquisition, Writing - review & editing, Supervision, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111550.

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