DNA – Cyanobacterial Hepatotoxins Microcystin-LR and Nodularin Interaction: Electrochemical Evaluation

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Abstract
Microcystin-LR (MC-LR) and nodularin (NOD), two potent cyanotoxins with strong hepatotoxic, genotoxic and carcinogenic potential have been associated with the induction of deoxyribonucleic acid (DNA) damage in vitro and in vivo. Electrochemical studies were performed to understand the DNA interaction mechanisms with MC-LR and NOD using a dsDNA-electrochemical biosensor and incubated solutions. The decrease of the dsDNA oxidation peaks with increasing incubation time due to aggregation of DNA strands and the liberation of adenine residues, causing the occurrence of DNA abasic sites, was observed, which may introduce mutations in the dsDNA during the replication process.

Keywords: Microcystin-LR, Nodularin, DNA-electrochemical biosensor, DNA oxidative damage

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1 Introduction

The occurrences of cyanobacterial blooms in aquatic environments are of increasing concern in many lakes, rivers and brackish waters worldwide [1]. More than 40 genera of cyanobacteria are known to produce a variety of harmful compounds as secondary metabolites, called cyanotoxins [2].

Microcystin-LR (MC-LR) and nodularin (NOD) are among the most commonly reported toxins produced by cyanobacteria [3], Scheme 1. MC-LR is a heptapeptide with the chemical structure cyclo(d-alanine-l-leucine-d-MeAsp-l-arginine-Adda-d-glutamate-Mdha), Scheme 1A, where d-MeAsp represents the d-erythro-beta-methyl aspartic acid and Mdha is N-methyldehydroalanine [3]. The designation of MC-LR arises from the two variable amino acids in positions 2, l-leucine (L), and 4, l-arginine (R). The chemical structure of NOD is very similar to the one described for MC-LR, and consists of a pentapeptide with the structure cyclo(p-MeAsp-l-arginine-Adda-d-glutamate-Mdbh), Scheme 1B, in which Mdbh is 2-(methylamino)-2-dehydrobutyric acid [4]. In the chemical structures of MC-LR and NOD, Adda corresponds to the unique C20 beta-amino acid (3-amino-9-methoxy-2,6,8-trimethyl-10-phenydeca-4,6-dienoic acid) [5].

MC-LR is mainly produced by Microcystis aeruginosa cyanobacteria, but may also be produced by other species, such as Anabaena, Nostoc, Phormidium and Planktothrix. On the other hand, NOD is produced only by Nodularia spumigena cyanobacteria [6].

Although MC-LR and NOD are produced by different cyanobacteria species, they are both monomeric peptides with strong hepatotoxic activity [7], causing severe liver injury and death to humans and animals, after consumption of cyanobacteria-contaminated water [8,9]. These hepatotoxins act mainly through the binding and consequent inhibition of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) inside the liver cells [10,11]. Although MC-LR and NOD are chemically and toxicologically very similar, NOD does not bind covalently to PP1, as in the case of MC-LR [12]. Beyond protein inhibition, other adverse toxicological effects have been reported concerning MC-LR and NOD exposure, such as intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation [13,14].

The induction of deoxyribonucleic acid (DNA) damage has also been observed [15,16]. Furthermore, the genotoxicity and the potential carcinogenicity of MC-LR and NOD have been extensively studied in vitro and in vivo [17]. However, some studies suggest that MC-LR and NOD genotoxicity and carcinogenicity arise mainly from the secondary effects of these toxins rather than direct toxin-DNA interactions [18–21].

The most frequent biomarker of DNA damage is 8-oxo-deoxyguanosine (8-oxoGua), which results from the addition of a hydroxyl radical to deoxyguanosine, causing misreading by DNA polymerase and G:C to T:A substitutions [22]. The detection of 8-oxoGua after exposure to MC-LR and NOD has been reported in vitro in cultured rat hepatocytes and in vivo in rat liver [23].
The detection of cyanotoxins is usually carried out with biosensing devices that use enzymes [24,25], antibodies [26] and DNA probes [27]. However, the DNA-electrochemical biosensor using a glassy carbon electrode transducer has never been applied for the study of cyanotoxins. The DNA-electrochemical biosensor is a very good model for the evaluation of nucleic acid damage, taking advantage of the high sensitivity and selectivity of electrochemical detection and allowing the investigation of specific DNA-drug interactions [28]. In this context, the aim of this study was to investigate the interaction of the cyanotoxins MC-LR and NOD with the double stranded DNA (dsDNA), using two approaches: (a) the DNA-electrochemical biosensor, i.e., the study was performed with immobilised dsDNA on a glassy carbon electrode surface, and (b) incubated solutions of dsDNA with MC-LR or NOD.

2 Experimental

2.1 Reagents

Calf thymus single stranded DNA (ssDNA) and dsDNA was obtained from Sigma-Aldrich. MC-LR and NOD were obtained from Enzo Life Sciences, Inc. (former Alexis Biochemicals). All compounds were used without further purification. All solutions were prepared using analytical grade reagents and purified water from a Milli-Q system (resistivity 18.2 MΩ cm, conductivity ≤0.1 µScm⁻¹). The supporting electrolyte used in all experiments was pH 4.5 0.1 M acetate buffer. Stock solutions of 400 µM MC-LR, 242 µM NOD and 300 µg mL⁻¹ dsDNA were prepared in deionised water and kept at 4°C until further utilization. The dsDNA solutions were diluted to the desired concentration by mixing buffer supporting electrolyte.

The pH measurements were carried out using a Crison micropH 2001 pH-meter (Barcelona, Spain) with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1°C).

2.2 Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using a μAutolab Type II potentiostat running with GPES 4.9 software (Metrohm-Autolab, Utrecht, The Netherlands). The experimental conditions for Differential Pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mVs⁻¹. Measurements were carried out using a glassy carbon electrode (GCE, d = 1.5 mm), with a Pt wire counter electrode, and an Ag/AgCl (3 M KCl) electrode as reference, in a one-compartment 0.5 mL electrochemical cell, all from Cypress System, Inc., USA.

The GCE was polished using diamond spray (particle size 1 µm, Kemet International Ltd, UK) before every electrochemical assay. After polishing, the electrode was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and various DP voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 2 mV, included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing artefacts, although the peak current intensity is in some cases reduced (<10%) relative to that of the untreated curve.

2.3 dsDNA-Electrochemical Biosensor Preparation

The dsDNA-electrochemical biosensor was prepared by successively covering the glassy carbon surface with three drops of 5 µL containing 50 µg mL⁻¹ dsDNA dissolved in pH 4.5 0.1 M acetate buffer. After placing each drop on the surface, the biosensor was allowed to dry. After modification, the GCE was washed with Millipore Milli-Q water to ensure the removal of non-adsorbed molecules.
2.4 Procedures for the Evaluation of dsDNA Interaction with MC-LR and NOD

The evaluation of the dsDNA interaction with MC-LR and NOD was undertaken using three experimental procedures:

Procedure 1: The dsDNA-electrochemical biosensor, prepared as described in Section 2.3, was immersed and allowed to incubate in 30 μM MC-LR or NOD solutions during different periods of time. Then the biosensor was removed from the solution, washed with deionised water in order to remove the unbounded MC-LR or NOD molecules and placed in the electrochemical cell containing only the supporting electrolyte, where the transduction was performed by DP voltammetry.

Procedure 2: Mixed solutions of 50 μg mL\(^{-1}\) dsDNA with 30 μM MC-LR or NOD in pH 4.5 0.1 M acetate buffer were prepared and incubated at room temperature during different periods of time. The DP voltammograms were recorded with the clean GCE in these solutions after each incubation period.

Procedure 3: Mixed solutions of 100 μg mL\(^{-1}\) dsDNA with 60 μM MC-LR or NOD in deionised water were prepared and incubated at room temperature during different periods of time. After each incubation period, the mixed solution was diluted in pH 4.5 0.1 M acetate buffer supporting electrolyte and the DP voltammogram recorded immediately with the clean GCE.

The reproducibility and the reliability of the methodologies to prepare the multilayer DNA-electrochemical biosensor have already been investigated [29].

3 Results and Discussion

The dsDNA oxidation studies were carried out in pH 7.0 0.1 M phosphate buffer and in pH 4.5 0.1 M acetate buffer [30]. Although the dsDNA oxidation peaks occurred at lower potentials at physiological pH, in pH 4.5 the DNA oxidation peak currents were several times higher. This allows lower detection limits for the identification of DNA oxidative damage and for this reason all experiments were carried out in pH 4.5 0.1 M acetate buffer, Figure 1.

The DP voltammogram in 50 μg mL\(^{-1}\) dsDNA solution, in pH 4.5, showed two small peaks corresponding to the oxidation of deoxyguanosine (dGuo), \(E_{pa} = +1.05\) V, and deoxyadenosine (dAdo), \(E_{pa} = +1.27\) V, residues in the polynucleotide chain [30], Figure 1. In the DP voltammogram in 50 μg mL\(^{-1}\) ssDNA solution, in the same pH conditions, the peaks were several-fold higher, Figure 1, thus meaning easier oxidation of dGuo and dAdo. The small peaks obtained in solutions of dsDNA showed the greater difficulty for electron transfer from inside the rigid dsDNA structure to the electrode surface than from the more flexible ssDNA conformation where the bases can be in close proximity to the electrode surface.

3.1 Microcystin-LR

3.1.1 Electrochemical Behaviour of MC-LR

The electrochemical oxidation behaviour of MC-LR at the GCE surface was investigated in order to be able to identify the peaks occurring after MC-LR interaction with dsDNA.

The DP voltammograms obtained in pH 4.5 immediately after addition of 30 μM MC-LR to the supporting electrolyte showed one anodic peak at \(E_{pa} = +1.09\) V, Figure 2.

In a new experiment, the voltammogram was recorded in the same conditions but after 24 h incubation of 30 μM MC-LR in acetate buffer. The initial oxidation peak of MC-LR occurred with a lower current and at a slightly lower potential value, \(E_{pa} = +1.07\) V and a new oxidation peak, at \(E_{pa} = +0.80\) V, appeared. The occurrence of this peak is explained by the chemical degradation of MC-LR in solution and the formation of chemically degraded MC-LR (cdMC-LR). Upon chemical degradation, the main MC-LR oxidation peak decreased in a time dependent manner while a new peak occurred at lower potential value, showing the electroactivity of the cdMC-LR homogeneously formed in solution.

A mechanism proposed for the chemical degradation after incubation in aqueous acidic buffer solutions consists in the separation of the Adda side chain from the peptide ring structure giving rise to an electroactive degradation product [31,32] and a non-electroactive degradation product formed by the MC-LR remaining peptide ring structure.

3.1.2 In Situ Sensing with a dsDNA-Electrochemical Biosensor

The dsDNA-electrochemical biosensor was used in order to investigate in situ and in real time the changes occur-
ring to the dsDNA immobilised on the electrode surface during the interaction with MC-LR. The multilayer dsDNA-electrochemical biosensor completely covers the electrode surface [33], avoiding non-specific binding of MC-LR molecules to the electrode surface.

The interaction between dsDNA on the GCE surface with MC-LR was detected by comparing the changes in the purine base oxidation peak currents of dsDNA recorded after incubation with the results obtained using a control dsDNA-electrochemical biosensor without incubation.

The DP voltammograms obtained with newly prepared dsDNA-electrochemical biosensors in buffer following Procedure 1 for different incubation times of 10, 20 and 30 min, Figure 3, showed a decrease with time of the oxidation peak currents of dGuo and dAdo residues in the polynucleotide chain. On the DP voltammograms recorded, no oxidation peak of MC-LR was observed due to the fact that its oxidation potential is close to the oxidation of dGuo residues and it is difficult to clarify these two contributions.

These experiments were repeated, always using newly prepared dsDNA-electrochemical biosensors. The dGuo and dAdo peaks decreased eventually reaching a constant value after 30 min incubation. A higher decrease was observed in the case of dAdo peak. In order to confirm that the decrease of DNA voltammetric signals was due to the MC-LR-dsDNA interaction, a control dsDNA-electrochemical biosensor was also prepared according to Procedure 1 and held in buffer solution for 30 minutes.

The experiment described above showed that structural modifications occurred in the dsDNA on the GCE surface upon interaction with MC-LR. The decrease of dsDNA oxidation peaks with increasing incubation time is explained considering the formation of a more rigid DNA structure due to the aggregation of dsDNA strands which causes a greater difficulty for the transition of electrons from the purine residues, than from a more flexible non-aggregated dsDNA structure.

The greater decrease observed in dAdo oxidation peak is an indication that the interaction between MC-LR and dsDNA occurs preferentially at DNA adenine residues.

### 3.1.3 dsDNA-MC-LR Incubated Solutions

The dsDNA-electrochemical biosensors enabled complete coverage of the electrode surface, which reduces the non-specific adsorption of MC-LR molecules to the GCE surface, but the immobilised dsDNA strands are immobilised. The analysis in incubated solutions, in which the dsDNA strands are allowed to move freely and adopt the better conformation for and after the interaction with MC-LR was carried out.

The first set of experiments regarding the interaction of MC-LR with DNA, in incubated solutions, was carried out in pH 4.5 0.1 M acetate buffer following Procedure 2, Figure 4A. The DP voltammograms recorded immediately after the addition of MC-LR to the dsDNA solution showed an increase of the peak at +1.05 V, Figure 4A. The peak has two contributions, one from the dGuo and another from the MC-LR molecules since their oxidation potential is similar.

The decrease of dAdo peak occurred in agreement with the experiments carried out with the dsDNA-electrochemical biosensor due to the structural changes caused in the DNA conformation by MC-LR interaction.

In a new experiment, the DP voltammograms were recorded with the clean GCE in the same solution but after 6 hours of incubation. Both peaks, at +1.05 V due to the
oxidation of dGuo and MC-LR and the peak due to dAdo residues decreased, and the peak at +1.05 V, corresponding to both dGuo and MC-LR charge transfer reactions, became wider, Figure 4A. For longer incubation times, i.e. 24 hours, both peaks reached a constant current but a new peak occurred at $E_{pa} = +1.12$ V, Figure 4A. Also, due to the chemical degradation of MC-LR in solution the occurrence of the oxidation peak of cdMC-LR, at $E_{pa} = +0.82$ V, was observed. The new oxidation peak at $E_{pa} = +1.12$ V corresponds to the oxidation of free adenine molecules [30] liberated upon cleavage of the bond between phosphate-sugar backbone of dsDNA, leading to the formation of DNA abasic sites, a type of DNA damage which, if left unrepaired can lead to mutations during the replication process. These experiments showed that the interaction of MC-LR with DNA caused strand aggregation, and the interaction occurred preferentially at adenine residues.

The experiments following Procedure 2 were carried out in pH 4.5 0.1 M acetate buffer where occurs MC-LR chemical degradation. In order to avoid MC-LR chemical degradation, the second set of experiments in incubated solutions, regarding the interaction of MC-LR with DNA, followed Procedure 3, in which the incubation was carried out in deionised water and the transduction in acetate buffer after dilution.

The DP voltammograms recorded in pH 4.5 0.1 M acetate buffer after incubation in deionised water showed a similar behaviour for the interaction of MC-LR with dsDNA, Figure 4B. Increasing the incubation time, the decrease of the peak current occurred mainly for the dAdo residues, Figure 4B. Also, the peak corresponding to dGuo and MC-LR charge transfer reactions, at +1.05 V, became wider and with higher current due to superposition with the near peak of the adenine free residues, at +1.10 V, Figure 4B. However, there was no clear separation between these peaks and all peaks reached a constant current value after 24 hours incubation.

### 3.2 Nodularin

#### 3.2.1 Electrochemical Behaviour of NOD

The DP voltammograms in pH 4.5 0.1 M acetate buffer immediately after addition of 50 $\mu$M NOD to the supporting electrolyte showed one anodic peak, at $E_{pa} = +1.05$ V, Figure 5. The DP voltammogram, in the same solution with a clean GCE, after 24 h incubation in buffer showed the oxidation peak of NOD with a lower current, and the oxidation of the products of NOD chemical degradation (cdNOD), at $E_{pa} = +0.82$ V.

NOD underwent chemical degradation in buffer with increasing incubation time [31], the NOD oxidation peak decreased in a time dependent manner and cdNOD occurred. The mechanism proposed for this process suggested the separation of the Adda side chain from the peptide ring structure giving rise to electroactive degradation products and a non-electroactive one formed by the NOD remaining peptide ring structure [31,32].

#### 3.2.2 In Situ Sensing with a dsDNA-Electrochemical Biosensor

The interaction between NOD and DNA was studied using a dsDNA-electrochemical biosensor incubated in the NOD solution as described in Procedure 1, Figure 6. The DP voltammograms recorded in these conditions showed the decrease of both DNA oxidation peaks, Figure 6. This experiment has been repeated with a new biosensor for longer incubation times and the progressive decrease of dsDNA bases oxidation peaks with increasing the incubation time showed that structural modifications occurred in the dsDNA upon interaction with NOD. The decrease of the DNA oxidation peaks is correlated with the aggregation of dsDNA strands.
The interaction between NOD and DNA was also carried out in incubated solutions. The incubation was carried out in buffer following Procedure 2, Figure 7. The DP voltammograms recorded immediately after the addition of NOD to the DNA solution showed an increase and a positive shift of the peak at +1.05 V due to the contribution of dGuo and NOD molecules, Figure 7. Increasing the incubation time, both peaks decreased in a time dependent manner. After 24 hours of incubation almost a complete disappearance of dAdo peak was observed. These results are explained by the preferential interaction between NOD and dsDNA at adenine residues, leading to the formation of dsDNA aggregates which impede the electron transition from inside this rigid structure to the GCE surface. Also, the peak corresponding to dGuo and NOD charge transfer reactions, at +1.05 V, became wider and with higher current due to superposition with the near peak of the adenine free residues, at +1.10 V, Figure 7.

Similar results were observed when the incubation was carried out in deionised water following Procedure 3, showing the decrease of dAdo oxidation peak but complete disappearance of the dAdo oxidation peak was never observed.

4 Conclusions

MC-LR and NOD are among the most commonly reported toxins produced by cyanobacteria. Several previous studies have brought evidence for the possibility of direct induction of dsDNA damage in vitro and in vivo upon interaction with any of these toxins whereas other studies suggested that MC-LR and NOD genotoxicity and carcinogenicity arise mainly from the secondary effects of these toxins rather than direct toxin-DNA interaction. The interaction between DNA and MC-LR and NOD was investigated using dsDNA-electrochemical biosensors and in incubated solutions and it was confirmed the decrease of the dsDNA oxidation peaks with time, showing that MC-LR and NOD interact with the dsDNA causing the aggregation of DNA strands. The analysis of dsDNA interaction with MC-LR or NOD in incubated solutions, where dsDNA strands are allowed to move freely and adopt the better conformations for and after the interaction, enabled the detection of adenine free residues.
sugar backbone of DNA, which, if left unrepaired, can lead to mutations during the replication process.

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