Redox Mechanisms of Nodularin and Chemically Degraded Nodularin

Paulina V. F. Santos, Ilanna C. Lopes, Victor C. Diculescu, Mário César U. de Araújo, Ana Maria Oliveira-Brett

Abstract

The electrochemical behaviour of Nodularin (NOD), a hepatotoxic cyclic pentapeptide, was studied at a glassy carbon electrode. NOD electrochemical oxidation is an irreversible, pH-independent process, involving the transfer of one electron. Upon incubation in different pH electrolytes, chemical degradation of NOD was electrochemically detected by the appearance of a new oxidation peak. The chemically degraded NOD (cdNOD), undergoes an irreversible, pH-dependent oxidation, and its redox products are reversibly oxidised. The charge transfer properties of cdNOD as well as of its redox metabolites were investigated. Mechanisms for NOD oxidation, NOD chemical degradation and oxidation of cdNOD and its metabolites were proposed.

Keywords: Nodularin, Cyanobacteria, Degradation products, Oxidation, pH effect

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1 Introduction

Nodularin (NOD) is a potent hepatotoxin produced by Nodularia spumigena, cyanobacteria found in brackish waters (e.g. Baltic Sea), like saline lakes and estuaries, throughout the world [1–4]. NOD is a monocyclic pentapeptide with a similar structure to microcystins – cyclic hepatotoxic heptapeptides most frequently encountered, produced by freshwater cyanobacteria. NOD consists of Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), n-glutamic acid (n-Glu), N-methyldehydrobutyrye (MeDhb), d-erytro-β-methyaspartic acid (d-MeAsp), and l-arginine (l-Arg) [5, 6], Scheme 1. There are different forms of NOD, varying usually by the variable amino acid arginine, and the degree of methylation and stereochemistry [7]. However, only nine different forms of this toxin have been documented to date [8].

The harmful effects of NOD have been reported since 1878 and can induce toxic effects similar to those induced by microcystins [5, 9]. These hepatotoxins have also caused mortality in animals [10, 11] and illness in humans [12, 13] or even death when exposed through hemodialysis [14, 15]. The problem of toxicity of N. spumigena to animals and people is of increasing concern, as the incidences of its blooms grow [8].

The major target for NOD toxicity is the liver, where it enters through the bile acid multi-specific organic anion transporters located at the hepatocytes membranes.
longing to the hydrolytic capabilities of the B-9 (a bacterial strain be-

than the dissolved toxin, and also that NOD degradation made, leading to the conclusion that NOD contained in cyanobacterial cells is more resistant to photodegradation. The aim of this study was to investigate the electrochemical approach. Therefore, this study brings new data to the cyanotoxins degradation field. To our knowledge, no data concerning NOD electrochemical behaviour under these conditions have been reported to date. The aim of this study was to investigate the electrochemical behaviour of NOD and of the products of NOD chemical degradation with time, in aqueous solution at different pHs, using cyclic, differential pulse and square-wave voltammetry, at a glassy carbon electrode.

2 Experimental

2.1 Materials and Reagents

Nodularin (C$_{34}$H$_{60}$N$_{8}$O$_{10}$, MW 825.0) was purchased from Alexis Biochemicals (Lausen/Switzerland). Acetonitrile (HPLC grade) and formic acid were purchased from Sigma. A stock solution of 240 μM NOD was prepared in water and kept at 4°C until further utilization. During the night, NOD solutions were transferred from the electrochemical cell to 1.5 mL microtubes (Eppendorf) and kept at 4°C.

The composition and the correspondent pH values of all the supporting electrolytes used are listed in Table 1. Supporting electrolyte solutions were prepared using analytical grade reagents and purified water obtained from a Millipore Milli-Q system (conductivity < 0.1 μS cm$^{-1}$). The pH measurements were performed with a Crison microPH 2001 pH-meter (Barcelona, Spain) with an Ingold combined glass electrode.

All experiments were done at room temperature (25 ± 1°C). All incubations were done at room temperature in solutions with dissolved oxygen.

Table 1. Supporting electrolytes, 0.1 M ionic strength.

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition</th>
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<tbody>
<tr>
<td>1.1</td>
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</tr>
<tr>
<td>2.0</td>
<td>HCl + KCl</td>
</tr>
<tr>
<td>3.3</td>
<td>HAcO$_2$ + NaAcO</td>
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<tr>
<td>4.3</td>
<td>HAcO$_2$ + NaAcO</td>
</tr>
<tr>
<td>5.3</td>
<td>HAcO$_2$ + NaAcO</td>
</tr>
<tr>
<td>6.1</td>
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</tr>
<tr>
<td>7.0</td>
<td>NaH$_2$PO$_4$ + Na$_3$HPO$_4$</td>
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<tr>
<td>8.1</td>
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</tr>
<tr>
<td>9.2</td>
<td>Na$_3$BO$_3$ + NaOH</td>
</tr>
<tr>
<td>10.2</td>
<td>Na$_3$BO$_3$ + NaOH</td>
</tr>
<tr>
<td>11.8</td>
<td>KCl + NaOH</td>
</tr>
</tbody>
</table>

2.2 Voltammetric Measurements

Voltammetric experiments were carried out in a μAutolab Type II potentiostat in combination with GPES 4.9 Software (Eco Chemie B.V., Utrecht, The Netherlands). A glassy carbon electrode (GCE, d = 1.5 mm) was used as working electrode, an Ag/AgCl (3 M KCl) as reference electrode and a Pt wire as counter electrode, in a 2 mL one-compartment electrochemical cell.

For cyclic voltammetry (CV), the scan rate was 50 mV s$^{-1}$. For differential pulse (DP) voltammetry, the experimental conditions applied were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s$^{-1}$. For square wave (SW) voltammetry, a frequency of 50 Hz, a potential increment of 2 mV, and a pulse amplitude of 50 mV, corresponding to an effective scan rate of 100 mV s$^{-1}$ were used.

The GCE was mechanically cleaned by polishing with diamond spray (particle size 1 μm, Kemet International Ltd, UK) and rinsed thoroughly with Milli-Q water before performing the experiments. Before testing NOD samples with dissolved oxygen.

solutions by CV, DP and SW voltammetry, various voltammograms were recorded only with supporting electrolyte buffer solution, in order to obtain a stable baseline voltammogram.

2.3 Acquisition and Presentation of Voltammetric Data
All differential pulse voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 2 mV included in the GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artefact, although the peak current is in some cases reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all graphs were determined from the original untreated voltammograms after subtraction of the baseline.

2.4 UV-Vis Spectrophotometry
UV-vis measurements were performed using a spectrophotometer SPECORD S100 running with Aspect Plus Version 1.5 (Analytik Jena GmbH, Jena, Germany). All experiments were done with a quartz glass cuvette of optic path of 1 mm.

2.5 HPLC Measurements
Samples of 5 μM NOD solutions were prepared in pH 5.3 0.1 M acetate buffer and analyzed on an Alliance Waters 2690 Separations Module HPLC system with a C18 reverse-phase column ODS-3 V from Inertsil and an isocratic mobile-phase mixture of acetonitrile/water (35:65, v/v), with 0.05% formic acid, pH 2.8. Injections of 50 μL were performed. The eluate was monitored at 239 nm and at 210–600 nm with a Waters 996 Photodiode Array Detector (PDA 996) controlled by Waters S.A., USA.

3 Results

3.1 Nodularin Electrochemical Behaviour

3.1.1 Cyclic Voltammetry
The electrochemical behaviour of NOD at a GCE was first investigated by CV in 100 μM NOD solution, prepared in pH 7.0 0.1 M phosphate buffer. CVs were recorded by scanning the potential starting from −1.00 V to +1.35 V. In order to remove dissolved oxygen, a constant flux of N2 was bubbled through the solutions during 10 minutes, and over the solution surface during the CV measurements. These experiments showed one anodic peak, at $E_{p1a} = +1.15$ V, meaning that NOD can only undergo oxidation at the GCE surface.

The CVs obtained in a 75 μM NOD solution in pH 5.3 0.1 M acetate buffer, presented one anodic peak 1α, at $E_{p1α} = +1.10$ V, Figure 1A. Successive scans recorded in the same solution, without cleaning the electrode surface, showed a decrease in peak 1α current, due to the adsorption of NOD and/or NOD oxidation products onto the GCE surface. No other peaks were observed suggesting that the oxidation of NOD does not involve the formation of electroactive products. A similar behaviour was observed in other electrolytes.

The influence of the scan rate (v) on peak 1α current was investigated in pH 7.0 0.1 M phosphate buffer, and peak 1α current increased linearly with the square root of scan rate, which is the characteristic behaviour of a diffusion-controlled process. A plot of NOD oxidation peak current vs. square root of scan rate always refers to the first voltammetric scan obtained using a clean GCE surface.

3.1.2 Square Wave Voltammetry
The electrochemical oxidation behaviour of NOD was also investigated by SW voltammetry. The voltammograms recorded in a 50 μM NOD solution in different electrolytes showed similar features.

The SW voltammogram in a fresh solution of 50 μM NOD in pH 5.3 0.1 M acetate buffer showed on the first scan only peak 1α, at $E_{p1α} = +1.09$ V, Figure 1B. The deconvolution of the total current in forward and backward components confirmed the irreversibility of the oxidation reaction of NOD. The forward component showed the oxidation peak at the same potential and with the same current as the total current whereas on the backward component no peak occurred.

3.1.3 Differential Pulse Voltammetry
The electrochemical oxidation of NOD was investigated over a wide pH range, between 1.1 and 11.8, using DP voltammetry. All DP voltammograms were recorded in fresh 50 μM NOD solutions, in different supporting electrolytes.

The DP voltammograms recorded in 1.1 < pH < 9.2, immediately after the addition of NOD to each buffer, Figure 2A, showed peak 1α at $E_{p1α} = +1.06$ V, but for pH > 9.2, peak 1α did not appear. The Nernst equation enables to determine the ratio of the number of electrons and protons involved from a plot of peak potential vs. pH.

The potential of peak 1α is pH independent, Figure 2B, which indicates an oxidation mechanism involving only electron transfer, because NOD and/or NOD oxidation product undergo a chemical deprotonation after the rate-determining step. The peak width at half height, $W_{1/2} \approx 84$ mV, is close to the theoretical value of 90 mV for the transfer of one electron [27]. Therefore, peak 1α involves the transfer of one electron, and its current decreased
3.2 Nodularin Chemical Degradation and Its Electrochemical Behaviour

It was observed that NOD undergoes chemical degradation upon incubation in different buffer electrolytes. The chemical degradation of NOD (cdNOD) gave rise to electroactive degradation products and their electrochemical oxidation behaviour was studied.

3.2.1 Cyclic Voltammetry

The oxidation behaviour of cdNOD was studied in a 75 μM NOD solution incubated in different supporting electrolytes, during 7 days by CV at 50 mV s⁻¹, and in these conditions appeared a new anodic peak 2. All CV experiments for cdNOD showed similar features, independently of the pH of the supporting electrolyte solution. The results obtained at pH 5.3 0.1 M acetate buffer were chosen to be presented.

CV recorded in pH 5.3 showed the anodic peak 2, at $E_{p2} = +0.77$ V, Figure 3A. Reversing the scan direction, two new reduction peaks 4 at $E_{p4} = +0.47$ V, and 3 at $E_{p3} = +0.36$ V, occurred. These two peaks correspond to

![Diagram](image1.png)

**Fig. 1.** (A) CVs in 75 μM NOD immediately after addition to pH 5.3 0.1 M acetate buffer: (—) first scan and (⋯⋯) scan in supporting electrolyte (s.e.) solution; $v = 50$ mV s⁻¹. (B) SWV in 50 μM NOD immediately after addition to pH 5.3 0.1 M acetate buffer; $f = 50$ Hz, $\Delta E_p = 2$ mV, pulse amplitude 50 mV, $v_{eff} = 100$ mV s⁻¹; $I_t$: total, $I_f$: forward and $I_b$: backward currents.

with the increasing pH, showing maximum values in acid electrolytes, Figure 2B.

![Diagram](image2.png)

**Fig. 2.** Background-corrected DP voltammograms in 50 μM NOD immediately after the addition to buffer as a function of pH: (A) 3D plot of first scan; (B) Plot of (●) $E_p$ and (○) $I_p$ of peak 1 vs. pH.
the reduction of cdNOD oxidation products formed at the GCE surface during the anodic scan. On the second CV scan obtained in the same solution, without cleaning the GCE surface, the anodic peaks $3_a$ at $E_{p3a} = +0.39$ V, and $4_a$ at $E_{p4a} = +0.49$ V, were observed, confirming their reversibility. Moreover, on the second scan, peak $2_a$ current decreased due to the adsorption of cdNOD oxidation products onto the GCE surface.

### 3.2.2 Square Wave Voltammetry

SW voltammetry was also used to investigate the oxidation behaviour of cdNOD in different electrolytes. SW voltammograms in a 50 μM NOD after 53 h incubation in pH 5.3 0.1 M acetate buffer solution, showed the appearance of peak $2_a$, at $E_{p2a} = +0.73$ V, Figure 3B. The deconvolution of the total current showed that peak $2_a$ corresponds to an irreversible oxidation reaction.

On a second SW voltammogram recorded without cleaning the GCE peaks $3_a$ at $E_{p3a} = +0.37$ V, and $4_a$ at $E_{p4a} = +0.47$ V, occurred. The reversibility of these reactions is shown by the two peaks on the forward and on the backward components of the total current, where the oxidation and the reduction currents are equal, Figure 3C.

The voltammograms recorded in 50 μM NOD solutions after 53 h incubation in different electrolytes showed similar features.

### 3.2.3 Differential Pulse Voltammetry

The electrochemical oxidation behaviour of cdNOD and its oxidation products was also investigated using DP voltammetry. DP voltammograms recorded on the potential range $+0.0$ to $+1.4$ V, in 50 μM NOD after 5 h incubation in pH 6.1, showed both NOD oxidation peak $1_a$, at $E_{p1a} = +1.07$ V, and cdNOD peak $2_a$, at $E_{p2a} = +0.70$ V, Figure 4A. DP voltammograms in the same solution but after longer incubation times, 24 h, 29 h and 53 h, showed a progressive increase of peak $2_a$ current with increasing incubation time, and the decrease of NOD oxidation peak $1_a$ current, Figure 4A. This is in agreement with the decrease of NOD concentration with the increase of the concentration of cdNOD homogenously formed in solution.

Successive DP voltammograms were also recorded in a NOD solution after 53 h incubation in pH 6.1, Figure 4B. On the first scan, peak $1_a$, at $E_{p1a} = +1.07$ V, and peak $2_a$, at $E_{p2a} = +0.70$ V, occurred. On the second scan, the new oxidation peaks $3_a$, at $E_{p3a} = +0.34$ V, and $4_a$, at $E_{p4a} = +0.44$ V, were observed, corresponding to the oxidation of cdNOD oxidation product formed at the GCE surface during the first potential scan. Increasing the number of scans in solution both peaks $3_a$ and $4_a$ currents increased due to the formation of more adsorbed cdNOD oxidation products at the GCE surface.

The electrochemical oxidation behaviour of cdNOD and its oxidation products was also investigated using DP...
voltammetry in 50 μM NOD incubated during 53 h in buffer electrolytes 1.1 < pH < 11.8.

Peak 2a did not occur for pH 1.1. For 1.1 < pH < 9.2 its potential was shifted to less positive values with increasing pH, Figure 5A, following the relationship $E_{p2a} (V) = 1.081 - 0.059 \text{pH}$, Figure 5B. The slope of the line, 59 mV per pH unit, shows that the oxidation mechanism involves the transfer of the same number of electrons and protons [28]. Taking into consideration that the width at half height of peak 2a was $W_{1/2} \approx 65 \text{mV}$, the oxidation process at peak 2a involves the transfer of one electron and one proton. For pH > 9.2, peak 2a potential is pH-independent, indicating a mechanism involving the transfer of only one electron and no proton, occurring because cdNOD oxidation product undergoes chemical deprotonation in more alkaline electrolytes [29].

The pK_a was determined according to the Nernst equation by the intersection of the two dotted lines in Figure 5B. The value of pK_a ≈ 9.2 for cdNOD was determined.

On the second DP voltammogram in different pH buffer electrolytes, without cleaning the GCE surface, peaks 3a and 4a occurred, Figure 6A. For 1.1 < pH < 9.2, the peaks 3a and 4a potential variation, Figure 6B, was linear and the slope of the lines, 59 mV per pH unit, showed that the oxidation mechanisms involved the transfer of the same number of electrons and protons. The widths at half height of both peaks $W_{1/2} \approx 45 \text{mV}$ confirms that two electrons and two protons were transferred. For pH > 9.2, peaks 3a and 4a potentials were pH independent, Figure 6B, indicating a reaction with the transfer of only two electrons, with no protons involved.

DP voltammograms were recorded after different incubation times in different pH buffer electrolytes and the
variation of cdNOD oxidation peak 2, current with incubation time and pH. Figure 7, showed that NOD degradation occurred at a higher rate in electrolytes with $5.3 < pH < 6.1$.

3.3 UV-Vis Spectrophotometry and HPLC

Spectrophotometric and chromatographic measurements were carried out in order to complement the voltammetric studies.

For UV-vis spectrophotometry, 30 μM NOD solutions at pH = 5.3, 7.0 and 10.2 were prepared. The spectra were recorded immediately after their preparation and after incubation in buffer for different periods of time. The toxin in fresh solution was identified in the absorption spectrum with a maximum at 239 nm, due to the conjugated diene in the structure of the unusual amino acid Adda [1]. After 5 and 24 hours incubation, no significant variation in the intensity or wavelength of NOD absorption bands was found in the UV-vis spectra recorded.

Chromatographic measurements of 5 μM NOD solutions, immediately after preparation and also after 7 days-incubation, were carried out using HPLC with diode array detection. NOD was identified by the retention time of 4.31 min and characteristic absorption spectrum with a maximum at 239 nm.

The chromatogram obtained after 7 days incubation of NOD solution presented also only one peak. However,
the absorbance of the peak after this incubation time was slightly lower than the peak obtained for the fresh NOD solution. Since only one peak is visible in the chromatograms recorded for both times, it seems that the process of NOD degradation requires a longer incubation time to check the occurrence chemical degradation.

3.4 Degradation and Redox Mechanisms

The electrochemical oxidation of NOD occurs in an irreversible pH-independent reaction with the transfer of one electron. In order to determine the electroactive centres some NOD amino acids were investigated, and they were not electroactive.

The sorbic acid (SA) electrochemical behaviour was investigated, considering that mimics the Adda side chain of NOD. The electrochemical behaviour of SA [30] resembles that observed for NOD, SA also undergoing chemical degradation in buffer solutions. Upon degradation, the main SA oxidation peak disappeared while a new peak occurred at a lower potential, due to the oxidation of the homogenous formed product of SA chemical degradation in solution. The oxidation of the SA degradation products led to the formation of two electroactive species that underwent reversible redox reaction, similarly to NOD.

Based on the results, the proposed mechanism for NOD oxidation, peak 1, corresponds to the oxidation of Adda’s C7 in a process involving the transfer of one elec-

Scheme 3. Proposed mechanisms: (A) chemical degradation of NOD, (B) oxidation of cdNOD and (C) oxidation of cdNOD oxidation products.
tron and the formation of a radical cation in the rate-determining step. Subsequently, the addition of a hydroxyl group to position C7 occurs, Scheme 2, and the radical is chemically deprotonated [31]. A mechanism for NOD degradation and for the oxidation of cdNOD and its metabolites oxidation was proposed, Scheme 3.

Upon incubation in aqueous acidic buffer solutions, the Adda side chain is separated from the peptide ring structure. This process may occur in two different places of Adda moiety giving rise to two electroactive degradation products containing a hydroxyl group (1) and (2), Scheme 3A, and two other degradation products (1’) and (2’), consisting of the NOD remaining peptide ring structure. The cdNOD peak 2 is attributed to the electrochemical oxidation of products (1) and (2), in a one electron and one proton reaction, followed by a chemical oxidation reaction where a second hydroxyl group is incorporated into the molecule, Scheme 3B. The reversible redox reactions that lead to peaks 3, and 4, are attributed to the oxidation of the dihydroxylated products, Scheme 3C.

5 Conclusions

The electrochemical behaviour of nodularin over a wide pH range, using cyclic, square wave and differential pulse voltammetry, with a GCE, complemented by UV-vis spectrophotometry and HPLC was investigated. NOD undergos oxidation in an irreversible, diffusion-controlled and pH-independent reaction, which occurs with the transfer of only one electron.

Upon incubation in different pH buffer supporting electrolytes, NOD chemical degradation was electrochemically detected by the appearance of a new peak at lower potential. The cdNOD, homogeneously formed in solution, undergoes oxidation in an irreversible, pH-dependent process, with the transfer of one electron and one proton, giving rise to two electroactive products with pH-dependent two electron and two proton reversible reactions.

The electrochemical study of NOD showed that oxidation and cyanotoxin chemical degradation in aqueous media follow a complex redox mechanism, and the electroanalytical determination of NOD and cdNOD is foreseen, which will provide very important and useful data for toxicity evaluation.

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