Direct Electrochemistry of Native and Denatured Anticancer Antibody Rituximab at a Glassy Carbon Electrode

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Abstract
Rituximab (RTX) is a human/murine chimeric monoclonal antibody (mAb) that specifically targets the transmembrane protein CD20 of B-cells. The oxidation mechanism of native and denatured RTX was investigated on glassy carbon electrode. The denaturing agent sodium dodecyl sulfate and the redutancts tris(2-carboxyethyl)phosphine and dithiothreitol were used. Significant differences were observed for native and denatured RTX oxidation due to morphological changes and unfolding of the RTX native structure. Native RTX presented only one oxidation peak of tyrosine and tryptophan residues, whereas in denatured RTX were detected three peaks corresponding to the oxidation of tyrosine, tryptophan and histidine residues.

Keywords: Rituximab, Antibody, Denaturing agents, Voltammetry, Glassy carbon

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1 Introduction

Cancer is a pathologic condition that affects millions of people around the world and there has been great interest in developing new anticancer drugs to avoid chemical treatments and sensitize tumour cells [1].

Monoclonal antibodies (mAb) are a new class of drugs that provide specific target delivering. In 1997, the U.S. Food and Drug Administration approved rituximab (RTX), the first monoclonal antibody, for the treatment of patients with relapsed/refractory low-grade B-cell non-Hodgkin lymphomas (NHL). Since then RTX has been used in type-1 diabetes, autoimmune and hematologic malignancies and other types of cancer [2].

The RTX antibody is a genetically engineered chimeric human/murine monoclonal antibody directed against the CD20 receptor, a hydrophobic transmembrane protein with a molecular weight of ~35 kDa, expressed on more than 80% of B-cell non-Hodgkin lymphomas and on non-malignant B-cells, except on hematopoietic stem cells, pro-B cells, plasma cells, or other normal tissues [3,4].

The RTX antibody consists of a human kappa constant region, a human IgG1 FC portion, and a murine variable region. RTX is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids and has an approximate molecular weight of 145 kDa [3–5]. The Fab domain of RTX binds to the CD20 antigen on B-lymphocytes, and leads to significant depletion of peripheral B-cells, but the exact mechanism is still unknown and depends on the pathology type [3].

RTX has different medical applications and the investigation of its redox mechanisms using electrochemical techniques to clarify in vivo drug activity, stereochemistry, diffusion, solubility, metabolism, and redox reactions involved in physiological processes is of great relevance.

The electrochemical behaviour of proteins not containing redox centres at different types of electrochemical transducers has been investigated over the last years using solid electrodes, such as gold electrodes and carbon electrodes [6–12]. The oxidation occurs in the electroactive amino acids [10–12]. Among the 20 amino acids presented in proteins, only tyrosine (Tyr), tryptophan (Trp), histidine (His) [12,13], cysteine (Cys) and methionine (Met) residues [12,14,15] (Scheme 1) are oxidized on carbon electrodes in aqueous solutions and all present a pH-dependent electron transfer mechanism [12].

Tyrosine and tryptophan undergo irreversible oxidation at GCE and BDDE [12]. Tyrosine oxidation occurs in one-irreversible step at the hydroxyl group [12]. Tryptophan oxidation occurs in two-irreversible steps, in the first step occurs the oxidation of the pyrrole ring and in the second the electrochemical hydroxylation of the benzene moiety [12,13].

Cystein oxidation occurs in two steps on carbon surfaces, in the first oxidation step cysteine gives rise to cys-
Cysteine (Cys)  Methionine (Met)

Scheme 1. Electroactive amino acid chemical structures.

tine, and is followed by the oxidation of cystine at a higher potential [12,14,15]. Methionine oxidation occurs in two steps, involving the adsorption and the protonation/deprotonation of the thiol group [12,14]. Histidine oxidation occurs in a single step at a higher positive potential than the other electroactive amino acids in the same experimental conditions [12,13,15].

Electrochemical studies in qualitative and quantitative analysis in proteomics of individual protein domains and mutants of proteins, not containing a centre with fast-reversible redox reactions, are still very few [16]. The adsorption of native proteins without prosthetic groups on solid surface is weaker due to the rigidity of the 3D-structure, which makes more difficult the electron transfer between the electroactive amino acids in the interior of the proteins and the electrode surface.

The present knowledge in biochemistry offers great possibilities to study the direct electrochemistry of proteins on solid surfaces, using the denaturing agent sodium dodecyl sulfate (SDS) and the reductants tris(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT), to unfold the protein 3D morphological structure ensuring that no quaternary structure remained, and exposing more electroactive amino acids residues to the electrode surface.

In the present work, the electrochemical oxidation behaviour of native and denatured RTX using differential pulse (DP) voltammetry at a glassy carbon electrode (GCE) was investigated for the first time. The oxidation of native RTX was studied using a RTX monolayer film deposited on the GCE surface. A RTX-electrochemical biosensor, prepared depositing a thick multilayer RTX onto the GCE, was incubated for different time periods with SDS and the reductants TCEP and DTT, in order to unfold the RTX tertiary and quaternary structures, and the denatured behaviour of the RTX-electrochemical biosensor film was investigated.

2 Experimental

2.1 Materials and Reagents

Rituximab (RTX) 10 mg/mL was obtained from Roche Pharmaceuticals and stored at 4 °C. RTX is formulated for intravenous (IV) administration as a sterile product in 9.0 mg/mL sodium chloride, 0.7 mg/mL polysorbate 80, 7.35 mg/mL sodium citrate dihydrate, and water for injection, USP (pH 6.5).

Dithiothreitol (DTT), tris(5-carboxyethyl)phosphine (TCEP) and sodium dodecyl sulfate (SDS) were obtained from Sigma. Stock solutions of 0.2 M DTT and 0.2 M SDS in deionised water, were prepared and stored at 4 °C. A solution of 0.2 M TCEP was prepared directly in 0.1 M acetate buffer pH 5.0. Solutions of different concentrations of DTT, SDS and TCEP were prepared by dilution in 0.1 M phosphate buffer pH 7.0. All reagents used were of high purity and analytical grade.

The 0.1 M ionic strength supporting electrolyte solutions [17]: acetate buffer pH 3.4–5.4, phosphate buffer pH 6.1–8.0, NaOH/Na2B2O7 pH 9.1, were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 μS/cm).

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA). The pH measurements were carried out with a Crison micropH 2001 pH-meter 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 running with GPES 4.9 software, Metrohm/Autolab, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon working electrode (GCE) (d = 1.5 mm), a Pt wire counter electrode, and an Ag/AgCl (3 M KCl) as reference electrode, in a 2 mL one-compartment electrochemical cell. The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms, and scan rate 5 mV/s.

The GCE was polished using diamond spray (particle size 3 μm (Kement, Kent, UK), before every chemical 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 DP voltammograms were recorded until a steady state baseline voltammogram was obtained.
procedure ensured very reproducible experimental results.

2.3 Acquisition and Presentation of Voltammetric Data

All the DP voltammograms presented were baseline-corrected using the moving average application with a step window of 5 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. 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 Electrochemical Procedures

The oxidation behaviour of the native and denatured anticancer antibody RTX was investigated using two experimental procedures.

2.4.1 Procedure 1 – RTX-Monolayer Film

The RTX monolayer film used to investigate the oxidation of native RTX was prepared depositing one drop of 5 μL of 10 mg/mL RTX solution on the GCE surface and after drying under a constant flux of N₂. The GCE electrode modified with the RTX monolayer film was placed in the electrochemical cell containing only the supporting electrolyte and allowed to hydrate for 5 min before the DP voltammetry.

2.4.2 Procedure 2 – RTX-Electrochemical Biosensor

The thick multilayer RTX-electrochemical biosensor used to investigate the oxidation of denatured RTX was prepared depositing 4 times one drop of 5 μL of 10 mg/mL RTX solution on the GCE surface and after each drop drying under a constant flux of N₂. The RTX-electrochemical biosensors prepared were incubated in the denaturing agents 33.33 mM SDS, 10 mM TCEP or 10 mM DTT, in 0.1 M phosphate buffer pH 7.0 solutions, during different times: 60, 120, 180 and 300 min. Afterwards, the RTX-electrochemical biosensors were removed from the solution, washed with deionised water, in order to remove the excess of SDS, TCEP or DTT, and placed in the electrochemical cell containing only the supporting electrolyte 0.1 M phosphate buffer pH 7.0, where DP voltammetry was performed. To ensure that the recorded peaks are exclusively due to the denaturing or reductants effect on the RTX structure, control thick multilayer RTX-electrochemical biosensors were incubated in 0.1 phosphate buffer pH 7.0 for the same periods of time.

3 Results and Discussion

3.1 Oxidation of Native RTX

CV and DP voltammetric experiments in 1, 3 and 5 mg/mL RTX Roche Pharmaceuticals in 0.1 M phosphate buffer pH 7.0 solutions showed no oxidation peak. Therefore, it was concluded that polysorbate 80 and other excipients were not electroactive.

The oxidation behaviour of native RTX was investigated by solid state voltammetry with the RTX monolayer film deposited on the GCE surface, Procedure 1, in supporting electrolytes with 3 < pH < 9 and 0.1 M ionic strength (Figure 1). The solid state DP voltammograms showed two oxidation peaks corresponding to the oxidation of tyrosine and or tryptophan and methionine residues.

The first oxidation peak of the RTX monolayer film occurred at $E_{p,a} = +0.65$ V, in 0.1 M phosphate buffer pH 7.0, corresponding to the oxidation of monomeric tyrosine and/or tryptophan [12,13]. Increasing the pH, the potential shifted to more negative values and the highest peak current was in 0.1 M phosphate buffer pH 6.0 (Figure 1). This is explained considering that the RTX charge and molecule orientation causes an increase of tyrosine and tryptophan residues adsorption, at $E_{p,a} = +0.65$ V, on the GCE hydrophobic surface, therefore increasing the oxidation peak current in pH 6.0.

For $3 \leq \text{pH} \leq 9$ the pH-dependence was linear with a slope of $-60$ mV per pH unit and considering the half-height width of peak, the oxidation involves one electron and only one proton [10,12,13], and this oxidation peak corresponds to the oxidation of tyrosine and tryptophan amino acid residues in the native RTX structure.

The second oxidation peak of the RTX monolayer film only occurred in acid media 0.1 M acetate buffer pH 3 and 4 and the peak current was very small compared with the first oxidation process. In 0.1 M acetate buffer pH 3.5
occurred at $E_{pa} = +0.97$ V [14], and corresponds to the first oxidation of monomeric methionine.

Solid state DP voltammograms (Figure 1) show that the electrochemical oxidation of native RTX involves complex electron transfer reactions occurring at tyrosine, tryptophan and methionine amino acid residues.

3.2 Oxidation of Denatured RTX

Considering that RTX molecule contains all electroactive amino acids, tyrosine, tryptophan, histidine, cysteine and methionine [18], the results obtained clearly show the difficulty of the electron transfer processes, in the tertiary and quaternary RTX structures, in which the amino acids are organised according to polarity/hydrophobicity, making very difficult the contact between the more interior electroactive amino acids with the GCE surface.

Due to protein structural complexity, the use of the denaturing SDS, a detergent that dissolves hydrophobic proteins, and the reducing agents DTT or TCEP, to disrupt disulfide bonds, are essential for qualitative and quantitative analysis in proteomic mechanism investigation [19,20].

Protein destabilization with SDS, DTT or TCEP, causes morphological changes in the protein 3D structure which unfolds and is able to follow the contours of the electrode surface making the electrochemical oxidation of the electroactive amino acid residues easier.

The electrochemical oxidation mechanism of the reducing agents DTT and TCEP was investigated using cyclic and differential pulse voltammetry over a wide pH range on a glassy carbon electrode [19]. The denaturing agent SDS is not electroactive.

The native RTX was denatured after incubation in the denaturing agents 33.33 mM SDS, 10 mM TCEP or 10 mM DTT in 0.1 M phosphate buffer pH 7.0 solutions, Procedure 2, during different periods of time: 60, 120, 180 and 300 min. SDS, DTT and TCEP concentrations were chosen based on protocols for electrophoretic procedures usually applied to denatured proteins [21].

The oxidation mechanism of denatured RTX was investigated using thick multilayer RTX-electrochemical biosensors which completely covered the electrode surface, avoiding non-specific adsorption and binding of SDS, DTT or TCEP molecules to the electrode surface.

The effect of the denaturing agents on RTX electrochemical oxidation was followed by DP voltammetry comparing the RTX-electrochemical biosensor control with the changes observed with an RTX-electrochemical biosensor after the interaction with each denaturing agent. For each experiment a new multilayer RTX-electrochemical biosensor was prepared and after incubation washed with deionised water in order to remove the remained SDS, DTT or TCEP and after transferred to 0.1 M phosphate buffer pH 7.0 where the DP voltammograms for each new multilayer RTX-electrochemical biosensor were obtained.

The anodic behaviour of a RTX-electrochemical biosensor, DTT, TCEP and SDS, using DP voltammetry be-
between +0.2 V and +1.2 V, was also investigated in 0.1 M phosphate buffer pH 7.0 (Figures 2A, 2B and 3) as a necessary control to enable clear identification of the oxidation peaks occurring after the interaction of DTT-RTX, TCEP-RTX and SDS-RTX.

The oxidation of the RTX-electrochemical biosensor showed as expected, a single small oxidation peak, Figure 3 (gray curves ↓), corresponding to the oxidation of tyrosine and tryptophan amino acid residues, at \( E_{pa} = 0.65 \text{ V} \) [12], in agreement with the results obtained for the RTX monolayer film (Figure 1).

DP voltammograms of 50 \( \mu \text{M} \) DTT in phosphate buffer showed only one oxidation peak 1\( _a \) at \( E_{pa} = +1.15 \text{ V} \) (Figure 2A) due to the oxidation of the sulphhydryl group with lowest \( \text{pK}_a \) [19]. DP voltammograms of 50 \( \mu \text{M} \) TCEP in phosphate buffer showed also only one oxidation peak 1\( _a \) at \( E_{pa} = +0.94 \text{ V} \) (Figure 2B) due to the oxidation of the phosphate group in the molecule [19]. DP voltammograms were also recorded for 1 \( \text{mM} \) SDS solution in phosphate buffer pH 7.0, but no oxidation peak was detected.

DP voltammograms of the RTX-electrochemical biosensors after incubation in a SDS solution for different periods of time showed an increase of the oxidation peak current of tyrosine and tryptophan amino acid residues, at \( E_{pa} = +0.65 \text{ V} \) (Figure 3A), and was observed an enhancement of the peak width at half height, \( W_{1/2} \). This is due to the RTX molecule orientation enabling a greater contact between tyrosine and tryptophan residues and the electrode surface and the fact that the oxidation for both amino acid residues occurs at near potentials [12,13]. The peak current decreased after 180 minutes due to adsorption of RTX nonelectroactive amino acid residues on the electrode surface that reduced the available electrode surface area.

SDS is a denaturing agent that disrupts noncovalent interactions by electrostatic repulsion, transforming the protein into a linear polypeptidic chain. Besides, as an anionic surfactant, SDS changes the overall protein charge to negative, enhancing the interaction between RTX and the positive electrode surface. As a consequence, the number of amino acid residues exposed to the electrode surface is expected to be higher. However, no new peaks were detected when compared with the RTX-electrochemical biosensor control.

DP voltammograms of the RTX-electrochemical biosensors after incubation in the reductant DTT solution (Figure 3B) showed after 10 minutes only the RTX oxidation peak, at \( E_{pa} = +0.65 \text{ V} \), and for DTT no peak was observed. The DTT-RTX interaction was time dependent and was only completed after 60 min when three amino acid residues oxidation peaks were observed (Figure 3B). The first oxidation peak, at \( E_{pa} = +0.65 \text{ V} \), corresponds to the oxidation of tyrosine and tryptophan amino acid residues [12]. The second oxidation peak at \( E_{pa} = +1.00 \text{ V} \) corresponds to the second step of tryptophan amino acid oxidation [12,13]. The third peak at a higher potential, \( E_{pa} = +1.15 \text{ V} \), corresponds to the oxidation of histidine amino acid residues [12,13]. The peak currents of the RTX-electrochemical biosensors in the DTT solution increased with incubation time (Figure 3B) indicating slow structural and morphological modifications of RTX molecule on the electrode surface, and confirming the high reactivity of DTT to promote a total disruption of the RTX folded structure.

DP voltammograms of the RTX-electrochemical biosensors after incubation in the reductant TCEP (Figure 3C) showed only one peak, at \( E_{pa} = +0.67 \text{ V} \). Comparing with the RTX-electrochemical biosensor control, there was an enhancement of the peak current similar to the result obtained with SDS.

TCEP and DTT have been applied to protein samples with the aim to cleave the disulfide bond between cysteine residues [19], and TCEP is considered to have at \( \text{pH} < 8 \) a higher reducing power in comparison with DTT [22].

The influence of TCEP on RTX three-dimensional structure unfolding (Figure 3C) was not as extensive as with DTT (Figure 3B) due to TCEP inaccessibility to the hydrophobic protein core with selective reduction of the amino acid residues in contact with the GCE surface.

The results obtained for DTT denatured RTX showed a higher oxidation current for the peak at \( E_{pa} = +0.65 \text{ V} \) and two new oxidation peaks, corresponding to the oxidation of tryptophan and histidine amino acid residues, confirming the high reactivity of DTT to promote a total unfolding of the RTX structure, and being an ideal reductant agent for protein structural analysis.

The RTX denaturation by SDS, DTT or TCEP was investigated for different incubation times using RTX-electrochemical biosensors. DTT was the strongest agent, allowing the identification of the electroactive tyrosine, tryptophan and histidine amino acid residues. SDS and TCEP caused an enhanced of the oxidation peak current of tyrosine and tryptophan amino acid residues due to a better access to the GCE surface.

4 Conclusions

RTX is an anticancer drug used mainly in the treatment of B-cell non-Hodgkin’s lymphoma combined with chemotherapy. Despite being structurally complex, native and denatured RTX were characterized by DP voltammetry at a GCE surface.

The oxidation of native RTX showed a pH-dependent peak corresponding to the oxidation of tyrosine and tryptophan amino acid residues and at lower \( \text{pH} \) another oxidation peak occurred corresponding to the oxidation methionine amino acid residues.

A new methodology in the investigation of the direct electron transfer oxidation of denatured RTX at solid interfaces was used. The action of denaturing agents SDS, DTT or TCEP unfold the RTX molecule and improves the electrochemical response and sensibility. SDS and TCEP enhanced the intensity of the oxidation peak of ty-
rosine and tryptophan amino acid residues, whereas DTT enabled the detection of the oxidation peaks of tyrosine, tryptophan and histidine amino acid residues.

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