Bevacizumab anticancer monoclonal antibody: native and denatured redox behaviour

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ABSTRACT

Bevacizumab (BEVA) is a monoclonal antibody (mAb) used in clinical oncology to treat certain types of metastatic cancers. The electrochemical oxidation of native and denatured BEVA was investigated in solution over a wide pH range and using BEVA-thin film voltammetry on a glassy carbon electrode. For native BEVA, only one pH-dependent oxidation peak, corresponding to tyrosine and tryptophan amino acid residues oxidation, was observed. The interfacial behaviour and adsorption of BEVA at the glassy carbon surface were evaluated by voltammetry and electrochemical impedance spectroscopy. The unfolding of the protein 3D morphological structure occurred upon denaturation with chemical agents, denaturing agent sodium dodecyl sulphate, and the reductants tris(2-carboxyethyl)phosphine and dithiothreitol, was electrochemically characterized. The electrochemical oxidation of denatured BEVA-thin film on GCE showed additional cysteine and histidine residues oxidation peaks.

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1. Introduction

The improved understanding of the molecular mechanisms of cancer has opened the way to a plethora of new anticancer therapies potentially more effective and with diminished adverse side effects when compared to classical chemotherapy and radiotherapy. Monoclonal antibodies (mAb) have earned special attention due to their more specific and more effective therapeutic properties and have become one of the most promising strategies for cancer treatment [1]. Several different monoclonal antibodies have recently achieved remarkable clinical success and have been approved by the U.S. Food and Drug Administration (FDA) for specific cancer therapy, and some newer types are still in clinical trials [1].

Bevacizumab (BEVA) is a recombinant humanized IgG1 monoclonal antibody (93% human, 7% murine sequences) that targets vascular endothelial growth factor A (VEGF-A). By inhibiting VEGF-A molecule, BEVA blocks angiogenesis and limits the tumours' blood supply, thus slowing or interrupting its growth [1,2]. BEVA is an intravenous drug originally approved by the FDA in 2004 to treat advanced colorectal cancer [2]. It is presently employed in the treatment of other human solid tumours such as lung, kidney, ovarian and brain cancers [1,2], and also in the treatment of profilative diabetic retinopathy (PDR) [3], hereditary haemorrhagic telangiectasia (HHT) [4] and glaucoma [5].

Structurally, BEVA comprises six murine sequences grafted onto a backbone of heavy chains of 452 amino acids and light chains of 214 amino acids, having a molecular weight of ~149 kDa. Each light chain is covalently linked through a disulphide bond at cysteine 214 to a heavy chain at cysteine 226. The two heavy chains are covalently bound through two interchain disulphide bonds, consistently with the structure of a human IgG1 [6],Scheme 1.

In the development and application of monoclonal antibodies, as effective anticancer drugs, the study of the reactions involved in their physiological action mechanism is of utmost importance. The electrochemical methods have proved to be a valuable tool and have enabled to obtain sound insight, as far as the in vitro behaviour of these, and other drugs, used in clinical oncology, is concerned [7–12].

The electrochemical behaviour of amino acids has revealed that tyrosine, tryptophan, cysteine, histidine and methionine, are the only electroactive at carbon electrodes [13–23]. Direct
Stock solutions of 6 mg mL⁻¹ BEVA for solution experiments, and 10 mg mL⁻¹ BEVA to prepare the BEVA-thin film on the GCE surface, in deionised water were prepared daily. Stock solutions of 0.1 M DTT and 0.1 M SDS were prepared in deionised water, and of 0.1 M TCEP in 0.1 M acetate buffer pH = 5.4. The 10 mM K₃[Fe(CN)₆] stock solutions were daily prepared in 0.1 M phosphate buffer pH = 6.9.

All the supporting electrolyte buffer solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity < 0.1 µS cm⁻¹): 0.1 M HACO/NaAcO acerate buffer pH 3.4, 4.5, and 5.4; 0.1 M NaH₂PO₄/Na₂HPO₄ phosphate buffer pH 6.08 to 8.05; and 0.1 M NaOH/Na₂B₄O₇ buffer pH = 9.25.

Microvolumes were measured using P20, P200 and P1000 microliter pipettes (Gilson S. A., Villiers-le-Bel, France). The pH measurements were performed with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode.

All experiments were carried out at room temperature (25 ± 1) °C.

2.2. Electrochemical parameters and cells

Voltammetric experiments were carried out using a μLab running with GPES 4.9 software, Metrohm/Autolab, Utrecht, The Netherlands. Measurements were performed 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 volume of 500 µL in an one-compartment electrochemical cell.

Cyclic voltammery (CV) was performed at a scan rate of 100 mV s⁻¹. The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms, interval time 0.4 s, and scan rate 5 mV s⁻¹. For square wave (SW) voltammetry, the experimental conditions were frequency of 50 Hz, potential increment of 2 mV and pulse amplitude of 50 mV, effective scan rate of 100 mV s⁻¹.

Electrochemical impedance spectroscopy (EIS) was performed using an Autolab 10 running the FRA software version 4.9. The voltage perturbation was 5 mV r.m.s. over a frequency range from 60 kHz to 0.1 Hz, with six frequencies per decade. The EIS spectra were recorded at the midpoint potentials. From CV experiments, in solutions of 1 mM K₃[Fe(CN)₆], in 0.1 M phosphate buffer pH 6.9, the midpoint potentials, of +0.45 V for clean GCE and of +0.52 V for BEVA-thin film on GCE, were determined.

The GCE was polished using diamond spray (particle size 1 µm, Kement, Kent, UK) before each electrochemical experiment. After polishing, the electrode was rinsed thoroughly with Milli-Q water for 30 seconds. After this mechanical treatment, the GCE was placed in buffer supporting electrolyte and DP voltamograms were recorded. The procedure was repeated as many times as necessary until reproducible baseline voltamograms were obtained.

All voltammograms were always recorded immediately after the immersion of the electrode into the electrochemical cell containing the sample solution.

2.3. Procedures

Procedure 1—Solutions of 5 mg mL⁻¹ BEVA in 0.1 M buffer electrolyte with 3.4 ≤ pH ≤ 9.25, were prepared. The GCE was placed in the electrochemical cell containing the BEVA solution where CV, DP and SW voltammetry were performed.

Procedure 2—BEVA-thin film modified GCE—The immobilization of BEVA at the GCE surface was carried out by successively covering the GCE surface with 3 drops each of 5 µL from a 10 mg mL⁻¹ BEVA stock solution. After placing each drop the electrode surface was dried under a constant flux of N₂. The GCE with immobilized BEVA
was then placed in the electrochemical cell containing only the buffer supporting electrolyte and allowed to hydrate for 5 minutes before DP voltammograms were recorded.

Procedure 3—denatured BEVA-thin film modified GCE—The GCE with immobilized BEVA, Procedure 2, was incubated in 33.33 mM SDS, 10 mM TCEP or 10 mM DTT, during different time periods, ranging from 30 to 300 minutes. Then, the electrode was removed from the solution, washed with deionised water to remove the excess of SDS, TCEP or DTT, and placed in the electrochemical cell containing only the 0.1 M phosphate buffer pH 6.9 supporting electrolyte, where DP voltammograms were recorded. Each experiment was carried out with a newly prepared BEVA-thin film modified GCE. For control experiments, newly prepared BEVA-thin film modified GCE were incubated in 0.1 M phosphate buffer pH 6.9 for the same periods of time.

2.4. 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.

The hydropathicity plots [28] of BEVA, sequence DB00112 provided by DrugBank database [29], were obtained using the online tool ProtScale from ExPASy–Bioinformatic Resource Portal [30] with a window size of 9 amino acids.

The antibody structure was obtained using the Protein Workshop 4.2.0 [31] software from Protein Data Bank RCSB PDB [32,33].

3. Results

3.1. Native BEVA in solution

The redox behaviour of native BEVA was studied by CV, DP and SW voltammetry in buffer supporting electrolytes with 3.4 ≤ pH ≤ 9.25.

3.1.1. Cyclic voltammetry

CVs were recorded in solutions of 5 mg mL⁻¹ BEVA in electrolytes with different pHs between the potential limits of +0.0 V and +1.4 V, Fig. 1. One main oxidation peak was observed on the positive-going scan of the first CV, corresponding to tyrosine (Tyr) and tryptophan (Trp) amino acid residues electrochemical oxidation, Table 1. No peak occurred after changing the scan direction, indicating the irreversibility of BEVA electron transfer process. A second CV in the same conditions, without cleaning the GCE surface, was recorded, but no peaks were observed due to the electrode surface blockage with adsorbed non-electroactive products.

Increasing the supporting electrolyte pH the oxidation peak was shifted to less positive potentials, corresponding to the BEVA pH-dependent oxidation mechanism, Fig. 1.

3.1.2. Differential pulse voltammetry

DP voltammetry allows lower detection limits and a better visualization of all redox processes. DP voltammograms were recorded in solutions of 5 mg mL⁻¹ BEVA in electrolytes with different pH.

The DP voltammograms, Fig. 2A, presented only one oxidation peak, in all electrolytes. The peak potential variation with pH was linear, following the equation, $E_{pa} = 1.091 - 0.060 \times pH$. The slope of the line –60 mV per pH unit and the width at half height of the oxidation peak, $W_{1/2} \sim 90$ mV, indicated that the oxidation involves one electron and one proton transfer, Fig. 2A. BEVA oxidation peak currents were also pH-dependent, Fig. 2B. The highest peak current was observed in pH = 6.08 and the lowest in pH = 4.5.

3.1.3. Square wave voltammetry

The electrochemical behaviour of native BEVA was also studied using SW voltammetry in buffer electrolytes with 3.4 ≤ pH ≤ 9.25. The SW voltammogram, in 0.1 M phosphate buffer pH 6.9, Fig. 3, showed the BEVA oxidation peak. The irreversibility of BEVA oxidation was confirmed by plotting the forward and the backward components of the total current, and only the anodic reaction occurred. A second SW voltammogram was recorded in the same conditions and without cleaning the GCE surface, but no peak was detected.

3.2. BEVA-thin film modified GCE

The electrochemical oxidation of a BEVA-thin film modified GCE was investigated in 3.4 ≤ pH ≤ 9.25 electrolytes, using DP voltammetry, following BEVA immobilization Procedure 2, and BEVA immobilized denaturation using Procedure 3.

![Fig. 1. CVs with GCE in 5 mg mL⁻¹ BEVA: (−) pH 4.5, (−−−) pH 6.9 and (⋯) pH 9.3 buffer supporting electrolyte; $v = 100$ mV s⁻¹.](image)

Table 1

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$E_{pa}$/V</th>
<th>$E_{pc}$/V</th>
<th>$E_{pa}$/V</th>
</tr>
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<tbody>
<tr>
<td>Tyrosine (Tyr)</td>
<td>0.63</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tryptophan (Trp)</td>
<td>0.63</td>
<td>1.08</td>
<td>−</td>
</tr>
<tr>
<td>Histidine (His)</td>
<td>1.15</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Cysteine (Cys)</td>
<td>0.52</td>
<td>0.88</td>
<td>1.27</td>
</tr>
<tr>
<td>Methionine (Met)</td>
<td>1.12</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
3.2.1. Native BEVA-thin film modified GCE

The DP voltammograms of native BEVA-thin film modified GCE, Fig. 4A, presented one oxidation peak, in all electrolytes, corresponding to tyrosine (Tyr) and tryptophan (Trp) amino acid residues electrochemical oxidation, Table 1 [18–21].

The native BEVA-thin film oxidation peaks were better visualized when compared with the experiments in solution, presenting higher peak currents which increased until reaching a maximum in 0.1 M phosphate buffer pH = 6.9.

The oxidation peak potential showed a linear pH-dependence, according to the relationship $E_{pa} = 1.054 – 0.060pH$, with the supporting electrolyte pH for the whole range studied, Fig. 4B. The slope of $–60$ mV per pH unit and $W_{1/2} \sim 90$ mV, confirm that the immobilized BEVA oxidation involves as well the transfer of one electron and one proton.

The interfacial behaviour and adsorption of native BEVA-thin film on GCE surface was characterized by CV and EIS in solutions of 1 mM K$_4$Fe(CN)$_6$, in 0.1 M phosphate buffer pH 6.9, Fig. 5.

The CVs recorded in 1 mM K$_4$Fe(CN)$_6$, in 0.1 M phosphate buffer pH 6.9, with BEVA-thin film on GCE showed that the K$_4$Fe(CN)$_6$ oxidation and reduction become irreversible and the peaks potential were shifted to more positive and more negative when compared with the CVs recorded with the clean GCE, Fig. 5A. For recording the EIS spectra the midpoint potentials of $+0.45$ V for
clean GCE, and of +0.52 V for BEVA-thin film on GCE, were determined and applied.

The EIS experiments were then carried out with a clean GCE and with BEVA-thin film on GCE, in 1, 3 and 5 mM K₄Fe(CN)₆ in 0.1 M phosphate buffer pH 6.9. The complex plan impedance plots at the midpoint potentials of +0.45 V for the GCE and of +0.52 V for the BEVA-thin film are shown in Fig. 5B and 5C. For the GCE a semicircle corresponding to the charge transfer reaction was observed. Whereas, the EIS at BEVA-thin film on GCE was essentially capacitive probing the adsorption of BEVA at the GCE surface.

3.2.2. Denatured BEVA-thin film modified GCE

The native BEVA-thin film at the GCE surface was denatured, Procedure 3, after incubation in 33.33 mM SDS, 10 mM TCEP or 10 mM DTT, in 0.1 M phosphate buffer pH 6.9, during different time periods. The concentrations of the denaturing agents were chosen based on protocols for electrophoretic procedures commonly applied to destabilize and unfold protein structure [34]. Additional experiments were performed incubating the BEVA-thin film at the

Fig. 5. In 0.1 M phosphate buffer pH = 6.9: (A) CVs in 1 mM K₄Fe(CN)₆, with: (---) clean GCE and (——) BEVA-thin film on GCE, v = 100 mV s⁻¹; (B, C) Complex plan EIS: (●) clean GCE and BEVA-thin film on GCE: (square) 1 mM, (triangle) 3 mM and (△) 5 mM K₄Fe(CN)₆ between (B) 50500 and 0.100 Hz, and (C) 50500 and 50Hz.

Fig. 6. DP voltammograms base-line corrected in 0.1 M phosphate buffer pH = 6.9 of (——) BEVA-thin film on GCE; and (——) after incubation in: (A) 33.33 mM SDS, (B) 10 mM TCEP and (C) 10 mM DTT.
GCE in 0.1 M phosphate buffer pH 6.9, for the same periods of time, and used as a control to allow an unambiguous identification of the oxidation peaks occurring after the interaction of BEVA-thin film at the GCE surface with the different denaturing compounds.

The redox behaviour of DTT, TCEP and SDS, in 0.1 M phosphate buffer pH 7.0, was previously investigated [7], showing only one oxidation peak DTT, at $E_{pa} = +1.2$ V, and TCEP, at $E_{pa} = +1.1$ V, while SDS was not electroactive, and this was taken into account in the identification of the denatured BEVA-thin film modified GCE occurring peaks [27].

The effect of the denaturing agents on the electrochemical behaviour of BEVA-thin film at the GCE surface was followed by DP voltammetry. The results obtained for the control experiments, BEVA-thin film at the GCE surface incubated in buffer, were compared with the changes observed after incubation of BEVA-thin film at the GCE surface in solutions of the denaturing agents.

The DP voltammograms of the BEVA-thin film at the GCE surface after incubation in SDS solutions, Fig. 6A, showed three oxidation peaks, at $E_{pa} = +0.65$ V, $E_{pa} = +0.90$ V and $E_{pa} = +1.10$ V. The control experiment showed two oxidation peaks, at $E_{pa} = +0.65$ V and $E_{pa} = +0.90$ V. The first oxidation peak current, at $E_{pa} = +0.65$ V, increased when compared to the control.

Similar results were found for the BEVA-thin film on GCE surface after incubation in TCEP solutions, Fig. 6B, but the oxidation peak current of the first peak, at $E_{pa} = +0.65$ V, decreased, when compared to control, and higher currents for the second and third oxidation peaks were observed.

The DP voltammograms for BEVA-thin film on GCE surface after incubation in the DT solutions, Fig. 6C, showed two oxidation peaks with significantly enhanced currents, at $E_{pa} = +0.65$ V and $E_{pa} = +1.00$ V. No time dependency was observed for the DTT-BEVA interaction.

4. Discussion

Among the 20 amino acids composing the protein chains has been previously shown that only tyrosine (Tyr), tryptophan (Trp), histidine (His), cysteine (Cys) and methionine (Met) residues are electroactive and undergo oxidation on carbon electrodes, Table 1, according to pH-dependent electron transfer mechanisms [18–21]. Direct protein electrochemistry has been largely based on the detection of tyrosine and tryptophan although the oxidation of histidine, cysteine or methionine has been reported mostly in peptides [25] and more recently in proteins [7,23,26].

In order to identify the BEVA oxidation peaks, the primary structure of the antibody has been analysed [29]. BEVA has 452 amino acids on the heavy chain and 214 amino acids on the light chain, of this 80: 33 Tyr, 12 Trp, 16Cys, 14His and 5 Met, electroactive amino acid residues were identified. All these amino acids are randomly distributed along the polypeptide chain. Most electroactive amino acids are contained in the Fab region of BEVA, Scheme 1 and Fig. 7.

The heavy chains of BEVA contain one N-linked glycosylation site at asparagine 303. The oligosaccharides are of complex biantennary structures with a core fucose and with the two branches terminating mainly with zero (G0), one (G1) or two (G2) galactose residues. The G0 glycoform predominates at approximately 80% relative abundance. Variants such as oxidised, glycated and deglycosylated BEVA are fully active. Although the polysaccharides influence BEVA structure, they are not electroactive.

The interaction of proteins with electrode surfaces is directly influenced by surface properties. The GCE surface is hydrophobic favouring interaction with hydrophobic protein domains. The Kyte–Doolittle hydrophobicity scale was used for the identification of the hydrophobicity and/or polarity of a protein or protein sequence.
The Kyte–Doolittle plot for both light and heavy chains of BEVA, Fig. 7, showed a general hydrophilic character with intercalated hydrophobic sequences. Most of the electroactive amino acids are incorporated into the hydrophilic regions located at, or in close proximity, to the protein surface and mostly contained at the Fab region of BEVA, Scheme 1 and Fig. 7.

The electrochemical oxidation of BEVA in solution or immobilized at the GCE surface showed only one small pH-dependent anodic peak. These results clearly reflect the difficulty of the electron transfer process arising from the organization of the amino acids in the tertiary and quaternary structures of the antibody, which difficult the contact between the protein electroactive amino acids and the GCE surface. Considering the oxidation potentials, Table 1, and the peak potential pH-dependence, the native BEVA oxidation peak corresponded to charge transfer reactions of Tyr and Trp amino acid residues oxidation located at the Fab region.

A methodology employing denaturants that unfold the protein 3D morphological structure, enabling the exposure of more electroactive amino acids residues to the electrode surface was followed. SDS, a detergent that dissolves hydrophobic proteins by disrupting non-covalent bonds, and the reductants DTT or TCEP, that disrupt disulphide bonds, were used to investigate the direct electron transfer oxidation of denatured BEVA-thin film on GCE surface, Fig. 6.

After denaturation, three consecutive charge transfer reactions were observed, Fig. 6. The first oxidation peak at $E_{pa} = +0.65$ V, corresponds to the Tyr and Trp residues as previously observed in the native BEVA. The second peak at $E_{pa} = +0.93$ V is due to Cys residues which oxidation at GCE involves three consecutive charge transfer reactions, Table 1. The third oxidation peak, at $E_{pa} \sim +1.10$ V, is attributed to His residues, Table 1, Fig. 6.

SDS is an anionic surfactant that changes the overall protein charge to negative, enhancing its interaction with the positive electrode surface, and increasing the number of amino acid residues exposed to the electrode surface. In agreement, the DP voltammograms, recorded after different denaturation times, showed the occurrence of the three consecutive charge transfer reactions, and the increase of the Tyr/Trp oxidation peak, Fig. 6A.

The DP voltammograms recorded after denaturation of BEVA-thin film on GCE with TCEP showed also three consecutive charge transfer reactions, Fig. 6B. However, the incubation of the BEVA-thin film on GCE surface in DTT enabled the identification of two oxidation peaks with enhanced currents, Fig. 6C. The differences observed in BEVA electrochemistry after denaturation with TCEP and DTT is explained considering a higher reducing power of TCEP and that DTT cannot reduce solvent inaccessible or buried disulphide bonds.

The electrochemical oxidation of BEVA was also compared with the electrochemistry of rituximab (RTX) [7]. The monoclonal antibody RTX contains 76 electroactive amino acid residues: 31 Tyr, 13 Trp, 16Cys, 13His and 3 Met. All these amino acids are randomly distributed along the polypeptide chain. The denaturation of RTX with SDS and TCEP only caused the Tyr/Trp oxidation peak time-dependent increase while with DTT three consecutive charge transfer reactions occurred [7]. The changes between RTX and BEVA electrochemistry after denaturation are related to the differences on the primary structure of these antibodies.

Both BEVA and RTX are IgG1 type immunoglobulins sharing constant domains on both heavy and light chains. The analysis of their primary structures revealed that the variable domain of the heavy chains extents up to 120 amino acids while of the light chains extents up to 60 amino acids. The analysis of the hydrophathy graphs showed similar profiles of the heavy chains and large differences in hydrophobic/hydrophilicity of the light chains, Fig. 8. However, most of the electroactive amino acids, responsible for the anodic peaks observed after denaturation, are contained into the heavy chain Fig. 8(B). Thus, the differences observed on the voltammograms recorded after RTX and BEVA denaturation with different agents are due to differences in the amino acid sequences of the variable domains of the heavy and light chains that form the Fab region of the antibody and which are actually responsible for their different functions.

5. Conclusions

Bevacizumab is a recombinant humanized monoclonal antibody used in the treatment of certain types of metastatic cancers. The charge transfer reactions of the native and denatured BEVA-thin film on GCE surface, over a wide pH range, were investigated. The interfacial behaviour and adsorption of BEVA-thin film on GCE surface was also probed by electrochemical impedance spectroscopy.

The electrochemical oxidation of native BEVA-thin film on GCE surface showed only one pH-dependent oxidation peak, corresponding to tyrosine and tryptophan amino acid residues oxidation. The unfolding of BEVA-thin film on GCE surface after denaturation with chemical agents enabled the exposure of more electroactive amino acid residues to the electrode surface, and additional peaks, due to cysteine and histidine amino acid residues oxidation peaks, were identified.

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