In situ dsDNA-bevacizumab anticancer monoclonal antibody interaction electrochemical evaluation

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Abstract

The interaction of the anticancer monoclonal antibody bevacizumab (BEVA) with double-stranded DNA (dsDNA) was studied by voltammetry and gel-electrophoresis in incubated samples and using the dsDNA-electrochemical biosensor. The voltammetric results revealed a decrease and disappearance of the dsDNA oxidation peaks with increasing incubation time, showing that BEVA binds to the dsDNA but no DNA oxidative damage was detected electrochemically. Non denaturing agarose gel-electrophoresis experiments were in agreement with the voltammetric results showing the formation of compact BEVA-dsDNA adduct. The dsDNA-electrochemical biosensor in incubated solutions showed that BEVA also undergoes structural modification upon binding dsDNA, and BEVA electroactive amino acid residues oxidation peaks were detected.

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1. Introduction

Tumour cells, like most normal cells, have high diversity of receptors on their surfaces. Molecules on the outside of the cell can attach to these receptors, causing changes to occur within the cells. Monoclonal antibodies (mAb) have earned special attention due to their specific and effective anticancer therapeutic properties. Bevacizumab (BEVA) is a recombinant humanized monoclonal antibody (mAb) used for the treatment of certain types of metastatic cancers [1–3]. BEVA exerts its antitumoral activity by blocking vascular endothelial growth factor A (VEGF-A) [4–6], a glycoprotein considered to be the main inducer to the growth of blood vessels. As a result, the blood supply to tumour cells is reduced, therefore...
slowing or interrupting growth [5].

Due to its therapeutic efficacy and increasing use in clinical oncology [1,3,7], the understanding of the in vivo behaviour and physiological mechanism of action, as well as of the toxic and chemotherapeutic adverse side effects of BEVA is of utmost importance.

Studies on the interaction of anticancer drugs with DNA have enabled to elucidate their possible implications in DNA structural modifications, and the mechanistic and cytotoxic aspects of their physiological action [8–13]. Several methods such as UV–VIS, IR and Raman spectroscopy, NMR, mass spectrometry, capillary electrophoresis, surface plasmon resonance, femtosecond laser spectroscopy, HPLC, molecular modelling techniques and electrochemistry have been applied for that purpose [8,9,14–17].

Electrochemical methods offer fast response, high sensitivity and great selectivity [9,11,14–16]. A DNA-electrochemical biosensor consists of an electrode with dsDNA immobilized on the surface [9,15,16]. The extensive potential window of carbon electrodes allows electrochemical detection of both dsDNA conformational changes and oxidative damages caused to DNA [8–11,13,15,16,18–21]. AFM images have shown that a complete covering of the electrode surface is essential in order to avoid non-specific adsorption of DNA so that the DNA modifications detected are only due to the interaction with the compound [9,15,16].

The DNA-electrochemical biosensor has been used to study the interaction mechanisms of DNA with anticancer drugs [9–11] and the monoclonal antibody rituximab [13]. DNA structural modifications and oxidative damage were observed following changes in the DNA oxidation peaks of purine bases, nucleotides, nucleosides, and the oxidation products of guanine, 8-oxoguanine (8-oxoGua), and of adenine, 2,8-dihydroxyadenine (2,8-oxoAde), which are biomarkers of DNA base oxidative damage [9,15,16].

The voltammetric behaviour of native and denatured BEVA was investigated [22]. In native BEVA, only one pH-dependent oxidation peak, corresponding to tyrosine, first tryptophan and first cysteine, 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 upon denaturation with chemical agents denaturing agent, sodium dodecyl sulphate and the reductants tris(2-carboxyethyl)phosphine and dithiothreitol, showed additional second tryptophan and second cysteine residues oxidation peaks [22].

In this context, the aim of the present work was to elucidate the interaction mechanism between BEVA and dsDNA. Experiments have been carried out in incubated solutions and using the dsDNA-electrochemical biosensor. Gel-electrophoresis was also applied to support the BEVA-dsDNA interaction mechanism.

2. Experimental

2.1. Materials, reagents and solutions

Sodium salt double stranded calf thymus DNA (dsDNA), polyadenylc (poly[A]) and polyguanylic (poly[G]) acids from Sigma−Aldrich and bevacizumab (BEVA, 25 mg mL−1) from Roche Pharmaceauticals were used without further purification.

Stock solutions of 500 μg mL−1 dsDNA, 200 μg mL−1 poly[G] and poly[A], and 1 mg mL−1 BEVA were prepared in deionised water and kept at 4 °C. All solutions were diluted to the desired concentration in 0.1 M acetate buffer pH = 4.5 or 0.1 M phosphate buffer pH = 6.9 supporting electrolytes, prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 μS cm−1).

Microvolumes were measured using P20, P200 and P1000 μL pipettes (Gilion S. A., Villiers-le-Bel, France). The pH measurements were performed with a Crison microph pH-1, pH-meter with an Ingold combined glass electrode.

All experiments were carried out 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 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 500 μL one-compartment electrochemical cell.

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.

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 s. After this mechanical treatment, the GCE was placed in buffer supporting electrolyte and DP voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

2.3. Acquisition and presentation of voltammetric data

All the DP voltammograms presented were baseline corrected using the automatic function included in the software. The mathematical treatment of the original voltammograms was used in the presentation of all experimental data for a better and clearer identification of the peaks. The values for peak currents were determined from the original untreated voltammograms after subtraction of the baseline.

2.4. BEVA denaturation

The immobilization of BEVA at the GCE surface was carried out by covering successively 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 incubated in 10 mM DTT during 30 min. Then, the electrode was removed from the solution, washed with deionised water to remove the excess of DTT and placed in the electrochemical cell containing only the supporting electrolyte where DP voltammograms were recorded.

2.5. Gel electrophoresis procedure

Nondenaturing agarose (1%, ultrapure DNA grade from Sigma) gel was prepared in TAE buffer (10 mM Tris base, 4.4 mM acetic acid and 0.5 mM EDTA, pH 8.0). The solutions of 25 μL of 100 μg mL−1 dsDNA control, 25 μL of 500 μg mL−1 BEVA control, and 25 μL of 500 μg mL−1 BEVA incubated with 100 μg mL−1 dsDNA during different time periods (with 0.25% bromophenol blue in water) were loaded into wells. The electrophoresis was carried out in TAE buffer for 4.5 h at −100 V. After 0.5% ethidium bromide (EtBr) stained DNA was visualized and photographed under UV, λ = 312 nm, transillumination to visualize DNA mobility.

2.6. Procedures

Procedure 1 — BEVA-dsDNA in incubated solutions — BEVA-dsDNA solutions were prepared incubating 100 μg mL−1 dsDNA with 10, 100 or 500 μg mL−1 BEVA, in 0.1 M phosphate buffer pH = 6.9,
during 0 and 48 h. Control solutions of 100 μg mL⁻¹ dsDNA and of 500 μg mL⁻¹ BEVA, in 0.1 M phosphate buffer pH = 6.9, were also prepared, and stored during the same periods of time in similar conditions as the BEVA-dsDNA incubated solutions. DP voltammograms were recorded with a clean GCE after each incubation time period.

Procedure 2 — Preparation of the dsDNA-electrochemical biosensor

The dsDNA-electrochemical biosensors were prepared by covering successively the GCE surface with three drops each of 5 μL from a 50 μg mL⁻¹ dsDNA solution. After placing each drop on the electrode surface the biosensor was dried under a constant flux of N₂. A similar procedure was used to obtain the poly[G] and poly[A]-electrochemical biosensors.

The biosensor was then placed in the electrochemical cell containing only the supporting electrolyte buffer and allowed to hydrate for 15 min. Afterwards, the biosensor was incubated in solutions of 500 μg mL⁻¹ BEVA during different time periods. Then, the biosensor was washed with deionised water to remove the excess of BEVA, and placed in the electrochemical cell containing only the supporting electrolyte where DP voltammograms were recorded. For each experiment, new dsDNA, poly[G] and poly[A]-electrochemical biosensors were prepared. To ensure that the peaks were exclusively due to BEVA interaction with dsDNA, control dsDNA, poly[G] and poly[A]-electrochemical biosensors were incubated in buffered solutions for the same time periods.

3. Results and discussion

3.1. Voltammetric behaviour of BEVA and dsDNA revisited

The electrochemical behaviour of BEVA, DNA, guanine and adenine in 0.1 M phosphate buffer pH = 6.9 was revisited for control, Fig. 1.

The DP voltammogram of 500 μg mL⁻¹ native BEVA showed one peak, at $E_{B1} = +0.68$ V, due to tyrosine, first tryptophan and first cysteine, amino acid residues oxidation [23,24], Fig. 1A. The DP voltammogram after BEVA denaturation with dithiothreitol (DTT), showed two consecutive charge transfer reactions the first peak, at $E_{B1} = +0.65$ V, and the second peak, at $E_{B2} = +1.01$ V, due to second tryptophan and second cysteine residues oxidation peaks.

The DP voltammograms of 100 μg mL⁻¹ dsDNA showed two peaks corresponding to the oxidation of DNA purine bases nucleosides, guanine (Gua) residues, desoxyguanosine (dGuo), at $E_{dGuo} = +0.91$ V, and adenine (Ade) residues, desoxyadenosine (dAdo), at $E_{dAdo} = +1.17$ V [25–27], Fig. 1A.

The DP voltammogram, Fig. 1B, showed the oxidation peaks of 10 μM guanine (Gua), at $E_{Gua} = +0.67$ V, and 10 μM adenine (Ade), at $E_{Ade} = +0.94$ V.

3.2. Evaluation of BEVA-dsDNA interaction in incubated solutions

3.2.1. Voltammetry

To evaluate the ability of BEVA to cause conformational changes, hydrogen bonding cleavage and/or dsDNA base oxidative damage, the BEVA-dsDNA interaction was investigated using DP voltammetry, by comparing the changes on the dsDNA oxidation peaks, desoxyguanosine (dGuo) and desoxyadenosine (dAdo), in the presence and in the absence of BEVA, and by monitoring the occurrence of the oxidation peaks of free guanine (Gua); free adenine (Ade) and of guanine and/or adenine oxidation products, 8-oxoGua and 2,8-oxoAde.

The BEVA-dsDNA interaction, in incubated solutions in 0.1 M phosphate buffer pH = 6.9, Procedure 1, was investigated. It was found, in 0.1 M acetate buffer pH = 4.5, that in the mixture of BEVA and dsDNA occurred precipitation, and for this reason the BEVA-dsDNA interaction was only studied in pH = 6.9.

The DP voltammogram of 100 μg mL⁻¹ dsDNA with 100 μg mL⁻¹ BEVA, at 0 h incubation time, Fig. 2A, showed the decrease of dGuo oxidation peak while dAdo peak disappeared. Two new peaks attributed to the oxidation of DNA guanine (Gua), at $E_{Gua} = +0.68$ V, and BEVA, second tryptophan and second cysteine residues, at $E_{B2} = +1.07$ V, occurred. For longer incubation times, BEVA oxidation peak B1, at $E_{B1} = +0.63$ V, superimposed with Gua peak, at $E_{Gua} = +0.68$ V, also appeared.

The experiment was repeated for 10 and 500 μg mL⁻¹ BEVA, Fig. 2B and C. At 0 h incubation time, dGuo peak decreased and dAdo peak completely disappeared for both concentrations. For high BEVA concentrations the occurrence of Gu or B1 and B2 oxidation peaks, was observed. Increasing the incubation time both Gua and B1 oxidation peaks increased.

No peaks corresponding to 8-oxoGua and 8oxoAde oxidation were detected, on the DP voltammograms, showing that the BEVA-dsDNA interaction did not cause oxidative damage. A similar behaviour was observed for incubated solutions of BEVA with poly[G] and poly[A], Fig. 3. The DP voltammograms in incubated solutions of poly[G] and BEVA showed the time-dependent decrease of dGuo peak while the Gua peak appeared only for longer incubation times, Fig. 3A. The DP voltammograms in incubated solutions of poly[A] and BEVA showed the short incubation times BEVA peak B1, the time-dependent decrease of dAdo peak, and for longer incubation times, BEVA peak B2, always
appeared, but no Ade peak occurred, Fig. 3B.

The experiments described above showed that in incubated solutions BEVA-dsDNA interaction leaded to the formation of a complex adduct, and both DNA and BEVA undergo unfolding and conformational modifications, detected by DP voltammetry.

3.2.2. Electrophoresis

To clarify the BEVA-dsDNA interaction, gel electrophoresis of the products of the interaction of dsDNA with BEVA, was carried out. This technique enables the detection of conformational changes, comparing the relative migration profile with that of the dsDNA and BEVA controls, allowing the assessment of the destabilization degree. To visualize the DNA mobility and possible damage, the EtBr binding assay for DNA damage was used [28].

The electrophoretic migration profile of incubated samples of dsDNA with BEVA in 0.1 M phosphate buffer pH = 6.9 during 24 h (lanes 3, 4, 5) is shown in Fig. 4. The dsDNA (lane 1) and BEVA (lane 2) controls were incubated in the same experimental conditions as BEVA-dsDNA samples and analyzed after 24 h.

The bands intensity decreased in all experiments when compared to the results obtained for the dsDNA control (lane 1). This decrease indicated that EtBr was excluded from binding sites, due to the dsDNA conformational changes, promoted by the interaction of BEVA with the double helix.

3.3. Evaluation of BEVA-DNA interaction with the dsDNA-electrochemical biosensor

During the electrochemical evaluation of the BEVA-dsDNA interaction in incubated solutions, an incomplete network film of co-adsorbed free dsDNA, free BEVA and dsDNA-BEVA complexes at the GCE surface was formed. Complete coverage of the electrode surface was important to avoid the undesired adsorption to the electrode surface, and it can be achieved using the GCE modified with immobilized dsDNA, the dsDNA-electrochemical biosensor.

The BEVA-dsDNA interaction was investigated by following the changes observed on the DP voltammogram of the dsDNA-electrochemical biosensor after incubation in 500 µg mL⁻¹ BEVA in 0.1 M acetate buffer pH = 4.5, Fig. 5, according to Procedure 2. For each experiment a new biosensor was prepared.

The dsDNA-electrochemical biosensor showed the changes
occurring in the dsDNA immobilized on the electrode surface during the interaction with BEVA, in situ and in real time. The DP voltammogram recorded with the dsDNA-electrochemical biosensor, in 0.1 M acetate buffer pH = 4.5, showed both dGuo, at $E_{dGuo} = +1.02$ V, and dAdo, at $E_{dAdo} = +1.27$ V, oxidation peaks, Fig. 5.

The currents of both dGuo and dAdo oxidation peaks decreased with incubation time, particularly of dAdo oxidation peak, which disappeared after 15 min. After 10 min incubation, the peak corresponding to the oxidation of Gua and BEVA peak B1, at $E = +0.77$ V, increased in a time-dependent manner and occur superimposed.

This experiment was also carried out in 0.1 M phosphate buffer pH = 6.9. A similar behaviour, with the decrease of dGuo and dAdo, the occurrence of B1, Guo and B2 oxidation peaks, was observed.

In order to obtain more information on the mechanism of BEVA interaction with DNA, purine homo-polynucleotide single stranded poly[A]- and poly[G]-electrochemical biosensors were also prepared.

The DP voltammogram, in 0.1 M acetate buffer pH = 4.5, of the poly[G]-electrochemical biosensor showed one peak, at $E_{dGuo} = +1.01$ V, corresponding to the oxidation of dGuo residues, Fig. 6A. Newly prepared poly[G]-electrochemical biosensors were incubated in BEVA, and with increasing incubation time the dGuo oxidation peak decreased, and BEVA peak B1 and Guo oxidation peaks, at $E = +0.77$ V, appeared superimposed, Fig. 6A, confirming the results obtained with the dsDNA-electrochemical biosensor.

The DP voltammogram, in 0.1 M acetate buffer pH = 4.5, of the poly[A]-electrochemical biosensor showed one peak, at $E_{dAdo} = +1.27$ V, corresponding to the oxidation of dAdo residues, Fig. 6B. Increasing the incubation time in BEVA solution, the dAdo peak decreased, in a time-dependent manner, and BEVA peak B1 appeared, at $E_{B1} = +0.77$ V. For long incubation times, the dAdo peak disappeared and BEVA peak B2, at $E_{B2} = +1.26$ V, occurred, also in agreement with the results obtained with the dsDNA-electrochemical biosensor.

3.4. BEVA-dsDNA interaction mechanism

The experiments carried out in incubated solutions and with the dsDNA-electrochemical biosensor clarified the interaction between dsDNA, a negatively charged molecule, and the monoclonal antibody BEVA, positively charged for pH < 8.3 (BEVA pI ~ 8.3).

The interaction between BEVA and dsDNA is driven by electrostatic forces, and involves morphological conformational modification of both the polypeptide chain and the double helix, which were detected through by the appearance of oxidation peaks of
BEVA electroactive amino acid residues and dsDNA oxidation peaks (dGuo and dAdo) changes. The DP voltammograms have shown the time-dependent decrease of both DNA oxidation peaks (dGuo and dAdo), Figs. 2 and 5, in agreement with structural modifications of the DNA double helix, and the formation of a dsDNA-BEVA complex adduct that prevents the interaction of DNA bases with the electrode surface. For longer incubation times, the occurrence of Gua oxidation was observed. In agreement with local unwinding of the DNA double helix and exposure of some Gua residues to the GCE surface, Figs. 3A and 6A.

The results have shown that dsDNA-BEVA interaction caused changes in BEVA structure. In the native conformation BEVA is electroactive and peak B1 is due to tyrosine, first tryptophan and first cysteine, amino acid residues oxidation. Upon dsDNA binding, peak B2, due to second tryptophan and second cysteine residues oxidation peaks, also occurred, Figs. 2 and 6, and the B1 peak current increased, Fig. 2, explained by the BEVA 3D structure unfolding, enabling the exposure of electroactive residues to the electrode surface.

4. Conclusions

The interaction of the anticancer antibody BEVA with dsDNA was investigated by DP voltammetry and gel electrophoresis in incubated solutions and using the dsDNA-electrochemical biosensor. The voltammetric results showed the decrease/disappearance of the dGuo and dAdo oxidation peaks after incubation, indicating that the BEVA-dsDNA interaction led to the formation of a complex adduct that prevents the interaction of DNA purine bases with the electrode surface. For long incubation times the occurrence of the guanine residues oxidation peak was observed but no oxidative damage DNA was electrochemically detected.

The dsDNA-electrochemical biosensors confirmed the results obtained in incubated solutions, and also showed that BEVA undergoes 3D structural modification upon binding to DNA, which led to the exposure of BEVA electroactive amino acid residues to the electrode surface.

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