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ENZYME MODIFIED ELECTROCHEMICAL BIOSENSOR FOR THE STUDY OF ANTICANCER ACTION OF MITOMYCIN C

Sweety TIWARI, K.S. PITRE
Dr. Harisingh Gour University, INDIA

Abstract. NADPH, cytochrome-c enzyme modified Glassy Carbon Fiber Electrode (GCFE) was fabricated for the study of DNA-Mitomycin C interaction using DPV (Differential Pulse Voltammetric) technique. The study has also been performing using anticancer drug, Mitomycin C (MC) modified, ds(double strand)-DNA modified electrochemical biosensors and at bare GCFE (Glassy Carbon Fiber Electrode), using DPV (Differential Pulse Voltammetric) technique. Mitomycin C was activated electrochemically and also using NADPH, cytochrome-c reductase enzyme. This enzymatic activation of Mitomycin C is parallel to its in-vivo metabolic activation in the cell to bind and cross link DNA. The enzyme modified GCFE was also fabricated for the study of DNA-Mitomycin C interaction. The work was supplemented by HPLC study on the adducts formed in the electrolysed solution, which revealed reductive activation of Mitomycin C to form quinone methide which covalently links itself with ds-DNA to produce adduct at the N-2 position of guanine in the minor groove. Thus, indicating the DNA cross linking and allowing Mitomycin C to work as an anticancer drug. A suitable mechanism model for the ds-DNA-Mitomycin interaction has been proposed.

Keywords: Mitomycin-C; Glassy Carbon Fiber Electrode; Electrochemical Biosensor; Enzyme Electrode; Mitomycin-C-DNA Interactions

1. Introduction

Mitomycin C, a potent antibiotic antitumor agent, is known to interact covalently with DNA [1], following its bioactivation by a variety of intracellular reductases [2-4], it reacts as an alkylating species and binds to DNA through guanine sites [5,6]. It forms covalent cross links between complementary DNA strands [7,8]. This process requires reduction of Mitomycin C into transiently activated form which is thought to occur in cells and can be mimicked easily in-vitro, electrochemically or enzymatically. The major DNA damage caused by activated Mitomycin C is cross-linking of DNA [9-11]. The majority of cytotoxicity of Mitomycin C has been attributed to DNA alkylation and termination of DNA replication, sometimes leading to chromosome loss or cell death. Maria Tomaz and Co-workers [12] have proposed the formation of deoxyguanosine-Mitomycin C adduct as a result of drug DNA interaction, as major product. However, two minor adducts formation has also been reported by them. It becomes necessary to search for correlation between the biological effects of Mitomycin C and its covalent modification of ds-DNA, which is of considerable interest in cIn an effort to study and understand the mechanism of action of anticancer DNA alkylating drug Mitomycin C, the present work has been designed for Differential Pulse Voltammetric study on the Mitomycin-ds-DNA interactions using bare GCFE, drug modified and NADPH cytochrome c reductase enzyme modified electrodes. The work has been supplemented by HPLC studies to identify adduct formed in the solution as a result of Mitomycin-ds-DNA interaction. A suitable mechanism for drug DNA interactions has been proposed.

2. Materials and methods

2.1 Chemicals and reagents

ds-DNA sodium salt (Calf thymus) 99% pure was obtained from Himedia Lab. Pvt. Ltd., Mumbai, and was used without purification. Whereas, Mitomycin C was obtained from Across Organics, RFCL Limited, New Delhi. All other chemicals used were of Analytical Reagent/BDH grade. Doubly distilled water was used for the preparation of the solutions.

2.2 Instrumentation

All voltammetric experiments were carried out using an Elico-µp (micro-processor) based polarographic analyser model CL-362 (Hyderabad, India). NF-12, Sigti Eleititigafit, U.K. Glassy Carbon Fibers were used to construct the electrode (GCFE), which was used as working electrode. A coiled platinum wire was used as a counter electrode. All potentials measured and reported in the present work were versus Saturated Calomel Electrode (SCE). The voltammetric cell had arrangements to bubble nitrogen gas through the solution. All experiments were carried out at room tempera-

ture. The pH-measurements were made on a Systronics (India) digital µ-pH meter system-361.

2.3 Preparation of stock solution

The stock solutions of 500µg/mL DNA and 500µg/mL Mitomycin-C were prepared by dissolving the weighed quantity in doubly distilled water. The solutions of other reagents required for the experiment were prepared by dissolving a calculated amount in required volume of doubly distilled water.

3. Results and discussion

3.1 Mitomycin C analysis at bare GCFE

On Differential Pulse Voltammetric (DPV) analysis of a solution of Mitomycin-C (15µg/mL) in a 0.1M acetate buffer (pH 5.6) using a bare GCFE as working electrode, two well defined peaks with peak potentials (Ep1 and Ep2) = -0.94V and -1.41V versus SCE were obtained (Fig.1), which are due to the reduction of diquinone group of Mitomycin C to form dihydroquinone involving one electron each. However, if the solution is electrolyzed performing positive potential scanning, it did not produce any DPV response.

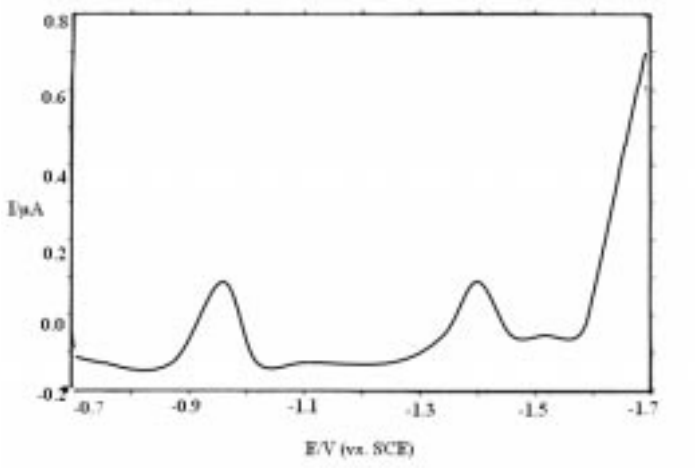

Fig. 1. Differential pulse voltammogram of 15µg/mL Mitomycin C at bare GCFE in 0.1M acetate buffer of pH 5.6, Pulse amplitude – 25mV, Scan rate – 12mV S^{-1}

3.2 Study of Mitomycin C-DNA interaction at bare GCFE

Looking at the possibility of the reduction of Mitomycin-C at the GCFE, attempts have been made to study the possible interaction of the anticancer drug with ds-DNA.

For which, experimental sets were prepared by taking a fixed concentration of Mitomycin C (15µg/mL) in 0.1M acetate buffer of pH 5.6 and the DNA concentration was varied from 00µg/mL to 150µg/mL, and the DPV response for each set was studied at the GCFE. The differential pulse voltammograms showed gradual negative shift in Ep values (Table 1) for both the Mitomycin C peaks with increasing DNA concentration. Thus, indicating complex formation between Mitomycin C and ds-DNA.

However, if the above solutions were electrolyzed, scanning the potential up to -1.20V only, the resulting DP Voltammogram showed a negative shift of the first peak (from -0.94 V to -1.0V). Thus complexation between DNA and Mitomycin C is confirmed. These results also indicated that only one electron reduction of Mitomycin C is sufficient to electrochemically activate Mitomycin to form ds-DNA-Mitomycin C complex.

Further, to ascertain the binding sites for complex formation between Mitomycin C and ds-DNA, the test solutions were prepared by taking 15µg/mL DNA in 0.1M acetate buffer of pH 5.6, and the Mitomycin-C concentration was varied from 00 to 150µg/mL and DPV response of each set at the GCFE was recorded with positive scanning of the potential. The first set without Mitomycin C showed a well defined peak at +0.75V versus SCE which shifted to less electropositive value Ep = 0.80V (Fig.2) with increasing Mitomycin C concentration and the peak height gradually decreased.

Table 1. DPV response of Mitomycin C [15 µg/mL] in acetate buffer of pH 5.6, with varying concentrations of ds-DNA

S. NO.	ds-DNA added (µg/mL)	Peak Potentials (Ep_1) (Volt)	Peak Potentials (Ep_2) (Volt)
1.	00	-0.94	-1.41
2.	05	-0.95	-1.41
3.	15	-0.96	-1.42
4.	25	-0.98	-1.43
5.	50	-1.00	-1.45
6.	150	-1.20	-1.48

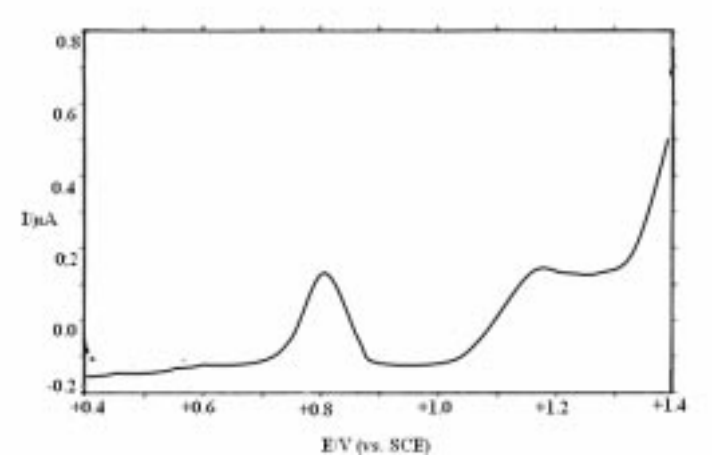

Fig. 2. Differential pulse voltammogram of complex between 15μg/mL DNA and 25μg/mL Mitomycin C at bare GCFE with positive potential scanning, in 0.1M acetate buffer of pH 5.6, Pulse amplitude – 25mV, Scan rate – 12mV S^{-1}

Since the peak at +0.75V is due to the oxidation of guanine of DNA [13,14]. The shift in Ep value and decrease in peak height clearly speaks the Mitomycin C combination with DNA through guanine site.

4. HPLC analysis of the adducts

The electrochemical observations were then supplemented by performing HPLC studies on adducts formed in the electrolyzed solution (Mitomycin C-ds-DNA complex at bare GCFE). For the HPLC analysis of adduct, Shimandzu High Performance Liquid Chromatograph LC-20AT was used. C-18 column was used with flow rate 2mL/min and eluant was used 8:92 methyl cyanide/0.02M potassium phosphate with pH 5.6.

The HPLC pattern produced a major peak at 48 minutes, due to adduct formed in the electrolyzed solution was compared with that reported by Tomasz et al. [12], in their study on Mitomycin C-DNA complexation. The observed HPLC data was found to be in close agreement with the reported literature, who have observed the major peak due to adduct i.e. Mitomycin C-DNA complex at 45 min. Thus, confirming Mitomycin C-DNA complex formation in the electrolyzed solution.

5. Enzyme modified electrode [15] for the Mitomycin-ds-DNA Interaction study

It has already been mentioned that NADPH cytochrome-c reductase causes reductive activation of Mitomycin-C which forms covalent complex with ds-DNA. The author has therefore fabricated the NADPH cytochrome-c reductase enzyme

modified electrode by preadsorption of promoter phospholipids at the surface of the GCFE and then dipping the electrode in a solution of NADPH cytochrome-c reductase for 300 s. It was then taken out of the solution and allowed to dry. The enzyme adsorbed GCFE was then dipped in a solution of Mitomycin C (15µg/mL) at deposition potential of -1.60V for 300 s. The Mitomycin C adsorbed enzyme modified GCFE was dipped in a solution of acetate buffer at pH 5.6 containing 15µg/mL ds-DNA and the solution was electrolyzed performing anodic potential scanning. The resulting differential pulse voltammogram showed an oxidation peak at +0.60V (Fig.3) due to oxidation of ds-DNA. A comparison of this observed peak in presence of Mitomycin C at +0.60V with that observed without Mitomycin C at +0.70V may be attributed to covalent complexation of Mitomycin ds-DNA at enzyme electrode.

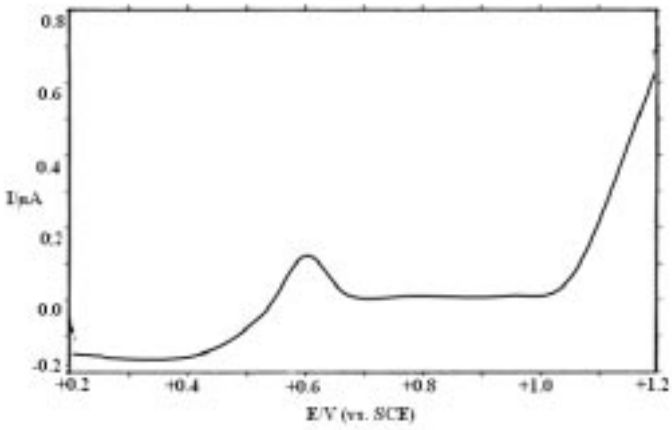

Fig. 3. Differential pulse voltammetric response of ds-DNA (15µg/mL) at Mitomycin C (25µg/mL) adsorbed, NADPH cytochrome c reductase modified GCFE in 0.1M acetate buffer of pH 5.6, Pulse amplitude – 25mV, Scan rate – 12mV S^{-1}

6. Proposed mechanism

On the basis of the above voltammetric studies and relevant literature a reaction mechanism scheme for the electrochemical activation of Mitomycin-C and its interaction with ds-DNA may be proposed as under (Fig. 4). Mitomycin at the electrode surface undergoes two electron reductions to form hydroquinone. This hydroquinone forms reaction intermediate (C). The quinone methide (C) reacts with DNA to produce mainly adducts at the N-2 position of guanine in the minor groove. The formation of guanine N-2 cross linked adducts has been confirmed by isolation of adducts from mouse mammary tumors following Mitomycin-C treatment by Tomaz et al. [16] and Bizanek et al. [17].

7. Conclusion

Electrochemical reductive activation and enzyme bioactivation of the anticancer alkylating agent Mitomycin-C and its interaction with ds-DNA at the electrochemical biosensors has been studied. The results show that Mitomycin C is covalently linked with DNA to form guanine N-2 cross linked adducts; thus inhibiting the multiplication of DNA. So the alkylating agent Mitomycin-C works as an anticancer drug.

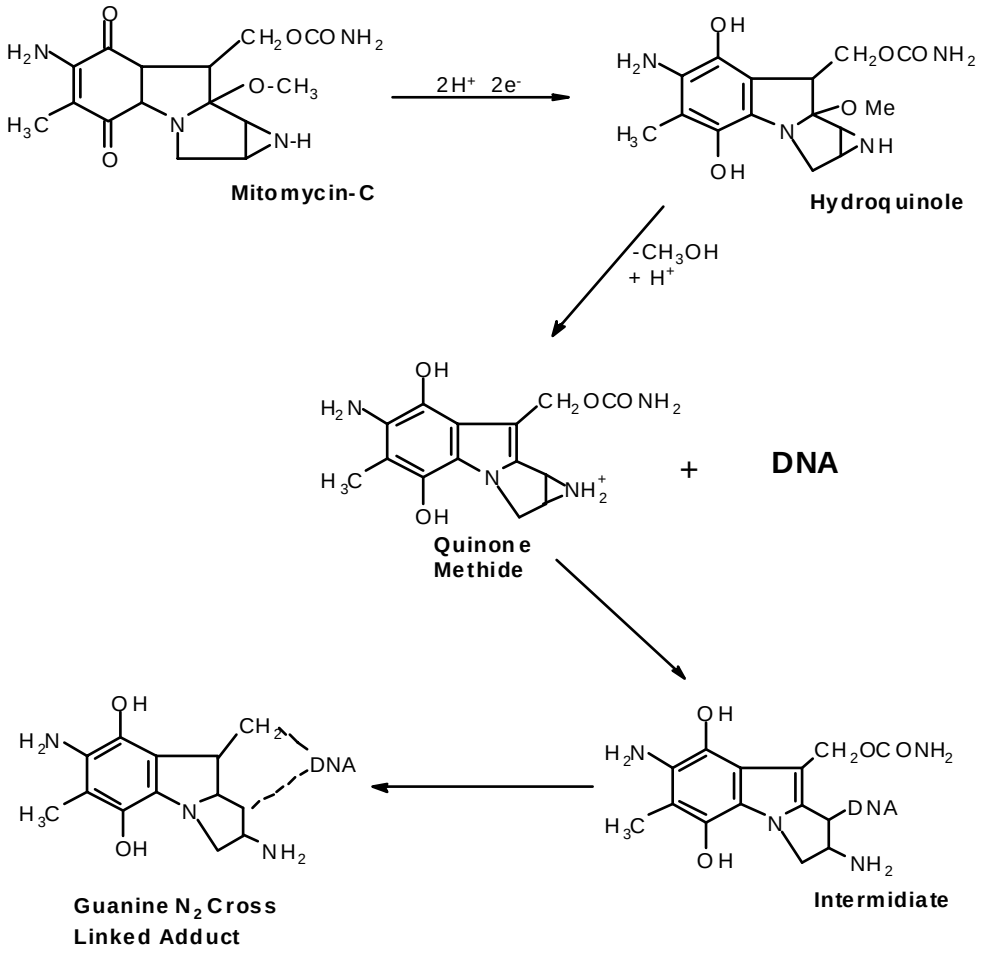

Fig. 4. Mechanism Model

The fabricated biosensors are of utmost relevance because the in-vivo mechanism of action of the DNA alkylating drug Mitomycin C to form ds-DNA-Mitomycin-C complex, is caused due to activation of Mitomycin-C by some enzyme activators in

the cell which reduces the quinone of the compound to form hydroquinone which in turn forms reactive intermediates as shown in Mechanism Model. It is parallel to the electrochemical reduction of Mitomycin-C to form reaction intermediates. Hence, the fabricated biosensors may prove to be a complementary tool for the study of biomolecular interaction mechanism.

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✉ **Prof. K.S. Pitre** (corresponding author)
Department of Chemistry,
Dr. Hari Singh Gour Vishwavidyalaya
Sagar (M.P.) 470 003
INDIA
E-Mail: kspitre@rediffmail.com