METRONIDAZOLE RADICAL ANION FORMATION STUDIED BY MEANS OF ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY

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Radiosensitizers belong to the most important class of the cancerostatic drugs. The electrochemical transfer of the first electron to the cytotoxic radiosensitizer Metronidazole (MET) and MET radical anion formation in aprotic medium was studied by means of electrochemical impedance spectroscopy. The heterogeneous electron transfer rate constant for the first reduction of MET (radical anion production) k^0 for the redox couple was determined. Similarly, the diffusion coefficient of MET in dimethylformamide was also calculated by impedance measurements using expression for Warburg coefficient and Warburg plot. Moreover, the equivalent circuit for the redox couple MET/MET$^{\bullet-}$ was proposed and its parameters were evaluated using a non-linear least square fitting. Our results, from the electrochemical point of view, also confirm the suitability of MET for the effective treatment of selected types of the cancer.

Keywords: Radicals; Radiopharmaceuticals; Electron transfer; Impedance spectroscopy; Metronidazole; Radiosensitizers.

Radiotherapy, one form of the cancer treatment that prevents malignant cells from growing and dividing, is closely connected with free radical production. Radiosensitizers are drugs that sensitize the malignant cells to radi-

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ation therapy. The cytotoxic properties of such compounds are due to the fact that in the cell these compounds undergo one-electron reduction to generate radical anions, which exhibit cytotoxicity towards cellular systems[1–4]. It is probable that nitro radical anions interact with cellular DNA. Another important property of several radiosensitizers is that they appear to radiosensitize hypoxic cells, but have no measurable effect in well-oxygenated cells[5]. This is probably not because of a remarkable inherent property, but because of simple kinetic competition between oxygen and drugs (chemicals) for reaction with key DNA base radicals. These compounds may also be useful as imaging agents for identifying hypoxic, drug-resistant regions of primary tumors or metastases[1]. Some electrochemical results indicate that several drugs would act in a two-step redox mechanism: the first step involving the oxidation/reduction of one moiety of a drug and then, the consecutive one, the reduction/oxidation of another moiety of the already oxidized/reduced molecule at the considerable lower reduction potential[1,3].

Crystallographic structural data of the selected hetero nitrogen compounds[6–8] were collected and the reactivity of radical anions from most of the nitrogen-containing compounds has been studied mainly by the pulse radiolysis technique[9,10]. Recently electrochemical techniques have been used to study the behavior of the nitro radical anions[3,11–21].

Pharmacological and toxic effects of the drugs are mediated by cytotoxic reactions and depend on the *in vivo* reduction/oxidation of the reactive group producing the radical anion or cation species. It is probably possible to modulate both the reduction ability and radical stability and consequently capability to be enzymically reduced. It is known that there is a good correlation between physico-chemical properties of some nitro-compounds, e.g. radical anion production, with their ability act as hypoxic cell radiosensitizers[22]. Among them electron affinity (EA) of atoms and molecules is one of the fundamental properties; e.g. the EAs of the DNA bases are of interest owning to their significance for understanding of DNA radiation damage[23]. Drugs with a high electron affinity (with a low reduction potential) are generally highly toxic and mutagenic. They are also more quickly metabolized in the cells. Furthermore, several studies[24,25] dealing with the relationship between reduction potentials and pharmacological activity of the drugs have proved that this parameter is of interest from electrochemical and pharmacological points of view.

Metronidazole (MET; Fig. 1), 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (CAS number 443-48-1), is a very important drug among the group of hetero nitrogen 5-nitroimidazoles and possesses high toxic properties to especially

anaerobic microorganisms and protozoa[26]. The mechanism of its biological action depends on the nitro group reduction process. It is the drug of choice in the case of mild-to-moderate Clostridium difficile infection. MET is sometimes utilized to treat infections of Giardia in dogs, cats, and other companion animals. It is also used to treat enteric and systemic infections[26].

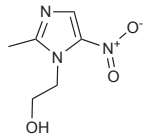

FIG. 1
Metronidazole structure

Electrochemical impedance spectroscopy (EIS) has been utilized to understand various biological phenomenons[1,27–32]. This contribution advances utilizing EIS for understanding of MET reduction process in aprotic medium because it is probable that mechanism of the *in vivo* reduction of radiosensitizers takes place at the cytoplasm|cell membrane interface[33]. The results obtained by EIS are presented. The fundamental parameters that describe the formation of MET radical anion are shown and their values are compared with those determined by other experimental techniques, mainly pulse radiolysis and cyclic voltammetry.

EXPERIMENTAL

Metronidazole (99%) was obtained from Acros Organics, France and was used without any purification. Dimethylformamide was utilized as an aprotic solvent and 0.1 M tetrabutylammonium hexafluorophosphate ($TBAPF_6$) as a supporting electrolyte. All chemicals were of p.a. or higher quality. Oxygen was removed from the solution by purging with argon (Messer, min. 99.998 vol.%) prior to the each measurement.

Electrochemical measurements were performed using AUTOLAB instrument PG STAT 30 equipped with FRA2 module (ECO Chemie, The Netherlands). Impedance measurements were carried out in the range 1 Hz to 25 kHz. AC amplitude of 10 mV was derived from an internal oscillator. Each impedance curve consisted of 100 measured points. A three-electrode electrochemical cell was used. The reference electrode (RE), Ag|AgCl|1 M LiCl, was separated from the test solution by a salt bridge. The working electrode (WE) was a valve-operated static mercury drop electrode (VA Stand 663, Metrohm). The counter electrode (CE) was a cylindrical platinum wire with area approximately 100 times higher than that of WE. Total volume of the measured solution was 5 ml. All experiments were carried out at 25 ± 1 °C. The electrochemical data from EIS measurements were analyzed by means of the ZSimDemo 3.22d program using a non-linear least-square fitting. Data consistency

was verified by Kramers–Kronig procedure. An estimate of initial values of parameters was obtained from high-frequency limiting values in combination with a vectorial subtraction of the solution resistance R_s (the high-frequency limit of Z′) and the double-layer capacitance C (the high-frequency limit of the solution resistance corrected plot of Y''/ω vs Y'/ω, where Y is the admittance and ω is the angular frequency).

RESULTS AND DISCUSSION

Similarly to other nitroimidazoles, MET exhibits several electron transfer processes in the potential range 0.0 to –2.8 V vs RE in aprotic medium. Here we focus on the first step which represents one-electron reductions of the nitro group of MET to nitro anion radical in the dimethylformamide (DMF). According to the literature[2,34,35] the first reduction step of the nitroimidazoles in aprotic solvents is related to the reversible transfer of the single-electron and consequently with the formation of respective radical anion. However, it is necessary to note that in some cases the protonation, dimerization and adsorption of reactants and/or products is/are coupled with the electron transfer step. If the vertex potential E_λ is set closely behind the potential of the first reduction peak, nitro radical production is reversible. The reversibility of this process in the case of MET in DMF is demonstrated in Fig. 2 where semi-integrated curves of the cyclic voltammograms (CV) of MET in DMF can be seen.

The ratio between cathodic and anodic peak currents is close to unity. However, with increasing scan rate the slight shift (tens of milivolts) of the reduction potential to the more negative values is observed. This could means that some chemical reaction and/or weak adsorption of MET on the electrode surface is coupled with the electron transfer step. This is similar to previous observations of some radiosensitizers[3,34–36].

According to Fig. 3, reduction peak current I_{pc} as a function of scan rate can be expressed as

$$I_{pc} = A^*|v - v_c|^p \qquad (1)$$

where v is a scan rate, A and p are the optional parameters and v_c is a difference between cross section of the experimental and theoretical curves with x-axis. In our case (Fig. 3), $v_c \to 0$, $A = 0.424 \pm 0.015$ and $p = 0.546 \pm 0.006$. Therefore the control of the overall one electron reduction process of MET in DMF by the diffusion of the reactive species is shown. A detailed investigation of the reduction of nitrosobenzene indicates that, similarly to the reduction of nitrobenzene, a rapid surface reaction of adsorbed compound

took place simultaneously with the diffusion controlled reduction[37]. This correlates with negative slight shift of the reduction potential of MET with increasing scan rate.

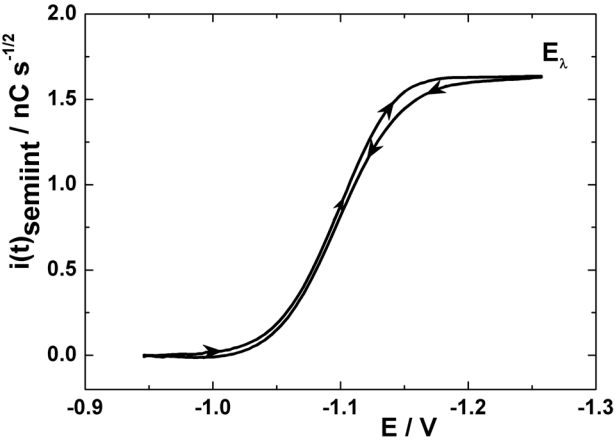

FIG. 2
Convolution of the experimental cyclic voltammogram of Metronidazole in 0.1 M $TBAPF_6$ as a supporting electrolyte in dimethylformamide at scan rate 250 mV/s; *c*(MET) = 4.2 mM

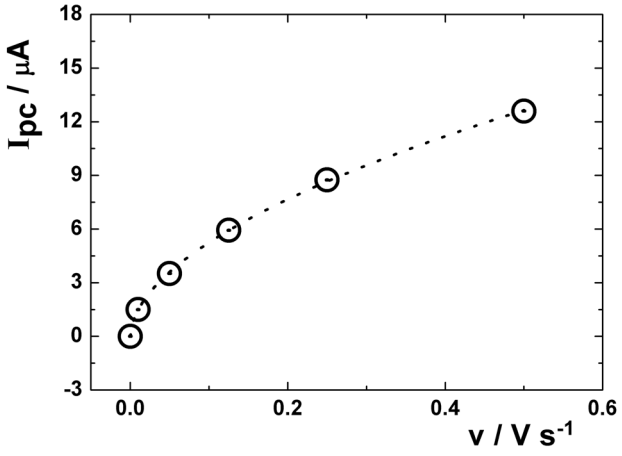

FIG. 3
Dependence of the peak current of Metronidazole on the scan rate

EIS Measurement

As stated in the Introduction part of this paper drugs with higher electron affinity are generally more toxic and mutagenic. This mutagenic potential can be characterized by two "electrochemical" parameters. The first one, E_7^1 value, accounts for the energy necessary to transfer the first electron to an electroactive group at pH 7 in aqueous medium to form a radical anion. Therefore, in the case of nitro compounds, the E_7^1 value represents the ability to form the nitro radical anion. This parameter was for MET discussed in our previous contribution[38].

Another parameter is the heterogeneous electron transfer rate constant, k^0, which also characterizes (quantifies) the ability of the compound to accept the electron. In this study we focused on the k^0 determination by EIS measurements.

Generally, the electrochemical behavior of the MET radical anion formation was represented by EIS as an equivalent circuit. The heterogeneous rate constant of the reaction MET–NO_2 + e^- $\rightleftarrows$ MET–$NO_2^{\bullet -}$ was determined using graphical complex plain representation of the cell impedance.

In Fig. 4, the typical impedance curves of MET in DMF at different potentials are plotted.

From Fig. 4 one can see that the system displays two processes having two time constants. The first one, in the high frequency region, may be ascribed to the adsorption of the reacting species[3,27] and second one is probably connected with radical anion formation. Modified Voigt circuit composed of one simple circuit with parallel capacitance C1 and resistance R2 and Randless circuit modified by constant phase element CPE1, defined by Eq. (*2*), instead of simple capacitance is the simplest equivalent circuit that fits our experimental data with acceptable low $\chi^2 < 2 \times 10^{-4}$ (Fig. 5).

$$Q = \frac{1}{Y_0 (\omega i)^n} = \frac{1}{Y_0 \omega^n} \mathrm{e}^{-\frac{\pi}{2} ni} \qquad (2)$$

where $\omega = 2\pi f$ is an angular velocity, i imaginary unit, Y_0 and $0 \leq n \leq 1$ are the parameters that characterize the constant phase element CPE1.

The accuracy of proposed circuit was checked using Bode plot (Fig. 6).

All values of respective circuit elements for MET radical anion formation are summarized in Table I.

From Table I, one can see that almost all elements reach the maximum or minimum around the reduction potential E = –1.14 V vs RE. Moreover, from the logarithmic dependence of the charge transfer resistance R3 on

the potential applied, E (figure not shown), one can estimate the electron transfer coefficient, α (as well as 1α). This dependence yields two asymptotic lines (slopes) corresponding to two limiting cases: (i) reduction rate is much larger than oxidation, (ii) oxidation prevailing over reduction[1]. The ratio of the slopes is very close to the unity. Therefore one can conclude that the transfer coefficient $\alpha \approx 0.5$. These calculations indirectly confirm the suitability of the proposed equivalent circuit (Fig. 5). Moreover, these results can be used to evaluate kinetic parameters of the system.

Under suitable experimental conditions it is possible to study the kinetics of the electrode reaction, and the heterogeneous electron transfer rate constant, k^0, can be evaluated from the impedance measurements.

The frequency dependence of the faradaic phase angle ϕ_F can be calculated according the equation as

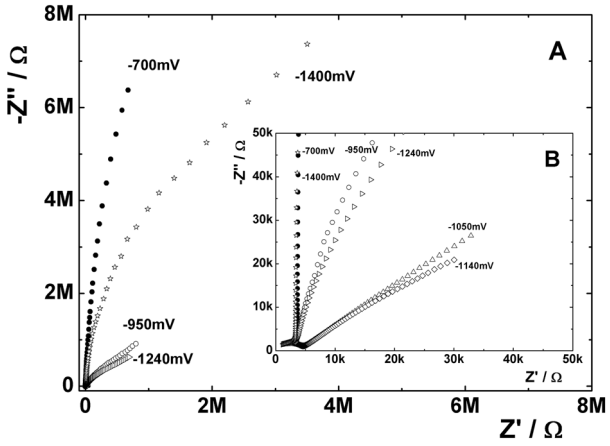

FIG. 4
Argand's diagram of MET in 0.1 M $TBAPF_6$ as a supporting electrolyte in dimethylformamide at various potentials vs. RE; (B) enlarged "low impedance" part of the diagram A

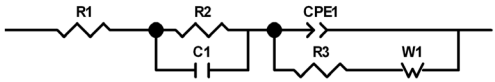

FIG. 5
Equivalent circuit representing the impedance of the cell containing MET in 0.1 M $TBAPF_6$ as a supporting electrolyte in dimethylformamide; R1 solution resistance, C1 capacitance; CPE1 constant phase element; W1 Warburg element; R2, R3 resistances of the respective electrode reactions

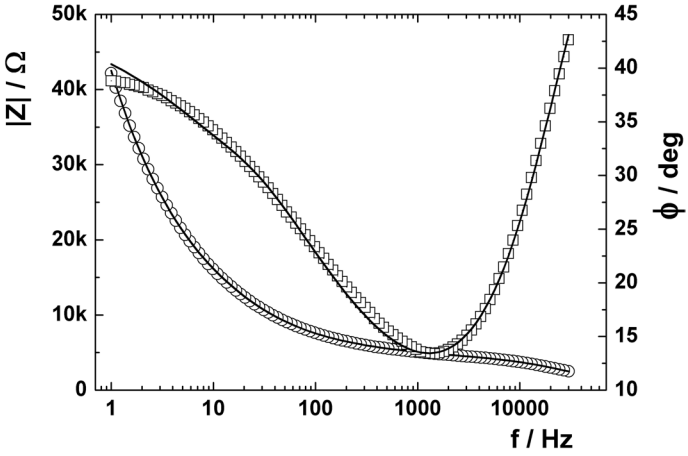

FIG. 6
Bode plot. MET in 0.1 M $TBAPF_6$ as a supporting electrolyte in dimethylformamide at –1.05 V vs. RE. Points represent experimental data, curves nonlinear regression analysis according to the equivalent circuit in Fig. 5

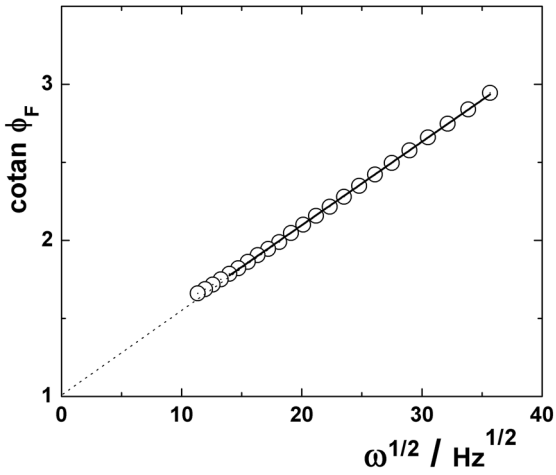

FIG. 7
The dependence of the cotangent of faradaic phase angle on square root of angular frequency $\omega^{1/2}$ ($\omega = 2\pi f$); MET in 0.1 M $TBAPF_6$ as a supporting electrolyte in dimethylformamide at –1.05 V vs. RE

$$\text{cotan}\,\phi_F = \frac{Z_F'}{Z_F''} = 1 + \frac{1}{\zeta} = 1 + \frac{(2\omega D^0)^{1/2}}{k^0} \qquad (3)$$

where D^0 is a diffusion coefficient, ζ is the rate parameter defined as $\zeta = k_f/(2\omega D_R)^{1/2} + k_b/(2\omega D_O)^{1/2}$; k_f, k_b are the potential dependent rate constants of reduction and oxidation, respectively, D_R and D_O are the respective diffusion coefficients; Z_F' and Z_F'' are the components of the faradaic impedance. Assuming that $D_R = D_O = D^0$ and $k^0 = k_f + k_b$ the heterogeneous electron transfer rate constant, k^0, for redox couple MET–NO_2 + e^- $\rightleftarrows$ MET–$NO_2^{\bullet -}$ can be estimated according to the Eq. (*3*). The plot of cotan ϕ_F vs $\omega^{1/2}$ should be a strait line and the rate constant can be evaluated from the slope[1].

For MET the diffusion coefficient D^0 was calculated from the expression for so called Warburg coefficient σ as

$$\sigma = \frac{RT}{n^2 F^2 A\sqrt{2}} \left(\frac{1}{D_{ox}^{1/2} c_{ox}^b} + \frac{1}{D_{red}^{1/2} c_{red}^b} \right) \qquad (4)$$

where R, T, n, F have a standard meaning, subscripts ox and red represent the oxidized and reduced form of the species, and superscript b denotes a bulk concentration. Warburg coefficient σ can be estimated from the slope of the Warburg plot (dependence of the imaginary part of the impedance versus reciprocal value of the square root of the angular velocity). The diffusion coefficient for MET in DMF was calculated to be 1.91×10^{-5} cm^2 s^{-1}

TABLE I
Simulated values of the electrical elements used in the equivalent circuit shown in Fig. 4

E V	R1 Ω	C1 F	R2 Ω	CPE1(Y0) Ω^{-1} s^n	CPE1(n)	R3 Ω	W Ω^{-1} $s^{1/2}$
–0.95	567	2.40×10^{-9}	246500	4.23×10^{-8}	0.91	221600	2.79×10^{-7}
–1.05	310	1.51×10^{-6}	613900	5.09×10^{-9}	0.91	3909	9.54×10^{-6}
–1.14	373	4.06×10^{-6}	184200	3.51×10^{-9}	0.94	3839	9.52×10^{-6}
–1.17	408	3.99×10^{-6}	99123	3.81×10^{-9}	0.90	19234	7.09×10^{-6}
–1.20	421	9.71×10^{-7}	30234	1.12×10^{-8}	0.94	123432	3.23×10^{-6}
–1.24	445	2.34×10^{-9}	2473	5.52×10^{-8}	0.87	295400	4.13×10^{-7}

which is close to the value 2.19×10^{-5} cm^2 s^{-1} obtained utilizing the different method by Barety et al.[35].

The heterogeneous electron transfer rate constant for the first reduction of MET (radical anion production) in aprotic medium at E = 1.14 V vs RE was according the Eq. (*3*) calculated to be $k^0 = (2.09 \pm 0.19) \times 10^{-2}$ cm s^{-1} (Fig. 7). This value is comparable with the value obtained for MET in the aprotic media by another approach[35]. Therefore, one can say, that our procedure for k^0 determination of MET–NO_2/MET–$NO_2^{\bullet -}$ couple in the DMF using EIS is correct. Moreover, our results also confirm that diffusion coefficient of the species dissolved in the solution can be successfully determined by EIS.

If our previous results[3,38] on E_7^1 are combined with heterogeneous electron transfer rate constant evaluation by EIS measurements in this paper, one can conclude that MET can easily acts as a good chemical radiosensitizer. However, both results indicate that higher energy compared to other possible chemical radiosensitizer Etanidazole is necessary for the system to transfer the first electron to MET. These results confirm the suitability of EIS as a suitable technique for the testing of the drug properties. Moreover, our results, from the electrochemical point of view, also confirm the suitability of Metronidazole for the effective treatment of selected types of the cancer.

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