INTERACTION OF NITROSOUREA SLENU WITH SUPPORTED LIPID FILMS AND ERYTHROCYTE MEMBRANES

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Abstract

Nitrosourea 1-ethyl-1-nitroso-3-[4-2,2,6,6-tetramethylpiperidine-1-oxyl (SLENU) is a spin labeled analogue of the clinically used non-labeled antitumor drug lomustine (CCNU). The objective of this study is to characterize the interactions of SLENU with supported lipid films and erythrocyte membranes. Thin lipid films prepared on the surface of a glassy carbon electrode are used as a model membrane system for studying the interaction between SLENU and the lipid fraction of biomembranes. The effects of SLENU on the structure of the lipid film are investigated by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). It is shown that due to the amphiphilic properties of SLENU it penetrates the lipid layers. The possible formation of defects in the lipid films was studied by the aid of hydrophilic ions - the electroactive couple ferri-ferrocyanide. The SLENU penetration in the lipid phase provokes a little decrease in the film thickness. Up to concentration of 1 mM SLENU there is no change of the redox currents of ferri-ferrocyanide suggesting absence of defects in the lipid structure. Acidic hemolysis of erythrocytes is also not influenced by the presence of SLENU, suggesting the lack of destabilizing effect of SLENU on erythrocyte membranes.

Key words: *Electrode-supported lipid film; drug-membrane interactions*; s*pin-labeled nitrosourea*

Introduction

Spin-labeled nitrosoureas are synthesized in the early 80s, as potential antitumour agents with the hope of overcoming some of the problems related to the toxicity of alkylating antitumor drugs of the class of nitrosoureas [1][2][3]. They contain a nitroxyl radical, which is spin-label, hence their name. Since then many of their physical - chemical and biological properties were studied *in vitro* and *in vivo* [1][4][5][6][7][8], proven the positive effect of inclusions in their structure nitroxyl radical in terms of its anti-tumor effect and reduced toxicity [9][10][11][12].

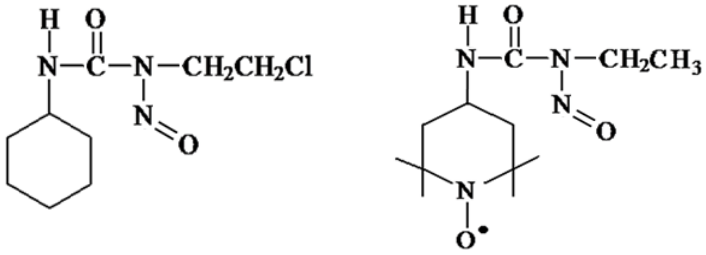
The nitrosourea used in this work is 1-ethyl-1-nitroso-3- [4-2,2,6,6-tetramethylpiperidine-1-oxyl (SLENU). SLENU is a spin-labeled analogue of the clinically used nitrosourea drug lomustine (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, CCNU). The chemical structures of CCNU and its spin-labeled glycine-containing analogue SLCNUgly are compared in Fig.1. In SLENU the cyclohexyl group of CCNU is substituted with a 1-oxyl-2,2,6,6-tetramethylpiperidinyl group, which determines the paramagnetic properties of SLENU

It was demonstrated that SLENU exhibited superoxide anion radical scavenging activity (SSA) in vitro, due to the presence of the nitroxyl free radical in its structure [4][12][13].

SLENU and CCNU are compared by means of their toxic effect on the growth of *Escherichia coli* (*E. coli*) и *Staphylococcus aureus* (*S. aureus*) [5]. It was found that while CCNU suppressed bacterial growth linearly with a concentration from 100 up to 500 μmol/ml, SLENU was not toxic to the bacteria within this concentration range. Besides this, SLENU has been shown to reduce the toxic effect of CCNU when the bacteria are co-treated with CCNU and SLENU in the same time. Since a similar effect was also found in the co-treatment of CCNU with the classic antioxidant vit. E., it was concluded that the lower toxicity of SLENU, as well as its toxicity reduction effect on

CCNU, are due to an antioxidant activity of SLENU. The protective effect of SLENU on the toxic effect of CCNU was also tested *in vivo* in mice, where a decrease in the level of oxidative stress' biomarkers was found after the co-treatment compared to the CCNU treatment alone [14].

The goal of this work is to study the effects and interactions of SLENU with the lipid layer of biomembranes, as well as with model membranes. Thin lipid films deposited directly on the surface of an electrode [15] are used as model membrane system.



CCNU **SLENU**

Fig. 1. *Chemical structures of CCNU and its spin-labeled analogue SLENU.*

Materials and methods

Reagents and film forming solution.

The spin-labeled nitrosourea 1-methyl-1-nitroso-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)] - urea (SLENU) was synthesized by the method of Gadjeva 1991 [2].

Due to the very low solubility of SLENU in water two types of 10 mM stock solutions were prepared - in hydroalcoholic mixture (50% ethanol) or in dimethyl sulfoxide (DMSO). The solutions were freshly prepared for each the experiment and stored permanently in a refrigerator at 4 ºC during the day.

The lipid films deposited onto the working electrode were prepared from natural lecithin (L-α-Phosphatidylcholine, from soybean, Type IV-S, SIGMA). It was dissolved in n-hexane (SIGMA Chemical Comp., USA) at a concentration of 1 mg/mL.

Cyclic voltammetry and impedimetric techniques.

In the present work voltammetry was used as an analytical tool for examination of charge transfer between the GCE and electroactive species in the electrolyte solution. The measurements were made with potentiostat/galvanostat/FRA (VERSASTAT 3F, Princeton Applied Research, USA). The sweep rates as well as the potential range are indicated in the figure captions.

The determination of passive electrical properties of the films, hence their thickness and compactness were made by a technique based on the concept of impedance. An equivalent circuit with parallel capacitance and resistance was used to evaluate the thickness of the films.

Experimental cell for preparation and investigation of lipid films.

Thin lipid films deposited directly on the surface of a *glassy carbon electrode* (GCE) are used as model membrane system. The method of deposition of the lipids on the electrode and the formation of thin lipid film with thickness of the order a monolayer was described earlier [15]

The electrochemical properties of the modified GCE were investigated in a three-electrode cell consisting of a working GCE (3mm diameter, 0.0707cm^2 face area, CH Instruments, USA), 0.1M solution of KCl, buffered with 4 mM Na_2HPO_4-NaH_2PO_4 – pH 7 (SIGMA Chemical Comp., USA) as electrolyte, a single junction Ag/AgCl reference electrode and an auxiliary Pt electrode (the electrochemical cell construction, the set-up and method of measurement were descibed in detail earlier (10, 13-15):

$$Ag / AgCl // 0.1\ KCl, 4\ mM\ Na_2HPO_4\text{-}NaH_2PO_4 // LF // GC \quad (1)$$

where LF is the lipid film formed onto the surface of the working GCE. Before each measurement the GCE surface was additionally conditioned by a fine polishing.

Isolation, treatment and acidic hemolysis of human erythrocytes.

Human erythrocytes were used as a model to study the interaction of SLENU with biological membranes. Venous blood anticoagulated with citrate was taken on the day of the survey. The erythrocytes were separated by centrifugation at 1300 gfor 4 min and washed three times with 150 mM NaCl at a ten-fold volume dilution.

The final suspension of washed erythrocytes was divided in two equal portions. One portion remained untreated and was used as a control. The second portion was treated with 100 μM of the anion exchange inhibitor 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) in isotonic NaCl solution containing 15 mM phosphate buffer, pH 7.6 for 10 min, at 4 ºC. The unbound reagent was removed by two washing steps in isotonic saline. Finally; the cells were resuspended in the washing solution at a hematocrit of 30% and kept at room temperature until further use.

Acid hemolysis was performed as follows: 6 μl of the starting erythrocyte suspension (containing DIDS-processed or control cells) were added to 1.5 ml of physiological saline, to obtain a working suspension having an extinction of 1at a wavelength λ = 650 nm, as measured with a spectrophotometer SPECOL10 (Carl Zeiss Jena, Germany). The active substance was added to this working solution to obtain the desired final concentration. After 30 sec, necessary to establish an equilibrium, 50 μl HCl (1%) were injected to reach a final pH 3.0 and the changes in extinction as a function of time E(t) were measured. The results were displayed as a percentage hemolysis (e) for a given period of time.

$$e = \frac{E(t) - E_f}{E_{in} - E_f}.100\% \quad (2)$$

where E_{in} and E_f are the initial and the final value of extinction, respectively.

The time required for 50% haemolysis is referred to as the acid resistance of the cells.

The analog signal from the output of the spectrophotometer is fed to a digital multimeter AX-594 (AXIO MET), which stores data in digital form using the program DMM Data Processor.

Results and discussion

SLENU-lipid film interaction

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were applied for the investigation of the effects of SLENU on the lipid film structure. In fig. 2 are shown two voltammogrames obtained with bare uncovered GCE (blue curve) and with GCE covered with a lipid monolayer (red curve). The measurements are performed in the range 0V to +1V vs. Ag| AgCl|0.1 M KCl. The comparison between the two voltammograms demonstrate the degree of the

drug penetration into the lipid phase of the films. The typical oxidation and reduction peaks of SLENU are clearly observed with a bare GCE, as well as, with lipid film modified GCE. The electrochemical reactions behind are the oxidation and the respective reverse reduction reaction of the nitroxyl radical from R-NO$^{\bullet}$ to oxoammonium cation R-NO^{+} [16]. It can be seen that the position of the peaks on the voltammograms obtained with the lipid-modified electrode and those obtained with the bare GCE is not shifted and the potential difference between the oxidation and reduction current peaks is the same. Such a behavior suggests incorporation of SLENU inside the hydrophobic zone of the lipid film leading to diffusion limited reactions close to the electrode surface. If SLENU did not penetrate the film, there would be no peaks in the same potential range as in the case with bare electrode.

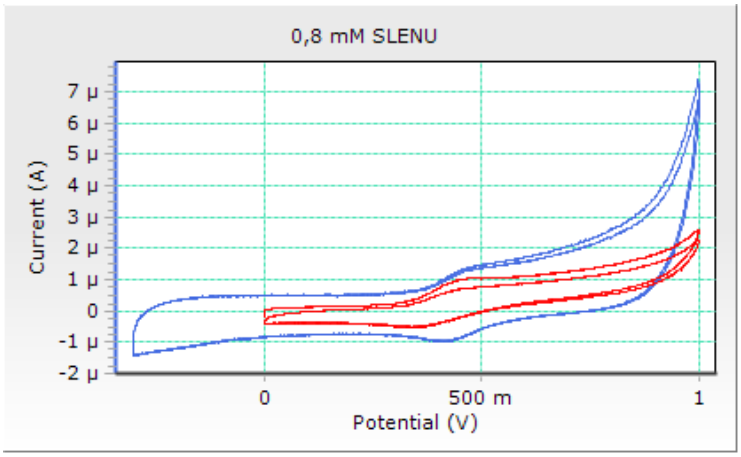


Fig. 2. *Cyclic voltammograms of SLENU obtained with bare uncovered GCE (blue curve) and with GCE covered with a lipid monolayer (red curve); 0.8 mM SLENU in 0.1 M KCl, 4 mM phosphate buffer (Na_2HPO_4–NaH_2PO_4), pH 7; scan rate 0.1 V/s.*

It worth mentioning that the presence of the two peaks in the obtained with the lipid film voltammogram could be a result of disruption of the film. In order to check this possibility the effect of penetration of the SLENU on the films structure is investigated for concentrations up to 1 mM. Representative results are shown in fig. 3, where the dependence of the oxidation peak current on the SLENU concentration is plotted, along with the estimated thickness of the film. In order to calculate the thickness of the film the impedance spectroscopy of the experimental cell was performed. The experimental data were modeled with an equivalent circuit with a resistance in series with a pair of parallel resistance and capacitance. The first resistance represents the bulk electrolyte solution between the lipid film-modified electrode and the counter electrode, and the pair of parallel resistance and capacitance represents the impedance of the lipid film-modified electrode. The impedance of the counter electrode is neglected because this electrode is with much bigger area, hence with much smaller impedance. The thickness of the film is calculated from the capacitance, using the equation for the capacitance of a capacitor with parallel plates.

$$C\left[\mu F/cm^2\right] = 8.85\frac{\varepsilon_l}{h\left[\text{Å}\right]} \tag{3}$$

where C is the calculated capacitance of the lipid film-modified electrode, h is the film thickness, and $\varepsilon_l \approx 2$ is the dielectric permitivity of the hydrofobic region of the lipid film.

The results presented in fig. 3 show that the peak current increases linearly with the SLENU

concentration. This suggests a gradual penetration of the drug's molecules into the lipid film. At the same time, the penetration of SLENU does not alter substantially the thickness of the film. The analysis of the results obtained from testing the dependence of the oxidative peak current (Ip) and the effective thickness of the lipid film from concentration of SLENU shows that spin-labeled nitrosourea does not cause damage to the model membrane.

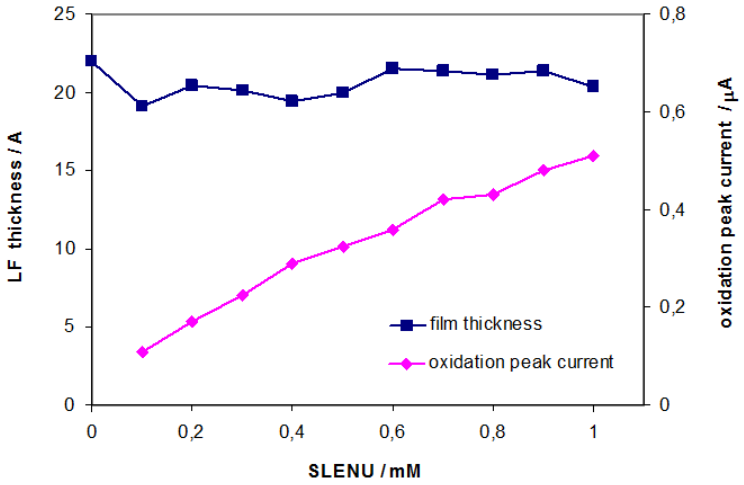


Fig. 3. *Dependence of the oxidation peak current Ip of the cyclic voltammograms and effective thickness h of the film on the SLENU concentration. Electrochemical cell conditions—same as in Fig. 2. The effective lipid film (LF) thickness is determined by the impedance at 1000 Hz).*

Additional assessment of the effect of SLENU on lipid structure of the films and their ion permeability was made by voltammetry tests in the presence of the hydrophilic electroactive couple ferricyanide/ferrocyanide. Results from these studies are presented in Fig. 4.

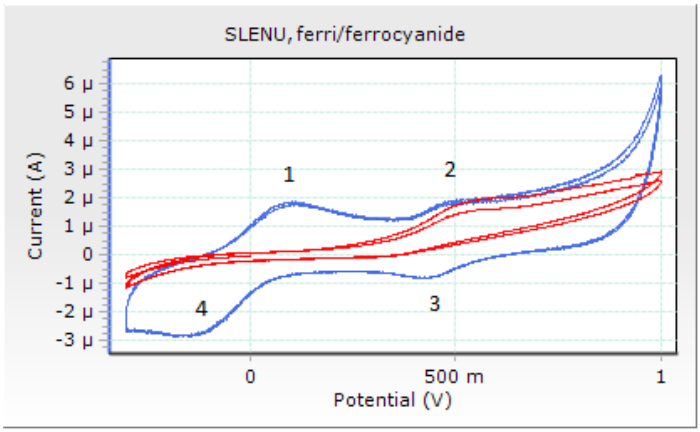


Fig. 4. *Cyclic voltammograms of 0,2 mM potassium ferri/ferrocyanide and 0,8 mM SLENU obtained with bare uncovered GCE (blue curves) and 1 mM potassium ferri/ferrocyanide and 0,8 mM SLENU with GCE covered with a lecithin monolayer (red curves); Peaks 1 and 4 – oxidation and reduction peaks of ferro/ferricyanide; Peaks 2 and 3 – oxidation and reduction peaks of SLENU.*

In fig. 4 four different peaks are observed in the voltammograms – two peaks for the oxidation of ferrocyanide and the reduction of ferricyanide (peaks 1 and 4), and two peaks for the oxidation and reduction of SLENU (peaks 1 and 4). The results clearly suggest that while SLENU penetrate the lipid films, the ferri/ferrocynide ions remain outside of the films, which is proven by

the lack of peaks 1 and 4 in the voltammogram obtained with the lipid film-covered electrode. Since ferri/ferrocyanide are hydrophilic these results are indication that the penetration of SLENU in the films doesn't produce any defects in the lipid structure.

Acid hemolysis of erythrocytes

Acidic hemolysis takes place when red blood cells are placed in a medium with low pH which leads to a cascade of processes finally leading to disturbance of the integrity of the membrane. According to [324] acid hemolysis takes place in three stages. The first stage includes transport of OH^- through the anion exchanger and H^+ through the lipid bilayer resulting in an acidification of the cytosol. During the second stage the cytosolic hemoglobin is oxidized and of free radicals are released. In the third stage the free radicals damage the membrane and pores are formed leading to hemolysis.

Substances which affect the permeability of the membrane will affect the acidic resistance (time needed for 50% hemolysis) of the red blood cells. In order to differentiate between the transport processes through the lipid bilayer and those through the anion exchanger, the latter can be inhibited by DIDS. In such a way the hemolysis is slowing significantly, and the processes in the lipid bilayer can be distinguished better.

Acidic hemolysis was investigated by the kinetics of lysis, which is measured photometrically in situ. After adding acid to the erythrocyte suspension the changes of its extinction due to the reduction of light scattering from the individual intact cells are monitored.

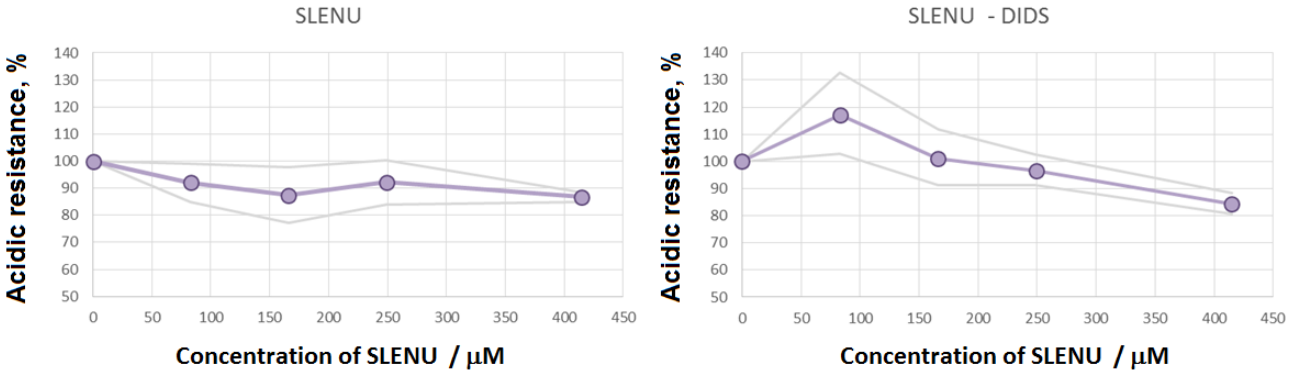


Fig. 4. *Acidic resistance of control and DIDS-inhibited erythrocytes at various concentrations of SLENU.*

Figure 4 shows the acidic resistance of control and DIDS-inhibited human erythrocytes in dependence on the concentration of SLENU.

Within the investigated concentration range, no significant effects of SLENU on the acidic resistance of control erythrocytes were observed at concentration lower than 400 µM. Only at the highest concentration a slightly reduced resistance (10 to 15%) was measured. Therefore, it can be concluded that at moderate concentrations the drug has no destabilizing effect on the erythrocyte membrane.

At low concentrations of SLENU (around 80 µM) a slightly increased acidic resistance was detected in the case of DIDS-inhibited erythrocytes. This effect could be explained by the antioxidant activity of the drug and inhibition of some destructive processes destabilizing the lipid bilayer. With increasing concentrations, however, the large amount of incorporated drug disturbs the stabilizing interactions between the lipid molecules in the hydrophobic zone of the lipid bilayer and a slight reduction of the acidic resistance (up to 20 %) can be observed.

Conclusions

In conclusion, using electrochemical techniques, such as CV and EIS, the interactions of the drugs and the lipid fraction of the biomembranes are easily investigated. SLENU penetrates the membranes without causing defects in the lipid structure. In full agreement with this result acidic hemolysis of erythrocytes is not influenced by the presence of SLENU. Electrode-supported lipid films have significant potential in the characterization of drug-membrane interactions of various chemical species and drugs of interest.

Acknowledgements

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