UNIVERSITY OF BUCHAREST
FACULTY OF CHEMISTRY
DOCTORAL SCHOOL OF CHEMISTRY

SUMMARY — PhD THESIS

ELECTROCHEMICAL AND SPECTRAL STUDIES
OF THE REACTIVITY IN REDOX PROCESSES OF
SOME ANTITUMORAL DRUGS WITH
ANTHRACYCLINE STRUCTURE

PhD student: ŞERBĂNESCU (ANGHELACHE) IULIANA FRANCISCA

Scientific coordinator: PROF. DR. ELENA VOLANSCHI

2012
CONTENT

Introduction.................................................................................................................................6

Experimental part – Original Contributions.................................................................31
Thesis objectives......................................................................................................................31
Materials, equipment and methods......................................................................................33

Objective 1. The study of the reduction mechanism of the anthracycline drugs......51

Doxorubicine...............................................................................................................................51
  1. Electrochemical studies – Cyclic voltammetry and linear voltammetry..........................51
  2. Spectral studies....................................................................................................................70
     2.1 UV-Vis absorption spectroscopy...................................................................................70
     2.2. Electron paramagnetic resonance spectroscopy.......................................................72
Epirubicine....................................................................................................................................74
  Electrochemical studies – Cyclic voltammetry and linear voltammetry............................74

Objective 2. The study of the micellar drug delivery system – Doxorubicine and mitoxantrone interaction with sodium dodecyl sulfate (SDS).................................81

Doxorubicine – SDS..................................................................................................................81
  1. Electrochemical studies – Cyclic voltammetry and linear voltammetry.........................81
  2. Spectral studies....................................................................................................................86
     2.1 UV-Vis absorption spectroscopy...................................................................................86
     2.2. Fluorescence spectroscopy.......................................................................................93
Mitoxantrone - Aqueous media (PBS/PB), deaerated .........................................................100
  Electrochemical studies – Cyclic voltammetry and linear voltammetry..........................100
Mitoxantrone – SDS................................................................................................................108
  1. Electrochemical studies – Cyclic voltammetry and linear voltammetry.........................108
  2. Spectral studies....................................................................................................................112
     UV-Vis absorption spectroscopy......................................................................................112
Objective 3. The analysis of the interaction of the micelle encapsulated drug with genetic material (double stranded DNA) at physiological pH...

Mitoxantrone – dsDNA interaction

Mitoxantrone - SDS micellar – double stranded DNA

1. Electrochemical studies – Cyclic voltammetry and linear voltammetry

2. Spectral studies - UV-Vis absorption spectroscopy

Part III – Conclusions

Bibliography

Appendix

List of the published articles
INTRODUCTION

Since their discovery about five decades ago anthracyclines are the main category of antitumoral drugs used for the treatment of different kind of localised or generalised cancer. Their mechanism of action is based on the interaction with the nucleic acids inducing apoptosis and free radical generation, along with side effects like influencing the helicases activity, membrane disturbances and mono- and bielectronic reductive activation of the drug [7]. These species can transfer the electron to molecular oxygen by forming superoxide anion and reactive oxygen species (ROS) [7].

Short time after it was clear that these drugs rise serious problems from therapeutical point of view meaning that the cancer cell develops an antibiotic resistance and toxicity dose – dependent in the normal tissue (especially in the cardiac tissue).

Delivery mechanism to the target cell implies passive transport (free diffusion) of the unionized drug through the cell membrane. For the multidrug resistant cells the major problem is the active transport of the drug against a concentration gradient.

Physico-chemical characterisation of drug delivery systems both \textit{in vitro} and \textit{in vivo} represents a major research direction in pharmaceutical chemistry with a great impact in cancer therapy. In the last years the treatment efficiency is substantially improved by encapsulation of the active compound in different delivery systems that ensures the transport towards the cancer cell minimizing drug degradation and side effects.

The use of micelles as drug carriers provides some advantages relative to liposomes and/or polymers: they are easy and reproducible to obtain in large quantities and different ligands can be attached on the external superficial layer because of their core-shell structure and water solubility optimising drug controlled release and pharmaceutical specificity.

Absorption and emission spectroscopy are the main methods used in the study of this type of interaction for several decades [58-66]. However, the electrochemical methods allowing the simulation of different biological media are frequently used in the last two decades. The results obtained from the two categories of methods are complementary: the electrochemical methods bring information about the stoechiometry
of the interaction and the diffusion coefficients of the free drug in solution and the drug attached to the micelle, whereas the spectral ones, much more sensitive to the structural or pH changes, allow the determination of absorption coefficient of different species involved and underline the contribution of different structural parameters to the interaction.

This thesis proposes a study of the interaction of some antitumoral drugs with anthracycline structure or structural analogues with an anionic surfactant, sodium dodecyl sulfate – SDS by coupled electrochemical and spectral methods (including absorption, emission and/or electron paramagnetic resonance spectroscopy), in the presence and absence of double stranded DNA.

The adopted strategy is based on the investigation of three model – compounds: doxorubicine, epirubicine (4’ – epimer of doxorubicine) and mitoxantrone, which have

Doxorubicine

Epirubicine

Mitoxantrone

the anthraquinonic structure in commun.

The objectives of the thesis are:

- *The study of the reduction mechanism of the anthracycline drugs* in aqueous and nonaqueous media in the presence and absence of oxygen by electrochemical methods (cyclic and linear voltammetry) and spectral (UV-Vis absorption and electron paramagnetic resonance spectroscopy). The radical intermediates of the drug and their role in cardiotoxic properties will be discussed based on simple experimental models
miming the \textit{in vivo} conditions taking into account the solvent nature, as well as oxygen content.

- \textbf{The study of the micellar drug delivery system:} the interaction of doxorubicine and mitoxantrone with an anionic surfactant (sodium dodecyl sulfate – SDS) was investigated by electrochemical (cyclic and linear voltammetry) and spectral methods (UV-Vis absorption and fluorescence spectroscopy). Because these drugs are positively charged at neutral pH an anionic surfactant was used as interaction partner justifying the use of phosphate buffer as media for our experiments. On the other hand, the micellisation properties of SDS are well known. The hydrophobic microenvironment of the drug encapsulated in the micelle is a simple model for the interaction biological membrane – drug. The improvement of intracellulary drug delivery / administration is the pourpose of this preliminary study. As well these results make up a base for the investigation of liposomal drug delivery.

- \textbf{The analysis of the interaction of the micelle encapsulated drug with genetic material (double stranded DNA) at physiological pH} by electrochemical (cyclic and linear voltammetry) and spectral methods (UV-Vis absorption spectroscopy). Differences / advantages of using micelles as drug carriers are discussed by comparison between this complex system and drug – micellar surfactant study (objective 2), as well as drug – DNA interaction previously investigated [120, 122, 151].

The main results of the thesis are presented in the followings. The numbering of Figures, Tables and literature references is that used in the thesis.
MATERIALS, EQUIPMENT AND METHODS

Reagents (chlorhydrates of doxorubicine, epirubicine and mitoxantrone) were used without further purification and were acquired from Sigma. Drug concentrations used are in the range $10^{-2} - 10^{-6}$ M. Tetrabutylammonium tetrafluoroborate, tetramethylammonium bromide (TMAB), phosphate buffer (PB) or phosphate buffer saline (PBS) were used as electrolytes in solution. In some case, DMSO was used as solvent with or without controlled water addition. Air was bubbled when oxygen was needed in the system; for removing oxygen when necessary, inert gas (argon) was bubbled in solution. Sodium dodecyl sulfate (SDS) was used in the concentration range $10^{-4} - 10^{-1}$ M prepared in distilled water or TMAB 0.1 M. Aqueous solutions of calf thymus DNA were used. In the study of SDS – drug mixture with DNA the last one was prepared in PBS such a way as the surfactant concentration would be far greater than the critical micellar concentration (CMC) in the presence of the drug and left for incubating for 30 - 40 minutes at room temperature insuring micelles formation and drug engulfment in the micelles.

For the electrochemical methods a three electrodes cell was used for Voltalab 32 and Voltalab 40 devices.

For spectroelectrochemistry the potential domaine was between -0.9 – -0.5 V with a three electrodes cell for C. Zeiss Jena and Unicam (Helios 4) spectrophotometer. For obtaining EPR spectra Jeol JES-3B spectrophotometer at the Institute of Physical Chemistry I.G.Murgulescu of the Roumanian Academy was used with the same three electrodes arrangement as in spectroelectrochemistry. EPR spectra were simulated using WinSim programme.

For UV-Vis absorption spectroscopy Unicam (Helios 4) spectrophotometer coupled with Vision programme were used.

For fluorescence spectroscopy JASCO FP-6300 spectrophotometer was used with $\lambda$ excitation of 480 or 610 nm. All experimental results were performed in the laboratories of the Department of Physical Chemistry, Faculty of Chemistry, University of Bucharest.
Objective 1. The study of the reduction mechanism of the anthracycline drugs [128]

**Doxorubicine** - 1. Electrochemical studies – Cyclic and linear voltammetry

a. Aprotic (DMSO) media, deaerated

**Fig.5 and 11** Cyclic voltamograms – doxorubicine 1.22*10⁻³ M (DMSO / 0.1 M TBABF₄) (v = 50, 100, 200, 400 and/or 600 mV/s)

In the potential range 0/-0.7V studied one reduction wave is observed; extending the potential range up to -1Va second reduction wave appears. Both waves correspond to EC systems and are characterized by the following values:

<table>
<thead>
<tr>
<th></th>
<th>Ist WAVE</th>
<th>IInd WAVE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV</td>
<td>RDE</td>
</tr>
<tr>
<td>kᵢ / cm/s</td>
<td>(2.6±0.1)*10⁻²</td>
<td>2.3*10⁻²</td>
</tr>
<tr>
<td>E° / V</td>
<td>-0.315±0.005</td>
<td>-0.330</td>
</tr>
<tr>
<td>k / s⁻¹</td>
<td>0.16</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td>Dᵦ / cm²/s</td>
<td>5.1*10⁻⁶</td>
<td>4.4*10⁻⁶</td>
</tr>
<tr>
<td>n / αᵦ</td>
<td>1.17</td>
<td>0.49</td>
</tr>
</tbody>
</table>

**Table** Electrochemical parameter values for the two waves
b. **Aprotic (DMSO) media, aerated**

The first cathodic wave is split in two waves and the anodic counterpart disappears when oxygen is added in the system with a negative displacement of the potential by comparison with deaerated solution. For the first wave the diffusion coefficient value is 

\[ D_0 = 3.5 \times 10^{-6} \text{cm}^2/\text{s}. \]

**Fig.16** Cyclic voltamogrammes – doxorubicine 1.22*10^{-3} M (DMSO / 0.1 M TBABF_4) in the potential range -0.7 / 0V (v = 50, 100, 200, 400 mV/s)

The second wave potential value is close to that of the molecular oxygen reduction potential in DMSO (-0.73V versus SCE [125]). So according to the literature data [129], the formation of doxorubicine - molecular oxygen complex may be supposed and could justify the irreversibility of the process and the apparition of a new wave at a more negative potential value. The possibility of electron transfer towards molecular oxygen competes with the protonation of the anion radical in the chemical step, as outlined by the dramatic diminishing of the I_p/p ratio corresponding to the first wave in aerated solutions.

c. **Protic (DMSO+water) media, deaerated**

In **Fig.18** are presented the cyclic voltamogrammes obtained in DMSO with controlled water addition. The system is characterized by the following values: the electron transfer constant \( k_f = (6.3\pm0.9) \times 10^{-3} \text{cm/s} \) (slower than in the absence of water, \((26\pm1) \times 10^{-3} \text{cm/s})\), formal potential \( E^0 = -0.571\pm0.006 \text{V} \), chemical reaction constant \( k = 0.19 \text{s}^{-1} \) and diffusion coefficient \( D_0 = 3.9 \times 10^{-6} \text{cm}^2/\text{s}. \)
In protic media with controlled water addition there is an EC mechanism, the most probable chemical reaction being the protonation of the species formed after the first electron transfer.

By comparing the informations for doxorubicine in aprotic media with protic impurities (commercially available DMSO) with protic media (DMSO+water 6/1, \(v/v\)) one can see that the values for diffusion coefficient and chemical reaction constant are approximately equal so it can be supposed that in both conditions the same species is formed, meaning the protonated semiquinonic radical (AH⁺).

### d. Protic (DMSO+water) media, aerated

By adding water and oxygen in solution the first wave is displaced towards more positive values by comparison with deaerated system.

The system is characterized by formal potential \(E^0 = -0.391\pm0.023\text{V}\), chemical reaction constant \(k = 0.13\pm 0.06\text{s}^{-1}\) and diffusion coefficient value \(D_o = 4.5\times10^{-5}\text{cm}^2/\text{s}\). The system corresponds to an EC mechanism.

The probable existence of some oxygen complexes in the reaction media can be observed (Fig.22).
The influence of dissolved oxygen upon the first reduction wave: A - aprotic media (DMSO, doxorubicine $1.22 \times 10^{-3}$ M, $v = 0.4$ V/s), B - protic media (PB, doxorubicine $10^{-2}$ M, $v = 0.1$ V/s); red - deaerated, black - aerated.

In aprotic media the first wave is split, displaced towards more negative potential values and without anodic counterpart indicating the involvement of semiquinonic anion radical in an irreversible chemical reaction either with molecular oxygen or with protic impurities of the solvent. In the same time a new wave appears at a more positive potential value close to that of the molecular oxygen reduction in DMSO. This wave can be assigned to the oxygen reduction in DMSO or oxygen – drug complex reduction formed according to literature data [129]. The absence of anodic counterpart seems to sustain the second mechanism being well known the reversibility of oxygen reduction in DMSO.

On the other hand in neutral protic media (Fig.22B) appears a new wave at more positive potential values by comparing with the first reduction wave of the drug in PB solution which is irreversible. This wave could be assigned to the molecular oxygen reduction which takes place at more positive values in protic media.

Two mechanisms can be proposed taking into account the literature data [129] for oxygen – drug complex formation:

**I:**  
$A + O_2 \rightarrow AO_2$ (C) or  
$A + e^- \rightarrow A^-$ (E)  
$AO_2 + e^- \rightarrow AO_2^-$ (E)  
$AO_2^- \rightarrow A + O_2^-$ (C)

**II:**  
$A + e^- \rightarrow A^-$ (E)  
$A^- + O_2 \rightarrow AO_2^-$ (C)  
$AO_2^- \rightarrow A + O_2^-$ (C)

meaning a CEC (I) or a ECC (II) type of mechanism. Our electrochemical results indicate as being more probable the mechanism II.
e. Aqueous media (PBS/PB), deaerated

In aqueous solution, doxorubicine presents a single bielectronic wave, followed most probably by protonation chemical reaction.

For higher sweeping rates there is a linear dependence between the current density and sweeping rate suggesting the overlapping of an adsorption wave over the diffusion one.

Fig.24 Cyclic voltamogrammes - doxorubicine $10^{-4}$ M (PBS pH = 7.09) in the potential range -1 / 0 V ($v = 500, 600$ mV/s)

From adsorption criteria [124], the superficial concentration value for the oxidized species adsorbed on the electrode can be obtained, $5.6 \times 10^{-10}$ mol/cm$^2$. Kinetic informations upon the process on the electrode surface can be obtained from Laviron analysis [98, 159]: transfer coefficient value $\alpha = 0.56$ and the electron transfer apparent rate constant $k_s = 8.7$ s$^{-1}$ (estimated from the cathodic reaction rate).
2. Spectral studies - 2.1. UV-Vis absorption spectroscopy

During the electrolysis at a controlled potential value (after the first reduction wave) UV-Vis absorption spectra were recorded at room temperature. It can be seen that the absorption bands at 480 and 500 nm are decreasing in time with almost complete disappearance of the one at 480 nm at prolonged electrolysis and red wavelength shift of the 500 nm band. New bands appear at 520 and 585 nm and the one at 357 nm increases in time. Stopping the electrolysis and exposing to the air the bands at 520 and 585 nm are diminishing and the band of the initial compound (A) is partially recovered. Therefore, the band at 520 nm to the radical anion (A^-) was assigned. As a similar behavior was observed when adding a base (tetrabuthylammonium hydroxide, TBOH) in DMSO, (Fig.28) the absorption band at 585 nm was assigned to the semiquinonic anion (AH^-) obtained either by dianion protonation or by phenolic dissociation. As the 640 nm band appears, the process becomes irreversible and the initial compound is only partially recovered.

**Fig.27** Absorption spectra on electrochemical reduction - doxorubicine $8 \times 10^{-5}$ M (DMSO / 0.1M TBABF$_4$ deaerated)

**Fig.28** Absorption spectra - doxorubicine (chemical reduction with TBOK)
2.2 electron paramagnetic resonance spectroscopy (epr)

fig.29 EPR spectra obtained by electrochemical reduction of doxorubicine 1.22*10^{-3} M (DMSO deaerated) (A) experimental, (B) simulated and the attribution of hyperfine splitting constants

The distribution of the hyperfine splitting constants attests for the odd electron delocalization on the anthaquinonic part of the molecule meaning a characteristic semiquinonic radical anion structure.

In conclusion, corroborating all the spectral and electrochemical experimental data, the following reduction mechanism for doxorubicine in aprotic deaerated media (using the solvent without special treatment it still contains traces of protons) can be proposed:

\[ A + e^- = A^- \quad \text{(E)} \]

\[ A^- + H^+ \rightarrow AH^+ \quad \text{(C)} \]

\[ A^- + e^- = A^{2-} \quad \text{(E)} \]

\[ A^{2-} + H^+ \rightarrow AH^- \quad \text{(C)} \]

The electron transfer towards molecular oxygen competes with protonation of the radical anion in the chemical step in aerated media.

There is another protonation step of dianion in protic media

\[ AH^- + H^+ \rightarrow AH_2 \quad \text{(C)} \]

to the final reduction compound corresponding to an ECECC mechanism.
**Epirubicine** - Electrochemical studies – Cyclic and linear voltammetry

*a. Aqueous media (PBS/PB), deaerated*

The system is characterized by:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_f$ / cm/s</td>
<td>$1.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>$E^0$ / V</td>
<td>$-0.685 \pm 0.021$ $-0.691$</td>
</tr>
<tr>
<td>$D_0$ / cm$^2$/s</td>
<td>$0.9 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

**Fig.30** Cyclic voltamograms - epirubicine 0.01M (PB pH=7.0) (v = 50, 100, 200, 400, 600, 800, 1000mV/s)

*b. Aqueous media (PBS/PB), aerated*

**Fig.35** Cyclic voltamograms - epirubicine 0.01M (PB pH=7.0) (v = 50, 100, 200, 400, 600, 800, 1000mV/s)

The system is characterized by the electron transfer constant $k_f = (1.9 \pm 0.4) \times 10^{-3}$ cm/s, formal potential $E^0 = -0.664 \pm 0.006$ V and diffusion coefficient $D_0 = 1.1 \times 10^{-6}$ cm$^2$/s.

In aqueous media the semichinonic radical anion $A^-$ is very reactive and can participate to the following competitive reactions:

- $A^- + O_2 \rightarrow A + O_2^-$
- $A^- + H^+ \rightarrow AH^+$
- $2A^- = A + A^2^-$

The most probable way of forming the drug- molecular oxygen complex seems to be mechanism II (ECC).
Objective 2. The study of the micellar drug delivery system – Doxorubicine and mitoxantrone interaction with sodium dodecyl sulfate (SDS) [135-137]

**Doxorubicine – SDS - Electrochemical studies – Cyclic and linear voltammetry**

The reduction process was studied after SDS addition in the potential range -1/0V at different sweep rates (Fig.37). at an ionic strength (0.25 M), the rest of the experiments being realized at an ionic strength between 0.11 – 0.15 M.

![Cyclic voltamogrammes - doxorubicine 10^-4 M (TMAB 0.1 M/TF pH = 7.09) in SDS presence 0.00 (dotted line), 3.8, 9.26 and 10.96 mM (v = 0.1 V/s)](image)

A displacement towards more negative potential values and the appearance of an anodic counterpart is induced by the addition of SDS to an aqueous solution of doxorubicine.

The small formal potential variation, ΔE' (20 mV), by comparison with the absence of SDS suggests a weak interaction between the drug and the surfactant. From the potential dependence on SDS concentration, the complexation constant K = 295 M⁻¹ and the number of ligands p = 1.025 corresponding to a drug – micelle, i.e. the complex stoichiometry of 1:1 can be determined. From the current dependence on SDS concentration, the complexation constant 274.13±104.87 M⁻¹ was determined, in good agreement with that from potential variation.

2. Spectral studies – 2.1 UV-Vis absorption spectroscopy

Addition of SDS to a doxorubicine solution of about 10⁻⁵ M results in a decrease of the absorption maxima at 480 and 500 nm until a certain value of the surfactant’s concentration corresponding to the critical micellar concentration (CMC) is reached.
At SDS concentrations higher than CCM an increase of both bands is observed (Process II, Fig.41B).

**Fig.41** Absorption spectra - doxorubicine 1.55*10^{-5} M (PBS pH = 7.09) A - process I (premicellar domain); B - process II (postmicellar domain)

The variation of the absorption maxima as a function of the SDS concentration is presented in **Fig.42**.

**Fig.42** The variation of A_{480} as a function of the SDS concentration added to a solution of doxorubicine 3.09*10^{-5} M (PBS pH = 7.09)

According to literature data [140], the CMC value of the surfactant is determined as the first point of increase after the minimum of the curve, in these conditions (7.97±0.62)* 10^{-4} M.

Each process was interpreted separately corresponding to a 1:1 interaction. For process I, an interaction constant of K = (9.66±0.67)*10^{3} M^{-1} and a molar absorption coefficient ε_{b} = 8 755.44±56.33 M^{-1}cm^{-1}; whereas for process II, K = (1.45±0.08)*10^{3} M^{-1} and ε_{b} = 13 325.1±87.78 M^{-1}cm^{-1} were obtained.

According to the literature data [64] the A_{500}/A_{480} ratio (Fig.45) gives informations about drug partitioning between aqueous (cytosolic) and micellar (membrane) phase: smaller values of the ratio correspond to a hydrophylic microenvironment for the drug, whereas greater values suggest a hydrophobic microenvironment for the drug (drug encapsulated in the micelle).

**Fig.45** The variation of A_{500}/A_{480} with SDS concentration
So a large value of the ionic strength determines a lowering of the interaction constant value as can be seen by comparing the values obtained for the second process by the two methods (electrochemical and spectral).

*Fluorescence spectroscopy*

The variation or the emission spectra of doxorubicin on SDS addition is similar, the same two processes being observed (Fig.46A, B).

**Fig.46** Fluorescence spectra – doxorubicine 8.4*10^-6 M (PBS pH = 7.09) A - process I (premicellar domain); B - process II (postmicellar domain)

**Fig.47** The variation of F Pr54 with SDS concentration

CMC value determined for the surfactant in these conditions is ~7*10^-4 M.

For the first process the interaction constant value obtained is $K = (5.06\pm0.72) \times 10^3$ M$^{-1}$ and for the second process $K = (1.63\pm0.17) \times 10^3$ M$^{-1}$. Working at a higher ionic strength (0.25 M) only the second process was observed corresponding to an interaction constant value of 274.83±12.5 M$^{-1}$, in good agreement with the value obtained from cyclic voltammetry results. This suggests that the differences between the spectral and electrochemical methods are rather due to the different ionic strength of the solution than to the different concentration range (10$^{-6}$ M for fluorescence, 10$^{-5}$ M for absorption and 10$^{-4}$ - 10$^{-3}$ M for cyclic voltammetry).

**Fig.48** The F F554/F587 variation with SDS concentration
The observed trend, similar to that of absorption, allows to distinguish between the two forms of the drug, in function of the SDS concentration: for concentrations smaller than CCM the drug in solution is predominant, whereas greater values suggest a hydrophobic microenvironment for the drug (drug encapsulated in the micelle).

**Mitoxantrone – Aqueous media (PBS/PB), deaerated**

*Electrochemical studies – Cyclic and linear voltammetry*

There are two reduction waves in the studied range. The analysis is rendered more difficult because of the overlapping of the adsorption wave over the diffusion one. From adsorption criteria the superficial concentration value of the oxidized \( \Gamma_O^* \), \((2.4\pm0.6)\times10^{-10}\) mol/cm\(^2\), and reduced \((0.54\pm0.08)\times10^{-10}\) mol/cm\(^2\) species were determined. The Laviron dependence [98, 159] leads to kinetic informations upon the process on the electrode surface: transfer coefficient value \( \alpha = 0.5\pm0.06 \) and the electron transfer apparent rate constant \( k_s = 12\pm0.19 \) s\(^{-1}\) were obtained.

**Fig.52** Cyclic voltamogrammes - mitoxantrone \(1.62\times10^{-4}\) M (PB pH = 7.4) (\(v = 50, 100, 200, 300\) mV/s)

Studying the adsorption wave and repeating the experiment we observed only one reduction wave in the same potential range (**Fig.53**).

**Fig.53** Cyclic voltamogrammes - mitoxantrone \(1.6\times10^{-4}\) M (PBS pH = 7.4) at \(v = 50, 100, 200, 300, 600, 700, 800, 900, 1000\) mV/s

The number of electrons transferred is \( n = 1.94\pm0.26 \) determined from the variation of \( \Delta E_{p/2} \) with sweeping rate in perfect agreement with the bielectronic reduction of the quinone. From adsorption criteria it can be evaluated the superficial concentration value
for the oxidized species \((2.4\pm 0.6) \times 10^{-10}\) mol/cm\(^2\) and \((0.54\pm 0.08) \times 10^{-10}\) mol/cm\(^2\) for reduced species. From Laviron dependence can be obtained kinetic informations upon the process on the electrode surface: transfer coefficient value \(\alpha = 0.5\pm 0.06\) and the electron transfer apparent rate constant \(k_s = 12\pm 0.19\) s\(^{-1}\).

Mitoxantrone has a single bielectronic reduction wave in aqueous solution, part of the drug being adsorbed on the electrode.

**Mitoxantrone – SDS - I. Electrochemical studies – Cyclic and linear voltammetry**

After adding SDS it can be observed that there are no more two reduction waves but one intermediate positioned on the potential axis by comparison with surfactant’s absence. The same two processes, in function of the surfactant concentration were observed (Fig.58a, b):

![Cyclic voltamogrammes - mitoxantrone (1.85 \times 10^{-4} M/PBS pH = 7.4) in the presence of increasing SDS concentrations: a - process I (premicellar domain); b - process II (postmicellar domain) at v = 0.2V/s](image)

These processes are better outlined representing the variation of the current as a function of the SDS concentration (Fig.59), and are separated by the CMC value of SDS of \(~4 \times 10^{-4}\) M

![The variation of the cathodic pic current with SDS concentration at v = 0.4V/s](image)

The formal potential variation \(\Delta E^0\) (~160 mV) with SDS concentration suggests a rather strong drug - surfactant interaction. From these data, the interaction constant \(K = 3.25 \times 10^8\) M\(^2\) was determined, as well as the number of ligands \(p = 1.85\) corresponding to a drug – micelle complex stoichiometry of 1:2 in agreement with the 2+ charge of the drug.
Each process was interpreted separately corresponding to a 1:2 interaction for the first process and a 1:1 interaction for the second one. Thus the values obtained are $K = (2.53\pm0.54)\times10^8 \text{ M}^{-2}$ for the first process and for the second one $K = (1.83\pm1.16)\times10^4 \text{ M}^{-1}$ and $D_b = 1\times10^{-6} \text{ cm}^2/\text{s}$ (for drug encapsulated in the micelle much smaller than $D_O = 8.7\times10^{-5} \text{ cm}^2/\text{s}$ obtained for the drug in PBS in the absence of SDS).

2. Spectral studies – 2.1 UV-Vis absorption spectroscopy

The two processes of decrease of the absorption maxima at 610 and 660 nm after adding SDS up to the CMC of $(8.16\pm0.92)\times10^{-4} \text{ M}$, followed by the increase (modifying the ratio $A_{660}/A_{610}$; the monomer’s band at 660 nm becomes more intense) were also observed (Fig.62A,B, Fig.63).

![Absorption spectra - mitoxantrone 2.86*10^{-5} M](#)

(PBS pH = 7.09) A - process I (precipellar domain); B - process II (postmicellar domain)

These processes are easy to be observed by representing the variation of the absorption maxima as a function of the SDS concentration

![The variation of $A_{660}$ with SDS concentration](#)

Analyzing the first process for a 1:2 interaction the parameters values are: for the interaction constant $K = (1.47\pm0.07)\times10^8 \text{ M}^{-2}$ and for the molar absorption coefficient $\varepsilon_b = 7427.1\pm105.3 \text{ M}^{-1}\text{cm}^{-1}$ For the second process, considering a 1:1 interaction, $K = (1.45\pm0.08)\times10^3 \text{ M}^{-1}$ and $\varepsilon_b = 36883.23\pm215.28 \text{ M}^{-1}\text{cm}^{-1}$ was obtained.
The partition coefficient \((k_x)\) characterizes also the drug – micelle interaction and represents the solute affinity for the micellar phase as compared with the aqueous one. The corresponding values of the partitioning coefficient for mitoxantrone solutions of different concentrations are presented in Table 16. A close dependence on mitoxantrone concentration was noted, (the- partition coefficient value decreases with increasing drug concentration), indicating that the mitoxantrone solubilization in SDS micelles is a competitive process which becomes more and more difficult as the drug is encapsulated in the micelle.

<table>
<thead>
<tr>
<th>(c_m \times 10^5 / M)</th>
<th>(k_x \times 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.21</td>
<td>7.85±1</td>
</tr>
<tr>
<td>2.86</td>
<td>87.82</td>
</tr>
<tr>
<td>1.87</td>
<td>135.73</td>
</tr>
</tbody>
</table>

Table 16 Mitoxantrone partitioning coefficient dependence on drug concentration between aqueous and micellar phase

As the ratio \(A_{660}/A_{610}\) increases from 0.78 to 1.27 for SDS concentrations larger than the CMC (Fig.67) drug dimers dissociation is caused by the interaction mitoxantrone – SDS micelle meaning that the drug is encapsulated as monomer.

Fig.67 The variation of \(A_{660}/A_{610}\) with SDS concentration

Comparing the drug’s spectrum in the presence of SDS with those in water and organic solvents of different polarities informations about the location of the drug in the micelle can be obtained. So an increasing in hydrophobicity induces drug’s dimers dissociation (Fig.67) the drug being encapsulated as monomer in the micelle. Taking into account octanol:water partitioning coefficient at pH = 7.4 (\(\log P = 0.79\)) mitoxantrone is clearly a lipophylic drug [13]. As micelle’s surface is a microenvironment with different properties from water (lower dielectric constant [14]) drug’s monomer is encapsulated in the micelle as the SDS concentration is greater than the CMC. The drug is located at the surface of the micelle most probably with the chromophore orientated towards the core.
and the lateral chains towards sulfate groups of SDS negatively charged. Both polar and electrostatic interactions play an important role in drug – micelle interaction.

For both investigated drugs the CMC value determined electrochemically and spectrally, about $10^{-4}$ M, is much lower than in pure water ($8.08 \times 10^{-3}$ M) or in PB 50mM ($1.99 \times 10^{-3}$ M) [15] because of the well known effect of lowering the CMC value induced by the presence of different ions and molecules in solution [16].

**Objective 3. The analysis of the interaction of the micelle encapsulated drug with genetic material (double stranded DNA) at physiological pH**

*Mitoxantrone - SDS micellar – double stranded DNA*

1. **Electrochemical studies – Cyclic and linear voltammetry**

To ensure drug’s encapsulation in the micelles, a mitoxantrone – SDS mixture in a concentration ratio corresponding to the second process (P/D = 149) was prepared and was titrated with DNA prepared in the same mixture. In these conditions, the drug and surfactant are kept constant, only the DNA concentration being varied during the experiment. The recorded voltamogrammes are presented in **Fig.68**.

![Cyclic voltamogrammes - mitoxantrone + micellar SDS (P/D = 149 / PBS pH = 7.4) in the presence of DNA: A - process I (0 - 6.34 µM/nucleotide dsDNA), B - process II (17.9 – 65.2 µM/nucleotide dsDNA) at v = 0.1V/s](Fig.68)

The evolution in **Fig.68**, for 0.00 M/nucleotide dsDNA, similar to that in **Fig.58** regarding the mitoxantrone – SDS system, allows the tentative assignment of the second wave at more negative potential values (~ -0.85V) to the neutral form of the monomer encapsulated in the micelle, whereas the first wave to the free drug in solution. By adding DNA, the second wave decreases and the first one increases, the shape of the
voltamogramme becoming similar to that of the drug at pH = 7.4 in PB (Fig.58a) and the two waves become almost equal (Fig.68B).

It may be inferred therefore, that small DNA concentrations extract the drug from the micelle until an equilibrium is reached and the cyclic voltamogramme becomes constant.

The current variation with DNA concentration for the first wave (Fig.71), outlines the existence of the same two processes: decrease (process I) and increase (process II) as in the case of drug – SDS systems, but there is an intermediate domain corresponding to the third process. The third domain is limited by the CAC and CMC values of SDS.

Fig. 71 The current density variation with DNA concentration for a mixture mitoxantrone + SDS (P/D=149 / PBS pH=7.4) at v = 0.1V/s

Each process was interpreted separately corresponding to a 1:1 interaction. For the first process the interaction constant is $K = (1.73\pm0.12) \times 10^6 \text{M}^{-1}$ and for the second one, $K = (8.84\pm1.31) \times 10^4 \text{M}^{-1}$.

DNA contribution to micellar aggregation is of 10.92 kJ/mol and the aggregation number of SDS in DNA presence is 90 both determined from the CAC and CMC values.

Moreover, there is a lowering of the DNA/drug ratio in the presence of SDS micelles ($\sim 0.07 - 0.15$) as compared with the absence of the surfactant (2.7, [17]) probably meaning that the administration of the drug encapsulated in the micelle is more efficient therapeutically than the drug alone.

2. Spectral studies – UV-Vis absorption spectroscopy

Spectral studies in presence of different amounts of dsDNA were performed in similar experimental conditions. It was observed that mitoxantrone – SDS spectra modify in time: the monomer band splits after ~ 1 hour of incubation becoming stable (Fig.73A – inset). The stable mixture obtained is titrated with DNA prepared in the SDS –
mitoxantrone mixture. Recorded spectra indicate the existence of the two processes: decrease and increase of the absorption maxima at 613, 649 and 665 nm.

![Fig.73](image)

**Absorption spectra** - mitoxantrone + micellar SDS (P/D = 149 / PBS pH = 7.4) in the presence of DNA: A - process I (0 - 2.06 µM/ nucleotide dsDNA), B - process II (2.87 - 36.4 µM/ nucleotide dsDNA); **inset** - mitoxantrone + SDS (P/D = 110): *continue line* - t\text{incubation} = 0, *dotted line* - t\text{incubation} > 1 hour

Spliting of the absorption maxima at 660 nm in the absence of the surfactant, as well as a shoulder at 649 nm and a maximum at 665 nm suggest the existence of two species of monomer.

![Fig.74](image)

**Fig.74** The variation of A\text{665} with dsDNA concentration

More over from the variation of the absorption with DNA concentration (Fig.74) a very narrow third domain, intermediate between processes I and II, can be seen.

Each process was interpreted separatly coresponding to a 1:1 interaction. For the first process the parameter values obtained are \( K = (9.54 \pm 1.96) \times 10^5 \text{M}^{-1} \) and \( \varepsilon_b = 1648.22 \pm 234.84 \text{M}^{-1} \text{cm}^{-1} \) and for the second one, \( K = (3.07 \pm 0.47) \times 10^4 \text{M}^{-1} \) and \( \varepsilon_b = 24484.58 \pm 605.78 \text{M}^{-1} \text{cm}^{-1} \).

The aggregation number of SDS in DNA presence is 70 determined from the CAC and CMC values.

Our electrochemical and spectral results have outlined three processes, tentatively assigned as follows: process I – extraction of the drug from the micelle by the DNA,
process II – interaction drug – SDS / DNA, the drug being deeper located in a hydrophobic environment, and process III – SDS molecules reorganisation near or on the DNA surface and the apparition of „free” micelles in solution for SDS concentrations higher than the CMC.

CONCLUSIONS

1. The study of the reduction mechanism of the anthracycline drugs

Analysis of the electrochemical results in correlation with the spectral ones (UV-Vis and EPR in situ techniques) in different experimental conditions, outlines:

• In aprotic deaerated solvents doxorubicine and epirubicine suffer a monoelectronic reversible reduction in two steps corresponding to the reduction of the central quinonic ring to radical anion and dianion. There is a protonation of these species in the presence of protic impurities (existing in commercially available solvents used without special anhidrization in concentration of the same order with the substrate) to free radical AH⁺ and semichinonic anion AH⁻, the global mechanism being ECEC.

• The radical anion was identified and characterised by EPR spectrometry – in situ indicating a delocalisation of the odd electron on C and D rings, the hyperfine splitting constant distribution attesting a characteristic structure of semiquinonic radical anion. Taking into account that the aromatic part is the one that intercalates to the DNA base paires, this spin distribution could explain the semiquinonic radicals action upon DNA.

• Diamagnetic intermediates (dianion A₂⁻ and nonradical anion AH⁻) were identified by UV-Vis spectroscopy on chemical / electrochemical reduction in situ.

• In the presence of oxygen, the first wave is split and the anodic counterpart disappears. Analysis according to electrochemical criteria outlines a chemical reaction following the electron transfer and supports the assignement of the process to the drug – oxygen complex as a first step of superoxide formation according to literature data [129].

• In protic media, similar to quinizarine, the redox behaviour is strongly pH dependent. In aqueous solutions at neutral pH there is a bielectronic reduction process according to the global stoichiometry A + 2e + 2H⁺ = AH₂.
• The reversible couple at -675 mV in deaerated solutions becomes irreversible in the presence of oxygen and is accompanied by a more intense irreversible wave at -490 mV [128]. According to the literature data [129] this behaviour suggests the formation of a drug – oxygen complex reduced at a more positive potential which facilitates electron transfer towards molecular oxygen generating superoxide anion according to the reactions:

\[
\begin{align*}
AO_2 + e^- &= AO_2^- \\
AO_2^- &= A + O_2^- 
\end{align*}
\]

which in the presence of water is responsible for ROS generation.

2. The study of the micellar drug delivery system

Micellar media is the simplest system miming biological membranes structure.

Doxorubicine – anionic surfactant (SDS) interaction was studied by cyclic voltammetry, absorption and emission (fluorescence) spectroscopy. Electrochemical results indicated:

• The displacement of the cathodic pic current towards more negative potential values attests the SDS – doxorubicine interaction. For lower SDS concentrations (premicellar domain) the electrostatic interaction between the positively charged drug at neutral pH and the anionic surfactant determines the neutralization of the drug charge. At higher concentrations than CMC, “free” micelles are forming in solution and drug – micelle interaction means the encapsulation of the drug in the micelle. A higher ionic strength (by using TMAB and PBS) induces the formation of larger micelles and promotes drug extraction from water into the micelle [140].

• As an extra information, the spectral method confirmed for the first process that after charge neutralisation by SDS drug dimerisation occurs as SDS concentration increases (by the lower molar absorption coefficient value than monomer aiming towards the dimer value) and for the second process – drug encapsulation into the micelle probably as monomer (by the higher molar absorption coefficient value than monomer caused by the hydrophobic microenvironment of the drug).
For SDS concentrations lower than CMC, the extinction of the fluorescence can be explained by drug – SDS complexes formation mainly through electrostatic interaction implicating most probably the nitrogen atom from the sugar moiety corresponding to a 1:1 stoichiometry. Drug charge neutralisation by the surfactants anions diminishes the repulsion forces favorising drug dimerisation. At higher surfactant concentrations, the second process characterised by fluorescence enhance aims to a drug saturation level and corresponds to doxorubicine encapsulation in SDS micelles, probably as a monomer.

- Binding parameters determined by fluorescence spectroscopy are in good agreement with those determined by absorption spectroscopy. For a higher ionic strength (0.25 M) electrochemical (from current and potential variation) and spectral results are also in good agreement confirming that discrepancies in interaction constant values estimated by different methods are rather due to ionic strength of the solution, than to the concentration domain used.

- The concentration domain makes the difference between the methods: cyclic voltammetry needs large drug concentrations about 10⁻³ M, whereas absorption and emission spectral methods smaller concentrations about 10⁻⁶ – 10⁻⁵ M.

- SDS – mitoxantrone interaction was studied by cyclic voltammetry and absorption spectroscopy for premicellar and micellar surfactant concentrations as mitoxantrone is not fluorescent.

The adsorption of mitoxantrone on the glassy carbon electrode in the absence of SDS was observed and characterised by electrochemical methods.

- Both methods indicated two different processes: in the premicellar domain (corresponding to process I) there is an electrostatic interaction between the positively charged drug and the negatively charged surfactant with a 1:2 probable stoichiometry. In the micellar domain (corresponding to process II) there is a drug – micelle interaction with a 1:1 stoichiometry resulting in drug encapsulation, most probably as a monomer, into the micelle.

- In our experimental conditions the CMC value of SDS determined is ~0.75*10⁻³ M smaller than in aqueous solution (8.1*10⁻³ M) or in PB 50mM (1.99*10⁻³ M) because of the different ions and molecules present in solution.
• Informations about the location of the drug into the micelle can be obtained by comparative analysis of the absorption spectra in different solvents with those in SDS. The results indicated as the most probable location of the drug in the superficial layer of the micelle with the heterocycle oriented towards the core of the micelle and the lateral chains towards the sulfate groups of SDS, both electrostatic and hydrophobic forces being involved.

3. The analysis of the interaction of the micelle encapsulated drug with genetic material (double stranded DNA) at physiological pH

Mitoxantrone – dsDNA interaction in the presence of higher SDS concentration than CMC was studied by cyclic voltammetry and absorption spectroscopy.

• The electrochemical results showed:
  
  ➢ the existence of two reduction waves in the –0,6 - –0,9 V range versus SCE;
  
  ➢ the first pic current density dependence on the DNA concentration indicate the existence of the same two processes of decrease (process I) and increase (process II) as in drug – SDS systems but there is an intermediate domain corresponding to a third process.

• Process I probably corresponds to mitoxantrone – DNA interaction meaning that in the presence of the biopolymer the drug leaves the micelle and interacts with the DNA. A region more or less constant, domain III is limited by the critical aggregation concentration (CAC) of the surfactant when drug – micellar aggregate complexes become detectable and the critical micellar concentration (CMC) marking the apparition of „free” micelles in solution (corresponding to the beginning of process II). It probably corresponds to SDS molecules reorganisation near or on the DNA surface, the most probable / possible explanation being that dsDNA compact structure is temporary disturbed by the micellar SDS hydrophobic microenvironment [171].

• Interaction constant values obtained by the two methods are in good agreement, as well the SDS aggregation number in the presence of DNA. The value obtained agrees with the literature data for a 0.1 M NaCl solution, micelles size being approximately the same in the presence or absence of the polymer [154].
• In the presence of SDS micelles drug / DNA ratio is displaced towards smaller values as compared with the absence of the surfactant from 2.7 [117] to ~0.07 – 0.15 which probably means that mitoxantrone encapsulated administration is more efficient than the drug itself.

• Taking into account electrochemical and spectral data for mitoxantrone – SDS and mitoxantrone – micellar SDS – DNA systems corroborated with previous results about mitoxantrone / quinizarine behavior in DMSO in the presence of tetrabuthyl ammonium hydroxide [120, 134], in aqueous solution at neutral pH there is an equilibrium between protonated and deprotonated species of mitoxantrone.

As perspectives, taking into account my working place, the cardiotoxic properties investigations could be continued / developed by ex vivo (cells), in vitro (cell lines) and/or in vivo (laboratory animals) experiments. This kind of experimental is much closer to reality because system complexity (the existence of cellular organelles, intracellular membranes, metallic ions, biomolecules – enzymes, nucleic acids etc). More over there are reproducible intra- and interdetermination conditions.

The biosensors are another research direction due to the physical and chemical drug adsorption properties on the electrode surface.

BIBLIOGRAPHY

[120] M. Enache, C. Bendic, E. Volanschi; *Bioelectrochemistry* **2008**, *72*, 10-20
[140] M. Sarkar, S. Poddar; *J. Colloid Interface Sci.* **2000**, 221, 181-185


