ELECTROCHEMICAL EVALUATION OF dsDNA - LIPOSOMES INTERACTIONS

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The aim of the present work was to evaluate the interaction between double-stranded DNA (dsDNA) and liposomes by voltammetric methods. The experimental results were analyzed considering the initial studies regarding the oxidation mechanism of dsDNA purine bases by cyclic and differential pulse voltammetry at the glassy carbon electrode (GCE). The interaction between dsDNA and 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was studied in a suspension containing both dsDNA and DMPC liposomes, prepared in pH = 7.0, 0.1 M phosphate buffer and using different incubation time periods. The formation of dsDNA–liposome complex was put in evidence by the decrease of the dsDNA oxidation peaks, dependent upon the incubation time. This behavior was explained considering the electroactive centers of dsDNA, guanosine monophosphate and adenosine monophosphate residues, part of them hidden inside the dsDNA–liposome complex structure and thus being unable to reach the GC electrode and preventing their oxidation. The electrochemical results are relevant for a better physico-chemical characterisation of the dsDNA and dsDNA-liposome complex, which can be important for the development of gene therapy vectors.

1. Introduction

Gene therapy is a rapidly progressing domain into clinical therapeutic procedures because it aims at eradicating cause rather than symptoms of diseases. The principle of gene therapy is simple: corrected copies of the malfunctioning genes are introduced into cells for the purpose of treating, curing or ultimately preventing disease. The key to success for any gene therapy strategy is to design a vector able to serve as a safe and efficient gene delivery vehicle [1,2,3].

Liposomes, spherical vesicles with lipid bilayer membrane, due to their diverse morphology and/or composition, have the ability to incorporate and protect many types of therapeutic biomolecules either in interior or in the lipid bilayer [4,5,6]. Complexes formed between cationic liposomes and nucleic acids represent an efficient vehicle for delivery and controlled release of DNA or RNA molecules to the target cells. Due to their opposite surface charge, cationic liposomes can form a positively charged complex with negatively charged DNA, leading to a positively charged complex called lipoplexes. This complex does not face any electrostatic barrier in penetrating the negatively charged biological cell surfaces [7].

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Many research studies have shown that the formation of liposomes – based DNA delivery system is a consequence of a self – assembly process spontaneously triggered by electrostatic interactions between lipids and DNA [8, 9, 10].

Electrochemical researches on DNA and liposomes are of great relevance for explaining many biological mechanisms. It is important to elucidate the role of lipid constituents in the self-assembly process that leads to formation of these complex composites, particularly because this information will be required to develop rationally designed formulations for therapeutic applications.

The main objective of this study was to investigate the electrochemical behavior of dsDNA – DMPC liposomes complex by using differential pulse voltammetry (DPV). The results were analyzed and discused considering the initial studies on the oxidation mechanisms of dsDNA purine bases by using cyclic voltammetry (CV) and DPV at the glassy carbon electrode (GCE).

2. Experimental

2.1. Materials and reagents

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), double-stranded calf thymus DNA (dsDNA), adenosine monophosphate (AMP) and guanosine monophosphate (GMP) were purchased from Sigma and used without any further purification. The chemical structure of DMPC, adenosine monophosphate and guanosine monophosphate are presented in the *Scheme 1*.
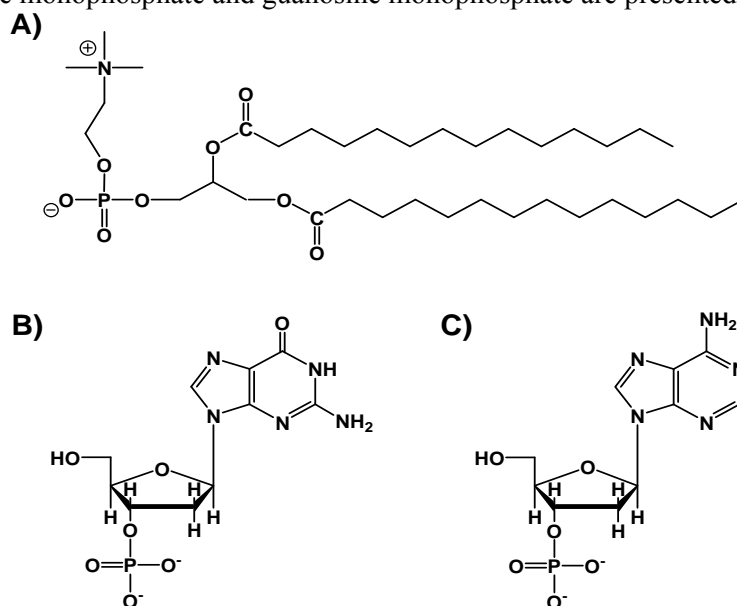
Scheme 1. Chemical structures of: A) DMPC, B) GMP and C) AMP

All supporting electrolyte solutions, *Table 1*, were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity < 0.1 µS cm^{-1}).

Table 1. Supporting electrolyte solutions, 0.1 M ionic strength

pH	Composition
2.1	HCl + KCl
3.4-5.5	HAcO + NaAcO
6.1-8.0	NaH_2PO_4 + Na_2HPO_4
9.25-10.5	$Na_2B_4O_7$ + NaOH
12.0	NaOH + KCl

2.2. Preparation of liposomes and dsDNA-liposomes complex

During these experiments, liposomes were prepared using the thin-film hydration method, appropriately adapted for our purposes. The liposomes were prepared in phosphate buffer pH 7.0, 0.1 M using deionized water. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (150 μg/mL as final concentration in liposome suspension) in ethanol solution was dried under nitrogen (~ 1 h), to leave a thin film on the wall of a round-bottom flask by removal of the organic solvent. After that, the lipid film was hydrated (~ 30 min) with phosphate buffer, followed by vigorous shaking (~ 1 h). Unilamellar liposomes were obtained by sonication for 30 minutes, four times, in a ultrasonic bath until the suspension became clear. To obtain a homogeneous population of small unilamellar vesicles (SUV), the suspension was centrifuged (30 min).

The complex between dsDNA and DMPC liposomes was prepared by gently stirring a solution of dsDNA with the SUV suspension; the molar ratio dsDNA:SUV suspension was 1:3. The Eppendorf tubes, used for sample storage at 4^0C for different time intervals, were rigorously shaken before each electrochemical experiment. This was done in order to prevent errors due to the lipid adhesion to the Eppendorf tube walls during the storage.

2.3. Apparatus

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Eco-Chemie, Utrecht, The Netherlands. Measurements were carried out using a three-electrode system in a 0.5 mL one-compartment electrochemical cell (Cypress System Inc., USA). Glassy carbon electrode (GCE, d = 1.5 mm) was the working electrode, Pt wire the counter electrode and the Ag/AgCl (3 mol L^{-1} KCl) reference electrode.

The pH measurements were carried out with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1 ºC) and microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA).

The redox behavior of AMP, GMP and dsDNA/lipids complex at a glassy carbon electrode was investigated using cyclic voltammetry (CV) and differential pulse (DP) voltammetry. The experimental conditions for CV were: potential increment of 2 mV and a scan rate of 50 mV s^{-1}. For DP voltammetry were: pulse amplitude 50 mV, pulse width 70 ms, interval time 400 ms and potential step 2 mV which corresponded to a scan rate of 5 mV s^{-1}.

The GCE was polished using diamond particles of 3 μm (Kemet, UK) before each electrochemical experiment. After polishing, it was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and voltammograms were recorded until steady state baseline voltammograms were obtained. This procedure ensured very reproducible experimental results.

2.4. Acquisition and analysis of voltammetric data

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improves the visualisation and identification of peaks over the baseline without introducing any artefact, although the peak intensity is, in some cases, reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all plots were determined from the original untreated voltammograms after subtraction of the baseline.

3. Results and discussion

3.1. Electrochemical behaviour of dsDNA bases

Electrochemical studies carried up to date [7, 12-15, 19] have shown that all dsDNA bases can be electrochemically oxidised at GCE surface, following a pH-dependent mechanism. Both sugar and the phosphoric residue are inactive at carbon electrodes and the electroactive centers are the bases. The purines, guanine (G) and adenine (A), are oxidized at lower positive potentials that the pyrimidines, cytosine (C) and thymine (T), which oxidation occurs only at very high positive potentials near the potential corresponding to oxygen evolution, and consequently are more difficult to be detected [15,16].

3.1.1. Guanosine monophosphate (GMP)

The electrochemical behavior of GMP was initially studied by CV in a solution of 20 μM GMP in pH 5.5 0.1 M acetate buffer, *Fig.1*. On the anodic component of the first voltammetric scan one oxidation peak was observed at E_{pa} = + 0.95 V. Changing the scan direction, no cathodic correspondent occurred in agreement with the irreversibility of GMP oxidation.

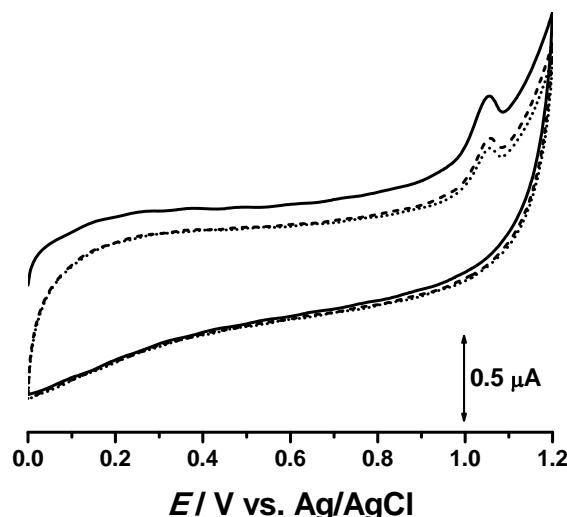*Fig. 1. Cyclic voltammograms in 20 μM GMP in pH = 5.5 0.1 M acetate buffer; (━) first, (- - -) second and (•••) third scans at v = 50 mV s⁻¹.*

After recording successive scan in the same solution without cleaning the working electrode surface, the oxidation peak of GMP decreased. This is due to the formation of oxidation products which can remain adsorbed on the electrode surface, so reducing the electroactive area.

Cyclic voltammograms were recorded in solutions of 20 μM GMP in supporting electrolytes with different pH values and a similar behaviour was observed but GMP oxidation peak potential was pH-dependent.

DP voltammetry allows lower detection limits and the pH effect on the electrochemical oxidation behaviour of GMP was investigated in electrolytes with different pH values between 3.0 and 12.0. All DP voltammograms were recorded in solutions of 10 μM GMP and in all electrolytes one oxidation peak occurred, *Fig. 2A*.

The potential of GMP oxidation peak is displaced to lower values with increasing the pH of the supporting electrolyte. The relationship is linear and the slope of the line E_{pa} vs. pH is 59 mV / pH unit, *Fig. 2B*, which shows that the oxidation mechanism involves the same number of protons and electrons.
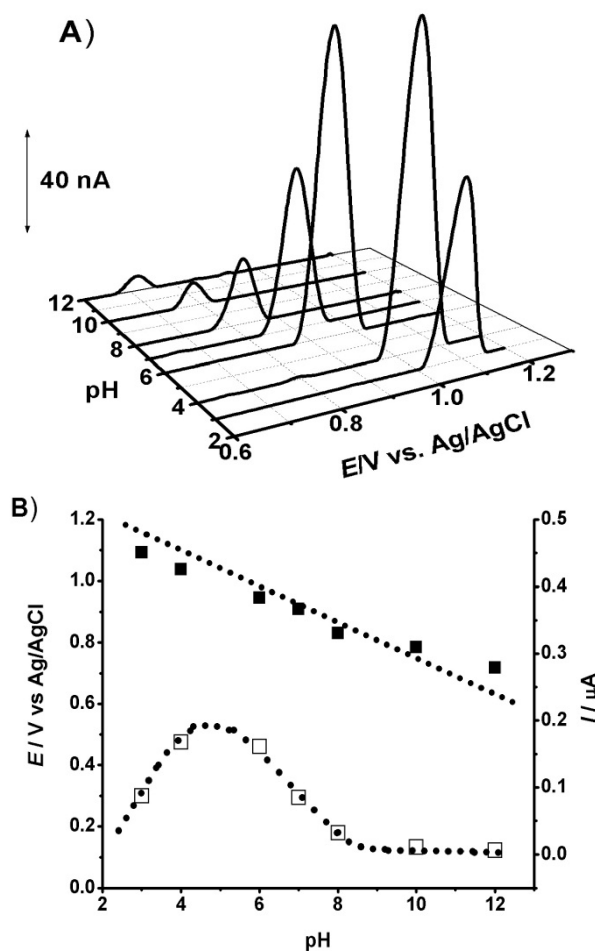
Fig. 2. A) 3D plots of DP voltammograms base-line corrected in 10 μM GMP vs. pH; and B) Plot of (■) E_{pa} and (□) I_{pa} of GMP oxidation peak vs. pH. Dotted line corresponds to 59 mV per pH unit.

Considering that the width at half height of the peak is $W_{1/2} = 47$ mV, close to the theoretical value for the transfer of two electrons, it can be concluded that the oxidation mechanism of GMP involve the transfer of two electrons and two protons. Higher oxidation peaks were recorded in electrolytes with pH values between 4.0 and 5.0.

3.1.2. Adenosine monophosphate (AMP)

CV was performed in a solution of 100 μM AMP in pH 5.5 0.1 M acetate buffer, *Fig. 3*, and showed on the anodic part of the first scan a single oxidation peak at $E_{pa} = + 1.38$ V.

Reversing the scan direction, no correspondent cathodic peak was observed, showing that the AMP oxidation process is irreversible.
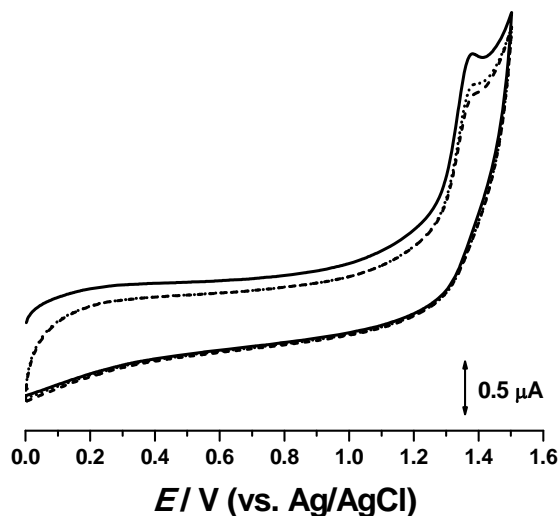

Fig. 3. Cyclic voltammograms in 100 μM AMP in pH = 5.5 0.1 M acetate buffer; (—) first, (---) second and (•••) third scans at v = 50 mV s⁻¹.

Recording successive scan in the same solution without cleaning the working electrode surface, the oxidation peak of AMP decreased owing to the adsorption of the oxidation products on the electrode surface reducing the electroactive area.

Cyclic voltammograms were recorded in solutions of 100 μM AMP in electrolytes with different pH values. A similar electrochemical behavior was observed in all the cases.

The pH effect on the electrochemical behavior of AMP was studied by DP voltammetry. Voltammograms were recorded in solutions of 20 μM AMP in electrolytes with different pH values between 2.0 and 12.0. In all electrolytes one oxidation peak was observed, *Fig. 4A*.

The potential of AMP oxidation peak is displaced to lower values with increasing the pH of the supporting electrolyte. The relationship is linear and the slope of the line E_{pa} vs. pH is 59 mV / pH unit which shows that the oxidation mechanism involves the same number of protons and electrons, *Fig. 4B*.
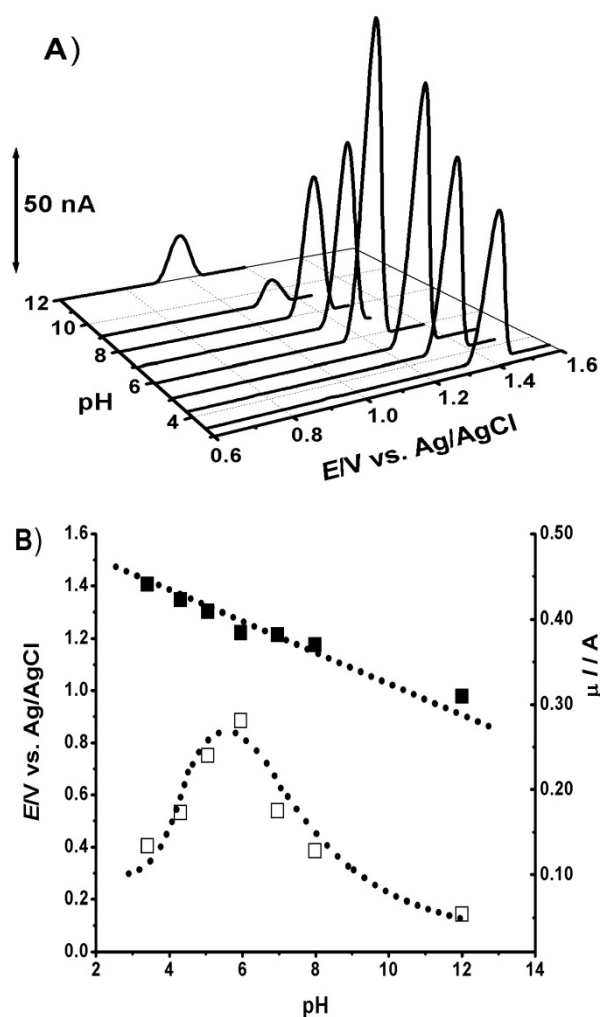
Fig. 4. *A) 3D plots of DP voltammograms base-line corrected in 20 μM AMP vs. pH; and B) Plot of (■) E_{pa} and (□) I_{pa} of AMP oxidation peak vs. pH. Dotted line corresponds to 60 mV per pH unit.*

Considering that the peak width at the half height is $W_{1/2}$ = 50 mV, close to the theoretical value for the transfer of two electrons, it can be concluded that the oxidation mechanism for AMP involve the transfer of two electrons and two protons.

3.2. dsDNA-liposomes interaction

The electrochemical behaviour of dsDNA as well as of liposomes has been briefly investigated in order to facilitate the identification of the peaks occurring during the lipoplex formation. The DP voltammograms of dsDNA were recorded in a solution of 50 μg/mL concentration in pH 7.0, 0.1 M phosphate buffer and two consecutive oxidation peaks were observed, at E_{pa} = + 0.90 V and E_{pa} = + 1.15 V, *Fig. 5*. These peaks correspond to the oxidation of dsDNA purinic bases. The peak at E_{pa} = + 0.90 V is attributed to GMP oxidation and the peak at E_{pa} = + 1.15 V is due to the oxidation of AMP. The peak current due to GMP oxidation is lower than that for AMP. This can be correlated with the lower content of GMP compared to AMP in dsDNA. The oxidation of thymidine monophosphate and cytidine monophosphate was not observed. This oxidation, as previously reported [6, 17, 18], can be detected at the pH 7.0, only at much higher positive potential, near the potential of oxygen evolution.
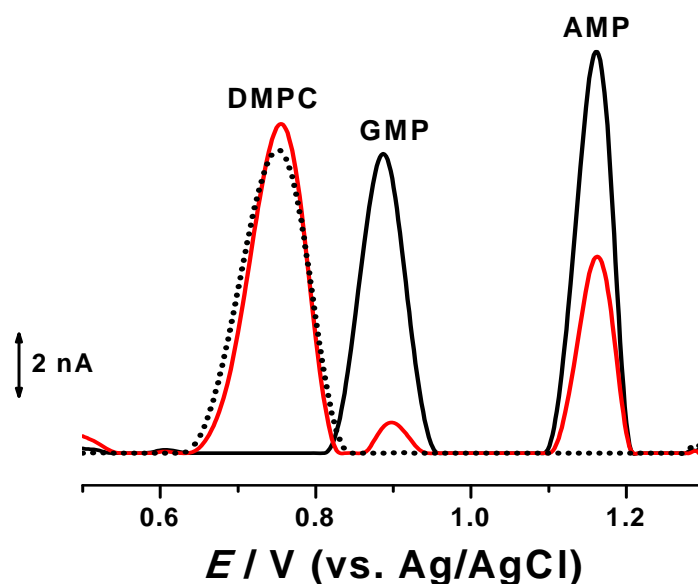
Fig. 5. Base line corrected DP voltammograms obtained for: (▬) 50 μg/mL dsDNA and (•••) 150 μg/mL DMPC liposomes before and (▬) immediately after the addition of dsDNA, in pH = 7.0 0.1 M phosphate buffer.

DP voltammograms were recorded also for suspensions of DMPC liposomes in pH 7.0 phosphate buffer, *Fig. 5*. An oxidation peak appeared at $E_{pa} = + 0.75$ V, attributed to the lipid DMPC oxidation at GCE surface.

The interaction between dsDNA and liposomes was studied in a suspension containing 50 μg/mL dsDNA and 150 μg/mL SUV in pH = 7.0, 0.1 M phosphate buffer. The oxidation peaks of dsDNA show a current decrease immediately after the addition of dsDNA to the liposomes suspension, *Fig. 5*. An interaction between the purine bases of dsDNA and the DMPC lipid composing the liposomes could be responsible for the process at the GCE surface. The interaction occurs almost instantly, and the lower intensity observed for peaks in the case of purine bases of dsDNA can be explained by the fact that only a part of the purine bases of dsDNA would reach the electrode surface.

Another observation is that the peak due to the GMP oxidation from the complex presents a significant decrease in current as compared with the peak obtained for AMP oxidation from complex. It could be explained by a preferential interaction of liposome lipids with dsDNA segments rich in GMP bases.

The very small current difference between the peaks observed for DMPC oxidation in the complex and in a solution containing only DMPC lipid, was attributed to the modification of the GCE available surface area between successive surface renewals.

Further, DP voltammograms were recorded in the mixed solution after different periods of time, *Fig. 6*. Between the measurements, the GCE surface was always polished in order to ensure a clean surface to avoid possible problems from the adsorption of dsDNA and/or lipid.
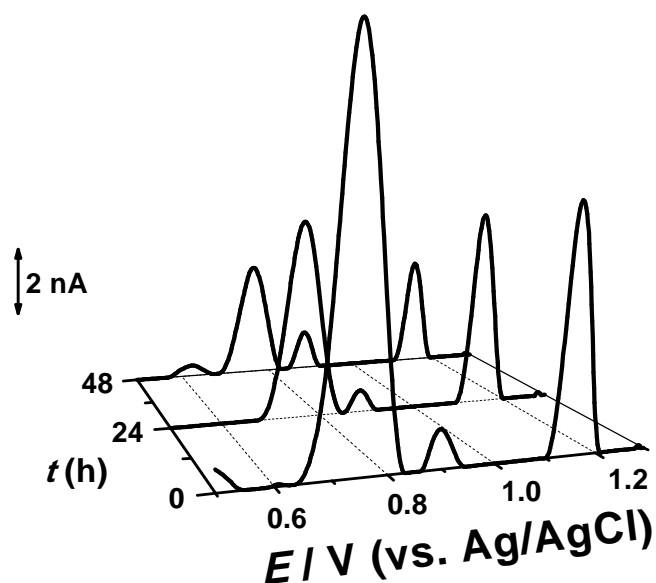
Fig. 6. 3D graph of DP voltammograms baseline-corrected in solution of 50 µg/mL dsDNA incubated with 150 µg/mL DMPC liposomes vs. incubation time, in pH 7.0, 0,1 M phosphate buffer

By increasing the incubation time, both DMPC liposomes oxidation peak current and purine bases oxidation peak currents continued to gradually decrease, reaching constant currents after 48 hours of incubation, as shown in the *Fig. 6.*

The decrease of the dsDNA oxidation peaks, within a 48 h interval, can be explained considering the dsDNA-SUV complex behaviour. The process is related to a decrease of the concentration of free/uncomplexed dsDNA, close to the electrode surface and available for oxidation *via* purine bases. After the formation of dsDNA-DMPC liposomes complex, a part of the guanosine monophosphate and adenosine monophosphate electroactive centers are hidden inside the structure being unable for electrochemical oxidation. By increasing the incubation time, more dsDNA strands can be involved in complex formation with the liposomes, thus determining a decrease of the free/uncomplexed dsDNA amount in solution.

At the same time, a decrease of the liposomes oxidation peak current was also observed. Less lipid electroactive centers will be exposed to the GCE surface because the lipids from liposomes are gradually involved in complexation with dsDNA.

4. Conclusion

Electrochemical methods proved to be promising tools for the study of dsDNA and the interaction mechanisms of lipids with dsDNA. In the present study, the electrochemical behavior of dsDNA and the interaction between dsDNA and DMPC liposomes has been investigated using cyclic voltammetry (CV) and differential pulse (DP) voltammetry. It was concluded that the oxidation mechanism for adenosine monophosphate and guanosine monophosphate, the purine bases of dsDNA, involve the transfer of two electrons and two protons. The interaction between dsDNA and DMPC lipid has been studied after different incubation time periods of dsDNA with SUV liposomes. The addition of dsDNA to the liposomes suspension resulted in a time-dependent decrease of guanosine monophosphate and adenosine monophospate oxidation peaks. This process is related to a concentration decrease of dsDNA bases available for oxidation and can be explained considering the formation of a dsDNA-SUV complex. Upon the formation of dsDNA-SUV complex, a part of the guanosine monophosphate and adenosine monophospate electroactive centers are hidden inside the structure being unable to reach the GC electrode surface.

Acknowledgements

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