Procaine Effects on Model Membranes with Chlorophyll *a*

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Chlorophyll a (Chla) was used as a sensor to monitor the changes, in the lipid bilayers, occurred by addition of procaine, a local anaesthetic. Different types of small unilamellar vesicles (SUVs) with chlorophyll a incorporated in the lipid bilayer, were obtained with Chla/lipid molar ratio = 1/100, in the presence or absence of an antioxidant (a-tocopherol acetate). In order to study the interactions of procaine with the lipid bilayers, various concentrations of this anaesthetic were added to each liposome sample (in the range $0 \div 10^{-2}$ M) and absorption and fluorescence spectra were recorded. Procaine addition to liposomal suspensions perturbed the structure of the artificial membranes. Chlorophyll a and procaine proved to be competitors in the lipid bilayer, in the case of the tocopherol-containing vesicles, resulting in the exclusion of chlorophyll in the aqueous environment.

Keywords: liposomes, chlorophyll a, procaine, local anesthetic, vitamin E

Procaine (Novocain) is a local anaesthetic used to reduce the pain of intramuscular injection of penicilin, and is also used in dentistry and geriatry. Procaine has protection effects on oxidative stress and toxicities caused by cisplatin *in vitro* [1]. The procaine molecule (fig. 1) consists of a lipophilic portion (an aromatic ring) and a hydrophilic amine separated by an intermediate chain (an ester group). The potency of the anaesthetic is related to its lipid solubility [2].

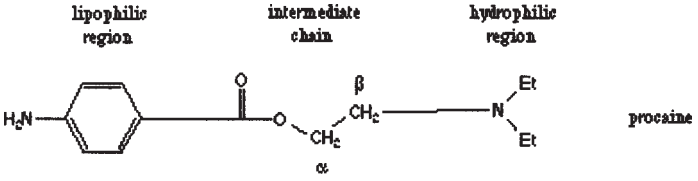

Fig. 1. Chemical structure of procaine [2]

The cell membrane is considered as the site of the action of local anaesthetics at cellular level [3]. The drugs with lipid solubility (such as anaesthetics) enter in the hydrophobic regions of both proteins and lipids, in the core of the membrane, and disrupt the structure of the lipid bilayer [4].

Local anaesthetics (LAs) produce molecular disordering in the lipid bilayers and an increase of the fluidity [5 - 9] and permeability of lipid membranes [10]. Hydrophobic and ionic interactions between LAs and lipid molecules predominate [11; 12]. Local anaesthetics influence also the electrostatic properties of the lipid membrane bilayers [13]. At physiological *p*H, tertiary amine local anaesthetics exist in both positively charged and neutral forms in proportions given by the Henderson Hasselbalch equation [14].

Liposomes mimic biological membranes and constitute valuable tools to study the influence of different agents on the bilayer structure, at molecular level. Liposomes are self-assembling lipid vesicles consisting of one or more lipid bilayers enclosing aqueous compartments.

Chlorophyll has antioxidant properties and antiseptic, antifungal and odor-absorbing qualities; it is also used as a natural coloring agent [15, 16]. This photopigment was successfully used for liposome characterization due to its spectral features [17, 18].

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The present work was aimed at using Chl*a* embedded into liposomes as a sensor to study the changes that occur in the lipid bilayer, by interaction of a local anesthetic (procaine) with liposomal membrane in the presence or absence of α-tocopherol acetate (α-TocAc), a thermal stable antioxidant [19, 20].

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Experimental part

Reagents

Procaine and the lipids: β-stearoyl-γ-oleoyl L-α-phosphatidylcholine (SOPC) and dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma Aldrich (Germany). α-Tocopherol acetate (α-TocAc), KH_2PO_4, Na_2HPO_4 and the organic solvents of analytical purity (chloroform, ethanol, petroleum ether, methanol, n-propanol) were supplied from Merck (Germany).

All solutions were prepared with bidistiled water.

Methods and applied procedures

Chlorophyll a extraction. The chlorophyll *a* (Chl*a*) was extracted from fresh spinach leaves according to the procedure of Strain and Svec [21]. The purity of the Chl*a* samples was checked by recording the absorption spectra.

Liposome preparation. The lipid model membranes were prepared using the thin-film hydration method [22]. The lipids were dissolved in chloroform together with Chl*a* (Chl*a*/lipid molar ratio = 1/100) in a round bottom flask and the solvent was removed in a rotary evaporator (BIOBLOCK SCIENTIFIC – Heildolph 94200, 60–90 rpm). The lipid films were hydrated in a phosphate buffer solution (Na_2HPO_4 - KH_2PO_4), *p*H = 7.2, using few glass beads of 3mm diameter. The multilamellar vesicles obtained in this way were subjected to mechanical stirring (VIBRAX stirrer, 200 rpm) and to ultrasound treatment using a titanium probe sonicator (15 min, Hielser, UP 100 H) above the critical temperature (T_c) of phase transition of lipids, resulting in clear suspensions of small unilamellar vesicles (SUVs). These suspensions were centrifuged (30 min, 20 000 g, SIGMA 2-16 K centrifuge) in order to remove the titanium traces. Only the supernatants were used in experiments. In some samples of liposomes, the vitamin E (as a-TocAc) was incorporated into liposomal membrane during the lipid film preparation in a percent of 2 mol% of phospholipids.

Each liposome system was divided in portions and the anaesthetic was added in the following manner: adequate aliquots of procaine stock solutions (10^{-1} M or 10^{-2} M) were added to 1 mL vesicle suspensions and the final volume of each sample was adjusted to 3 mL with phosphate buffer (*p*H = 7.2). A scale of procaine concentrations ($0 \div 10^{-2}$ M) was obtained.

The absorption spectra were recorded on a PERKIN-ELMER LAMBDA 2S spectrophotometer.

The fluorescence measurements of Chl*a*-liposomes were performed on a PERKIN-ELMER LS55 spectrophotofluorimeter.

Part of fluorescence quenching data (when procaine acted as a quencher of the Chl*a* fluorescence) was analyzed by using the modified Stern-Volmer equation [23]:

$$F_0/(F_0 - F) = 1/(f_a K_Q[Q]) + (1/f_a)$$

where:

F_0 is the fluorescence intensity of Chl*a* in the absence of procaine;

F- the fluorescence intensity of Chl*a* in the presence of procaine;

f_a - the quencher-accessible fraction;

K_Q - the Stern-Volmer quenching constant;

[Q] - the quencher (procaine) concentration.

Results and discussion

By using various concentrations of procaine, added to lipid vesicles, changes in the absorption and the fluorescence spectra of Chl*a* in liposomes were observed.

All the absorption spectra of Chl*a* in liposomes, recorded in the visible region, were normalized at 800 nm.

The addition of the anaesthetic has no effect on the position of the red maximum (668-669 nm), but variations of the optical densities in the Chl*a* absorption spectrum, at the main red peak and also changes in the Soret band (increase/decrease of the optical density values) are notable (figs. 2 ÷ 5).

The variations of the Chl*a* spectral parameters reveal the fact that the local anaesthetic perturbs the structure of the lipid bilayer and increases the lipid mobility. The Chl*a* molecule is sensitive to the increase in the mobility of the lipid moiety, being more mobile in the lipid bilayer. Chl*a* is located in the liposome lipid bilayer with the macrocycle at the interface with the water phase, near the lipid polar heads and the phytol in the hydrophobic lipid chains region [17]. Being more mobile, Chl*a* could be more exposed to the water phase having the possibility to form molecular aggregates, as water adducts or just could be expulsed from the bilayer. In these situations, the absorption of Chl*a* will decrease because its concentration in the bilayer decreases.

The SOPC-membranes present great variations in the optical densities at the main red peak of Chl*a* (fig. 2), comparatively with DMPC-membranes, which do not present significant variations (fig. 3). This fact could be explained by the lipid nature. The unsaturated lipid SOPC forms bilayers with high mobility and high susceptibility to oxidative degradation and to procaine effects. On the contrary, the saturated lipid DMPC forms more stable membranes, being less perturbed by procaine.

Previous studies [24] show that the increase of the procaine concentration results in a large absorption band in the 700-740 nm region in the case of the tocopherol-containing small unilamellar liposomes. As the absorption above 680 nm is characteristic for de Chl*a* aggregates and/or for Chl*a*-water adducts, this spectral modification could

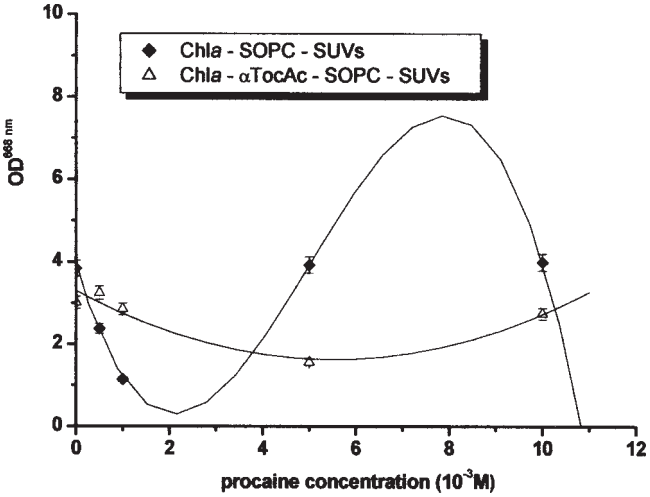

Fig. 2. The normalized optical density values at the main red peak of the Chl*a*-SOPC (0.5 mM) –SUVs (molar ratio Chl*a*: lipid= 1:100) with and without α-TocAc (2 mol% of phospholipids), versus procaine concentration (correlation coefficients: r = 0.99786 Chl*a*-SOPC –SUVs and r = 0.91306 for Chl*a*-α-TocAc- SOPC –SUVs)

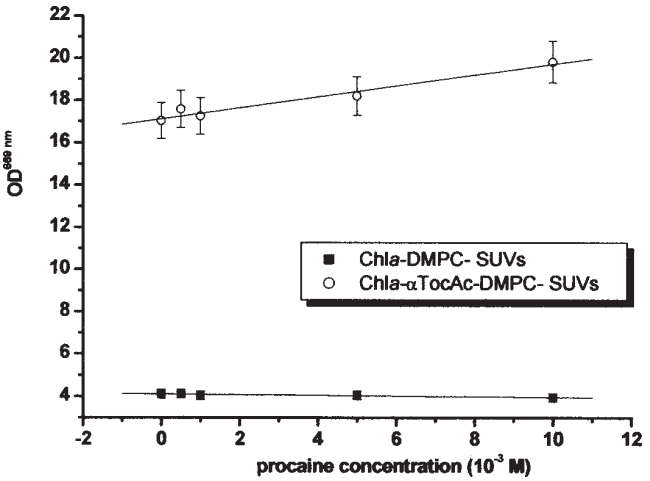

Fig. 3. The normalized optical density values at the main red peak of the Chl*a*-DMPC (0.5 mM)–SUVs (molar ratio Chl*a*: lipid= 1:100) with and without α-TocAc (2 mol% of phospholipids), versus procaine concentration (correlation coefficients: r = 0,98068 for Chl*a*-DMPC–SUVs and r = 0,91617 for Chl*a*-α-TocAc-DMPC-SUVs

be explained by a partial expulsion of Chl*a* from lipid bilayers. The absorption in the wavelength region from 700 to 740 nm reaches a maximum when procaine concentration is close to the lipid concentration ($5 \cdot 10^{-4}$ M). In this case, the concentration of procaine is 100 times larger than the concentration of Chl*a* ($5 \cdot 10^{-6}$ M), so procaine could dislocate Chl*a* from lipid bilayer.

The explanation is the following: in the case of the liposomes with vitamin E, which are smaller [25] and the curvature radius is smaller, the presence of procaine induces higher mobility and disorder and therefore, because of a higher tension in the lipid bilayer, the Chl*a* is partially expulsed in the aqueous medium. Thus, Chl*a* and procaine proved to be competitors in the tocopherol-containing bilayers.

The Soret band absorbance ratio of Chl*a* is another spectral parameter used in monitoring of the size and oxidation of liposomes with Chl*a*.

The variations in the Soret band absorbance ratio values are more pronounced in the tocopherol-containing bilayers (fig.4). This can be related to a high mobility in these cases

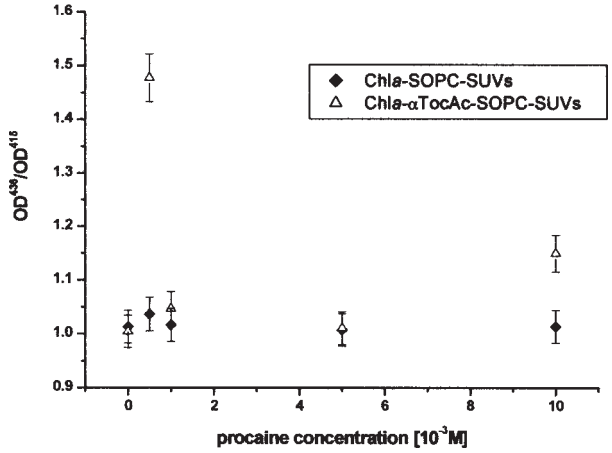

Fig. 4. Soret band absorbance ratio values of the Chl*a*-SOPC (0.5 mM)-SUVs, with and without α-TocAc (2 mol% of phospholipids), (molar ratio Chl*a*: lipid = 1:100) versus procaine concentration

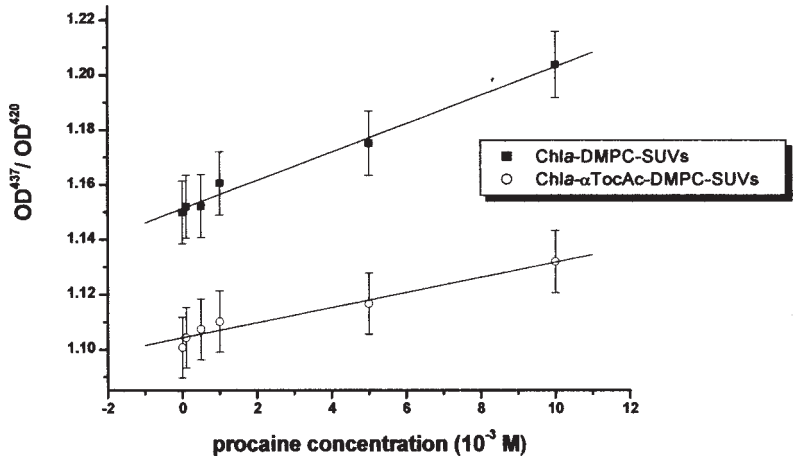

Fig. 5. Soret band absorbance ratio values of the Chl*a*-DMPC (0.5 mM) - SUVs (molar ratio Chl*a*:lipid = 1:100), with and without α-TocAc (2 mol% of phospholipids), versus procaine concentration (correlation coefficients: r = 0.994 for Chl*a*-DMPC–SUVs and r = 0.97763 for Chl*a*-α-TocAc-DMPC-SUVs)

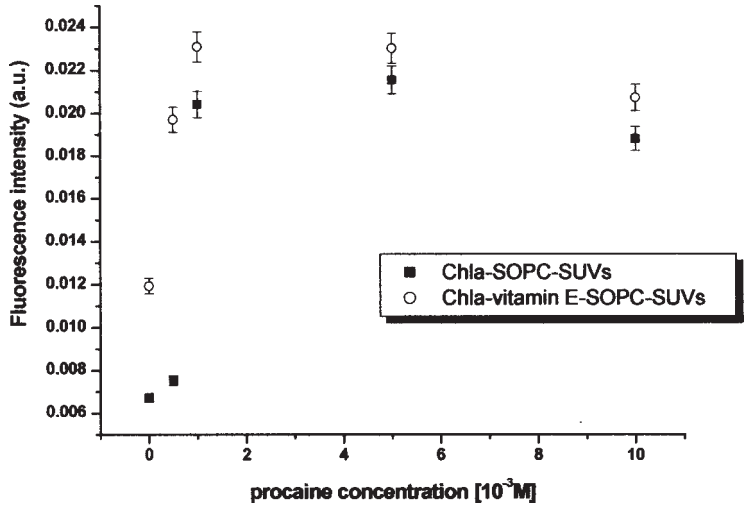

Fig. 6. Fluorescence emission maxima values observed at 685 nm ($\lambda_{excitation}$ = 430 nm) versus procaine concentration for Chl*a*-SOPC (0.5 mM) - SUVs, with and without vitamin E (2 mol% of phospholipids) inserted into lipid membranes (Chl*a*:lipid molar ratio = 1:100)

due to the small dimensions of these liposomes [25]. There is not a regular type dependence on procaine concentration for SOPC liposomes.

Figure 5 shows a linear increase in the Soret band absorbance ratio with the increase of procaine concentration, in the case of DMPC liposomes. This dependence indicates a better resolution in the Soret region of Chl*a* spectrum due to a new arrangement of Chl*a* macrocycle in the polar lipid moiety and procaine is responsible for the lipid changes.

Fluorescence emission spectra of Chl*a* in liposomes have been obtained, by using 430 nm wavelength for excitation. Figures 6 and 7 show an increase of the relative intensity at fluorescence emission maximum for low concentrations of procaine, followed by a decrease of fluorescence intensities for all samples except the system Chl*a*/DMPC (0.5 mM) SUVs.

A possible explanation of this behavior is the following: by increasing the procaine concentration, the mobility in the lipid bilayer is increased and Chl*a* becomes more mobile while the quenching by concentration is diminished. Thus, the relative intensity of fluorescence emission is increasing. At higher concentrations, procaine could perturb the lipid bilayer and penetrate in the lipid bilayer, resulting in a quenching of Chl*a* fluorescence by procaine. Another explanation could be: the higher concentrations of procaine could perturb the lipid bilayer and Chl*a* could be expulsed from the lipid bilayer and this explains the slow decrease of fluorescence.

It has been reported [26] that α-tocopherol acetate, contrary to α-tocopherol, is located in the lipid bilayer in a more hydrophobic position that does not perturb the

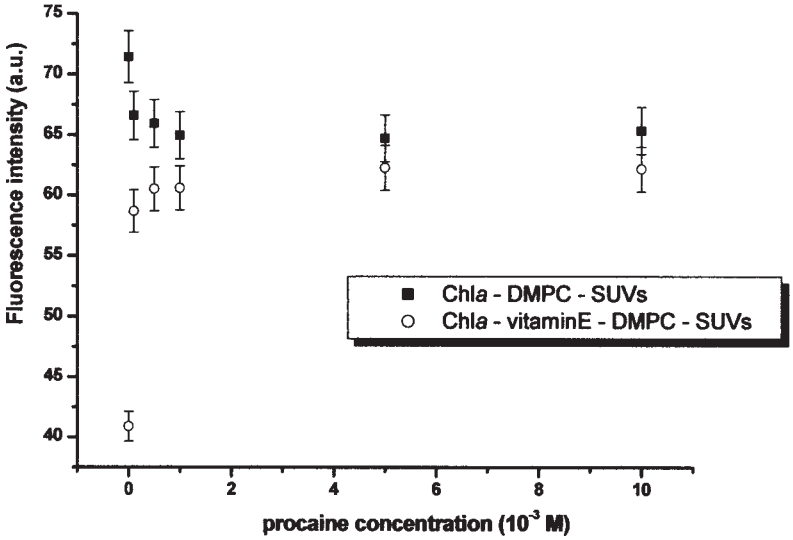

Fig. 7. Fluorescence emission maxima values observed at 685 nm ($\lambda_{excitation}$ = 430 nm) versus procaine concentration for Chl*a*-DMPC (0.5 mM) - SUVs, with and without vitamin E (2 mol% of phospholipids) inserted into lipid membranes (Chl*a*:lipid molar ratio = 1:100)

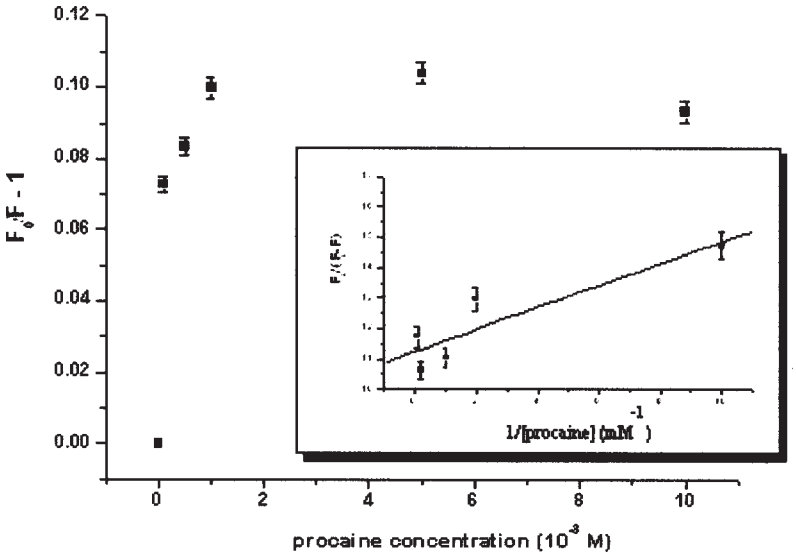

Fig. 8. The quenching by procaine of the fluorescence of Chl*a* embedded into liposome system: Chl*a*/ DMPC (0.5 mM)-SUVs; [Inset: the data have been treated according to the modified Stern-Volmer equation]

interface water/bilayer in saturated phosphatydilcholine membranes. Thus, there is a great probability that α-TocAc will contribute to the partial exclusion of Chl*a* in the aqueous environment.

The system Chl*a*-DMPC (0.5 mM) - SUVs presents a fluorescence quenching, procaine acting as a collisional quencher following the modified Stern-Volmer equation (fig. 8).

For the lipid membranes Chl*a*-DMPC (0.5 mM) SUVs, the quenching parameter f_a was calculated according to the modified Stern-Volmer equation as described in *Materials and applied procedures*. On the basis of this fluorescence quenching parameter, Chl*a* appears to be mostly partitioned in the inner phospholipid monolayer (f_a = 8.88%) of liposomes.

Conclusions

Chlorophyll *a* embedded into liposomal bilayers proved to be a good spectral marker for the liposome characterization and an useful molecular sensor. The variations in the spectral parameters (optical density values at the main red peak; Soret band absorbance ratio; relative fluorescence intensities) revealed modifications of Chl*a* environment in the lipid bilayer reflected in the increase in membrane fluidity caused by procaine treatment.

The results obtained using both absorption and fluorescence spectra of Chl*a* incorporated into different lipid membranes have shown that addition of procaine increases the mobility of membrane and possibly their disruption. The unsaturated lipid SOPC confers high mobility to liposomal bilayers.

The fluorescence data bring more arguments to the fact that procaine and Chl*a* are competitors in the tocopherol-containing bilayers. Further experiments using other experimental techniques, could bring more arguments in the favour of this hypothesis.

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