# Oxidative Stress Simulation on Chlorophyll *a* Lipid Model Membranes - Chemiluminescent Studies

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*Oxidative stress was simulated on small unilamellar liposomes with chlorophyll a as model membranes. Chlorophyll a incorporated in the lipid bilayer of liposomes was used as a spectral marker to monitor the oxidative damage on liposomes. Small unilamellar vesicles were obtained by the thin-film hydration method using the lipids: β-stearoyl-γ-oleoyl L-α-phosphatidylcholine and dimyristoyl phosphatidylcholine. The chemiluminescence assay was used to quantify the oxidative stress in model membranes. The antioxidative effect caused by vitamin E inserted into lipid bilayer of liposomes has been also monitored. Different lipid membranes were tested. The chemiluminiscence studies indicate that the liposomes system with a molar ratio Chla: α-toc: lipid = 1:1:100 exhibit a great increase of antioxidant activity.*

*Keywords: small unilamellar liposomes, lipid model membranes, chlorophyll a, vitamin E, oxidative stress, chemiluminescence*

Oxidative stress is defined as an imbalance between antioxidants and pro-oxidants in favour of the latter resulting in cell damage [1].

Vitamin E is one of the most powerful chain breaking antioxidants which can counteract oxidative membrane degradation [2]. The term vitamin E refers to a family of eight compounds divided into two classes: the tocopherols (α, β, γ and δ, which contain saturated phytol side chains) and tocotrienols (α, β, γ and δ, with three double bonds in the side chain). Of all the tocoferols, α - tocopherols display the most interesting antioxidant properties [3, 4].

Biological membranes have been experimentally modeled using model bilayer membranes called *liposomes*. Liposomes are phospholipidic vesicles that are made up of one or more lipid bilayers surrounding the internal aqueous compartment [5].

The chlorophyll *a* (Chla) is a very "valuable" molecule with antioxidant properties [6]. It was found that the derivatives of chlorophyll a are more effective radical quenchers than those of chlorophyll *b* [7]. The spectral features of Chla (strong visible absorption and fluorescence) allow its use as a sensor for the interactions at molecular level.

The aim of our paper is to study the liposomes stability under oxidative stress conditions using the Chla incorporated into lipid bilayers as a spectral marker. A connection between lipid oxidation (i.e. liposomes oxidative degradation) and Chla oxidation exists [8, 9].

It is known that the oxidative stress affects the cell membranes resulting in the lipid peroxidation reactions in membrane lipids.

The chemiluminescence was used to monitor the liposomes stability against oxidative damage. Two antioxidants were incorporated into liposome membranes: vitamin E (as α-tocopherol acetate) and chlorophyll *a*.

## Experimental part

*Reagents*

The lipids: β-stearoyl-γ-oleoyl L-α-phosphatidylcholine (SOPC) and dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma Aldrich (Germany). α-tocopherol acetate (α-toc acetate), $KH_2PO_4$, $Na_2HPO_4$, luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione), Tris, HCl, $H_2O_2$ and the organic solvents of analytical purity (chloroform, ethanol, petroleum ether, methanol, n-propanol) were supplied from Merck (Germany).

All solutions were prepared with bidistilated water.

As a chemiluminescence (CL) generating system we have used the reaction between $H_2O_2$ ($c = 10^{-5}$ M) and luminol ($10^{-5}$M) in Tris - HCl buffer (pH =8.6) as standard; in a final reaction volum of 1 mL. All CL measurements were performed on the Turner Designs TD 20/20-USA chemiluminometer.

*Methods and procedures applied*

Chlorophyll a extraction. Chla was extracted from fresh spinach leaves according to the Strain and Svec [2] procedure with little modifications. The phytopigments were separated on a powdered sugar chromatographic column, using different organic solvents (petroleum ether, n-propanol and ethanol) as mobile phase. The Chla samples absorption spectra were recorded on a PERKIN-ELMER LAMBDA 2S spectrophotometer (PECSS software) in order to check the purity.

Model membranes preparation. The liposomes were prepared using the thin-film hydration method [3]. A chloroform solution of the lipids was placed in a round-bottomed flask together with Chla (Chla/lipid molar ratio = 1/50 or 1/100). The solvent was removed in a rotary evaporator (BIOBLOCK SCIENTIFIC – Heildolph 94200, 60–90 rpm). Multilamellar vesicles (MLVs) were obtained by the thin-films hydration with a phosphate buffer solution ($NaH_2PO_4$ - $K_2HPO_4$), pH = 7.20, using few glass beads of 3 mm diameter. Small unilamellar vesicles (SUVs) were obtained from MLVs by mechanical dispersion (VIBRAX stirrer, 200 rpm) and bath sonication (BRANSON 1210), above the critical temperature of phase transition ($T_c$) of lipids. An antioxidant (α-toc acetate) was incorporated into some liposomal membranes during the lipid film preparation.

The liposome membranes prepared by hydration method are presented in table 1.

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**Table 1**
THE LIPOSOME MEMBRANES PREPARED

| Sample code | Signification |
|---|---|
| b30 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.25mM) - SUVs (1:2 dilution of b31) |
| b31 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.25mM) - SUVs |
| b32 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.5mM) - SUVs |
| b33 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.5mM) - SUVs(1:2 dilution of b32) |
| b10 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.25mM) - vitamin E (1 mol% of phospholipids)- SUVs (1:2 dilution of b11) |
| b11 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.25mM) - vitamin E (1 mol% of phospholipids)- SUVs |
| b12 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.5mM) - vitamin E (2 mol% of phospholipids)- SUVs |
| b13 | Chla (Chla/lipid molar ratio = 1/100) - DMPC (0.5mM) - vitamin E (2 mol% of phospholipids)- SUVs (1:2 dilution of b12) |
| b01 | Chla (Chla/lipid molar ratio = 1/50) - DMPC (1mM)- vitamin E (2 mol% of phospholipids)- SUVs |
| b02 | Chla (Chla/lipid molar ratio = 1/50) - DMPC (1mM) - vitamin E (2 mol% of phospholipids)- SUVs (1:2 dilution of b01) |
| b40 | Chla (Chla/lipid molar ratio = 1/100) – SOPC (1mM) - SUVs |
| b41 | Chla (Chla/lipid molar ratio = 1/100) – SOPC (1mM) – SUVs (1:2 dilution of b40) |
| b20 | Chla (Chla/lipid molar ratio = 1/100) – vitamin E (1 mol% of phospholipids) - SOPC (1mM) - SUVs |
| b21 | Chla (Chla/lipid molar ratio = 1/100) – vitamin E (1 mol% of phospholipids) - SOPC (1mM) – SUVs (1:2 dilution of b20) |

Calculation method of antioxidant activity (AA)

Time dependence of chemiluminescence intensity, I = f(t) allows to determine some process characteristics of which the most relevant is antioxidant activity [10]. The antioxidant activity was calculated using the relation:

$$\%AA = \frac{I_0 - I_s}{I_0} \cdot 100$$

where

$I_0$ represents the maximum CL for standard at t = 5s

$I_s$ the maximum CL for sample at t = 5s

The standard is the reaction mixture without the sample (the liposomes).

Calculation of the bleaching value

$$bleaching\ \% = [(OD_0\text{-}OD_i)/\ OD_0]\cdot 100,$$

where $OD_0$ is the absorbance at the main red peak for the unsonicated liposomes and $OD_i$ is the absorbance at the main red peak for the sonicated liposomes at different times.

The degradation by oxidation of Chla incorporated into lipid bilayer, is conducting to an increase in the bleaching values.

## Results and discussions

The oxidative stress was simulated on lipid model membranes using the luminol chemiluminescence. The oxidative degradation of luminol by $H_2O_2$ in alkaline buffer results in production of a large range of intermediate free radicals of oxygen. Different types of lipid vesicles were studied. A lower intensity of CL signal demonstrates a higher stability against oxidative stress.

The presence of the vitamin E reduced the CL intensity in Chla - DMPC (0.25mM) - liposomes and the %AA increase with 15% (fig.1). The effect of sample dilution results in the increasing of the CL signal.

The VIS spectra were recorded to observe the Chla oxidation and, therefore, the liposomes degradation.

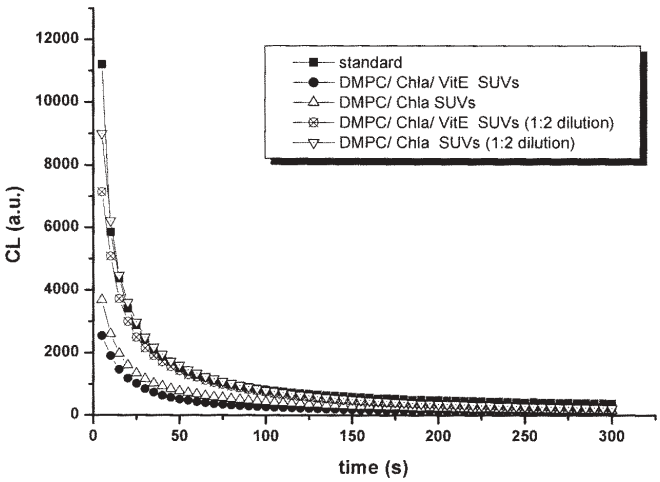


Fig.1. The CL intensities of DMPC (0.25 mM) - Chla- liposomes with and without α-toc acetate (1 mol % of phospholipids final concentration)< (Chla/lipid molar ratio=1/100)

The time evolution of the bleaching parameter for this type of liposomes is presented in figure 2. This parameter has lower values for the Chla-vitamin E- DMPC- SUVs as compared with Chla- DMPC- SUVs. This fact suggests that tocopherol- containing lipid bilayers are more stable in time.

The Chla- DMPC (0.5 mM)- lipid vesicles proved a prooxidant character. The samples dilution results in an increase in CL signal. The liposomes containing α-toc acetate (2 mol% of phospholipids final concentration) showed a diminution of the CL intensity (fig.3); therefore these systems are more stable.

The behavior of the Chla- SOPC- liposomes at lipid peroxidation is showed in figure 4. The sample dilution determined the increase of the CL signal. The tocopherol-containing bilayers have lower CL intensities.

Therefore, the liposomes stability against oxidative stress is higher in the case of the Chla -vitamin E – SOPC systems. This fact is indicated also in figure 5. The optical densities at the main red peak of the Chla incorporated into liposome

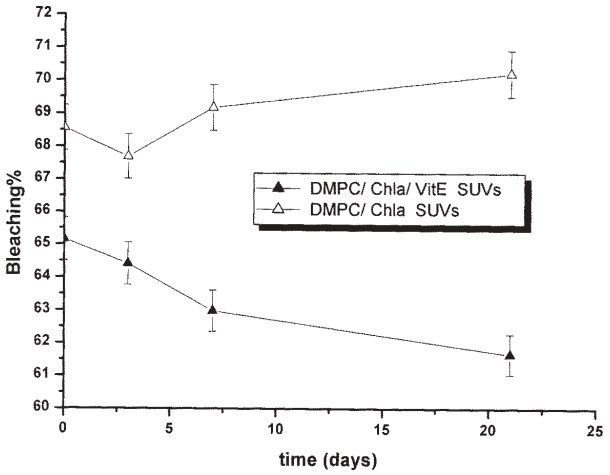


Fig.2. The time evolution of the bleaching values of the DMPC (0.25 mM)- Chla- liposomes with and without α-toc acetate (1 mol % of phospholipids final concentration); Chla/lipid molar ratio = 1/100

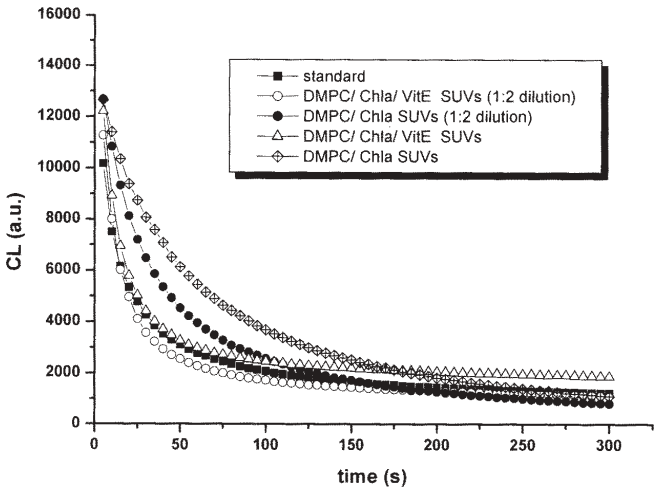


Fig.3. The CL intensities of DMPC (0.5 mM) - Chla- liposomes with and without α-toc acetate (2 mol % of phospholipids final concentration); Chla/lipid molar ratio = 1/100

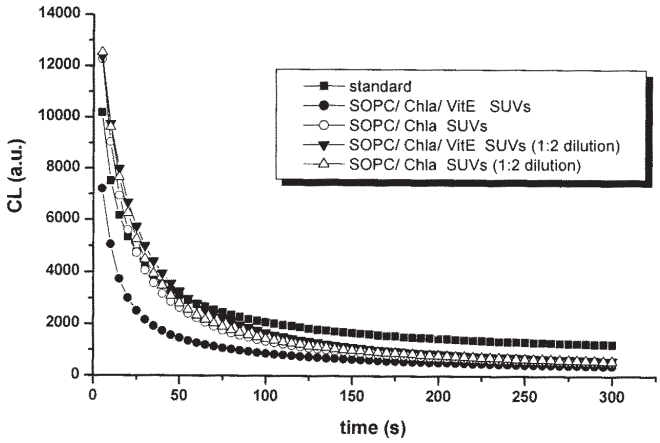


Fig.4. The CL intensities of SOPC (1 mM)- Chla- liposomes with and without α-toc acetate (1 mol % of phospholipids final concentration); Chla/lipid molar ratio = 1/100

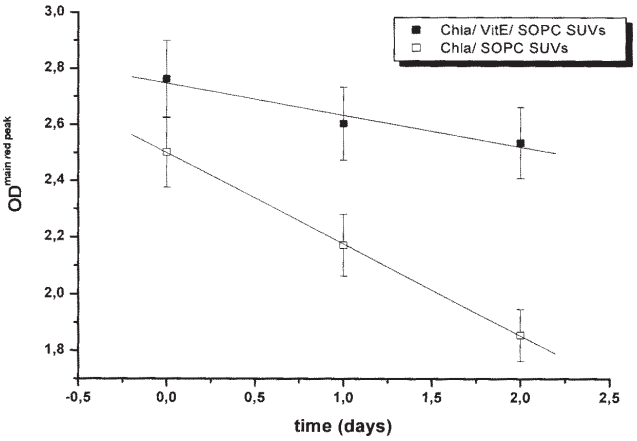


Fig.5. The time evolution of the main red peak optical densities for the Chla - SOPC (1 mM) liposomes and Chla - α-toc acetate (1 mol % of phospholipids) - SOPC (1 mM) liposomes (Chla/lipid molar ratio = 1/100)

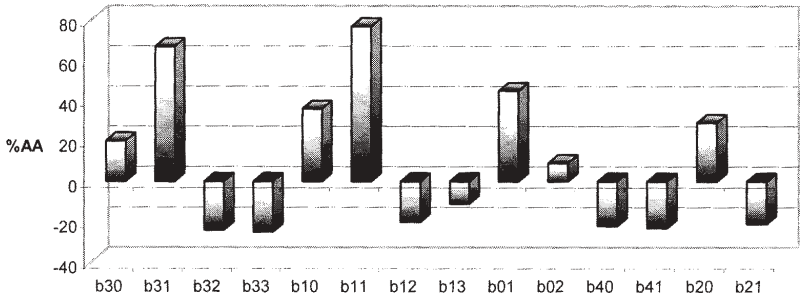


Fig.6. Antioxidant activity of different model membranes (signification of the sample cod is presented in table 1)

membrane decrease significantly in the SOPC- systems without vitamin E. So, the Chla degradation (and also the liposomes degradation) is higher in this case.

Generally, the antioxidant activity decreases with the sample dilution (fig. 6).

The tocopherol- containing bilayers have a higher antioxidant activity. An increase in lipid concentration is conducting to a prooxidant character. The Chla –DMPC – liposomes are more stable than Chla – SOPC – liposomes, the latters having unsaturated fatty acids which are more susceptible to oxidation than the DMPC system which contains only saturated compounds. The liposomes system with a molar ratio Chla: α-toc: lipid = 1:1:100 have strong antioxidative properties.

## Conclusions

The insertion of vitamin E into lipid bilayers played a protective role against oxidative damage on liposomes. The presence of the α-toc acetate reduced the amplitude of the chemiluminescence intensity. Chlorophyll *a* incorporated into liposomal membranes was used as a spectral marker to monitor the oxidative effects on liposomes. Under oxidative stress conditions, the model membranes with the molar ratio Chla:α-toc: lipid = 1:1:100 exhibit higher stability.

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Manuscript received: 17.09.2007