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CHLOROPHYLL *a* – LABELLED ARTIFICIAL LIPID MEMBRANES EXPOSED TO PHOTO-OXIDATIVE STRESS. SPECTRAL STUDIES

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Abstract. The chlorophyll stability in biomimetic environment is the precondition for its potential application in various fields of science and technology. Its unique role, based on the specific and very diverse chemical reactivity, justifies its use in industrial technology, pharmaceutical, food and cosmetic industry, but also in medicine.

In this work, two types of liposomes were prepared from saturated (dipalmitoyl phosphatidylcholine) and unsaturated (PHOSPHOLIPON 90®) lipids. The artificial lipid membranes were labeled with a natural photopigment – chlorophyll *a* (Chl*a*).

The stability of Chl*a*-based liposomes to *in vitro* induced oxidative stress by visible and UVB irradiation was examined by UV-Vis absorption spectra, and by monitoring two kind of lipid peroxidation biomarkers (conjugated dienes and TBA-MDA assay) which were quantified in order to evaluate the photodamage degree of artificial lipid membranes. Chl*a* proved to be an effective spectral sensor to detect any damages induced by visible light and UVB irradiation on liposomes. After applied Vis/UVB radiation, Chl*a*-liposomes suffered photodegradation which was more pronounced under UVB stress. The investigations demonstrated a closely connection between lipid peroxidation and chlorophyll degradation. The deterioration was higher in the case of biomimetic membranes made of unsaturated lipids exposed to photo-oxidative stress because of presence of double bonds in lipid molecules that decisively contribute to the formation of lipid radicals.

Based on these results it can be concluded that the chlorophyll behavior mechanism under the photo-oxidative stress is of crucial importance for further bio-applications.

Key words: Chlorophyll *a*, liposomes, photo-oxidative stress, UVB, lipid peroxidation biomarkers.

1. INTRODUCTION

Oxidative stress (OS) is defined as a "damage" which is caused by an imbalance between the overproduction of reactive oxygen species (ROS) and insufficient activity of antioxidant defense mechanisms [1]. ROS species damage the cell membranes, and react with the biomolecules (lipids, proteins, enzymes, carbohydrates and DNA) leading to dramatic biological impact. It should be noted that OS is universal phenomenon that affects not only people but also the plant metabolism that is even more vulnerable to this stress due to frequent changes in the environment. One of the OS consequences is chlorophyll (Chl) degradation which is mostly present during the autumn. According to its importance, Chl belongs to one of the most important bio-organic compounds primarily because of its central role in the process of *photosynthesis* – one of the most important global physical-chemical processes on the planet. The unique role of Chl is based on its specific and very diverse chemical reactivity which provide it applications in industrial technologies (*e.g.* in food, pharmaceutical and cosmetic industry), but also in medicine, as drugs in photodynamic therapy (PDT) for the treatment of certain types of cancer [2]. Perhaps one of the greatest potentials of Chl is due to its low toxicity in cancer prevention [3–5]. In food industry is usually used as a safe dye and it's declared as a food additive – E140 [6]. In the cosmetics industry, Chl is used as a leader's pigment, in production process of soaps, oils, fats, waxes, garments, canned liquid and as a deodorant [7–9].

Very often, Chl is used as incorporated into liposomes – which are lipid vesicles providing many advantages in terms of delivering bioactive components to a target site. Chl*a* inserted in biomimetic membranes acts as a sensor to detect modifications in the artificial lipid bilayers and to detect the possible interactions into the membranes at molecular level [10–12]. Chl-liposomes are also very popular for clinical sentinel lymph nodes (SLN) mapping due to the low toxicity, and brighter NIR fluorescence than the Chl alone [13].

Despite its sensitivity to light and oxygen, chlorophyll is considered a safe widely available and cost-effective photopigment, with good antioxidant and antimicrobial properties, and biocompatibility if used in biomedical applications [14–15]. For these reasons, a great importance for its further potential use has the knowledge of factors affecting the Chl stability, which can be internal (*i.e.* inherent in the system in which it is located), but also external (light exposure, temperature, various contaminants, the method of storage, or various enzymes may result in the Chl degradation). Thus, **the primary goal of this work** was to monitor the stability of Chl*a* –loaded small unilamellar liposomes (SUVs) under the oxidative stress induced by visible light and UVB irradiation. SUVs are nano-sized lipid vesicles made of one bilayer surrounding an aqueous compartment, structure very similar to that of natural biomembranes. Their structure confers them some advantages such as biocompatibility and the possibility of effective encapsulation of the active compounds. Incorporation of bio-active substances in liposomes is a challenge by itself as well as obtaining

satisfactory stability of those kinds of systems, and usually cannot be proved by direct methods. That's, the encapsulation of Chl*a* (used as a spectral marker) in liposomes prepared by thin hydration film is proven indirectly using absorption spectroscopy, as described in previous investigations [11, 12, 16]. By Chl*a* incorporation in lipid bilayers, the Chl*a* solubility will be increased [16–18], since its insolubility in aqueous medium. For that reason, the studies on Chl stability under the influence of photo-oxidative stress in biomimetic media (liposomes) may serve as useful tool for further research and applications of such systems in industrial, technological or biomedical field. As known, the electromagnetic radiation from the Sun reaching the Earth's atmosphere is composed primarily of visible, infrared and ultraviolet light. However, the largest part of the solar spectrum of radiation that reaches the Earth's surface belongs to visible light (about 90%). In the last years, the intensification of ozone depletion events resulted in more UVB (280–315 nm) rays reaching the Earth's surface, with dramatic consequences on living organisms. Some studies reported the negative effects of UVB rays on Chl [19–21]. **The second goal of this study** was to investigate the influence of lipid saturation on Chl*a*-liposomes stability to photo-oxidative stress (photo-OS), so the experiments were done on two types of lipids, DPPC (saturated) and PL-90 (unsaturated).

2. MATERIALS AND METHODS

2.1. MATERIALS

The saturated lipid dipalmitoyl phosphatidylcholine (DPPC, T_c = 41.4–41.7°C) was purchased from Sigma Aldrich (Germany) and used without further purification. PHOSPHOLIPON 90® (PL-90, a lipid mixture containing: 98% phosphatidylcholine, 2.1% lysophosphatidylcholine, 0.17% tocopherol; fatty acid composition of these lipids is following: palmitic acid 12%, stearic acid 3%, oleic acid 3%, 5% linolenic, linoleic 66%) was purchased from Nattermann Phospholipid GMBH, Germany. The phosphate buffer solution, PB (NaH_2PO_4-K_2HPO_4, pH 7.4) was prepared using analytical grade reagents (Centrohem, Serbia), and purified water from a Millipore Milli-Q system (conductivity $\leq 0.1\ \mu S \cdot cm^{-1}$).

2.2. CHLOROPHYLL *a* ISOLATION AND PURIFICATION

Chl*a* was isolated from fresh leaves of spinach (*Spinacia oleracea* L.) by slightly modified Svec method [22–23]. The extraction was carried out with organic solvent mixture of methanol and petroleum ether in a volume ratio of 2:1 (v/v). Re-extraction was carried out by mixture of petroleum ether and diethyl ether (1:1, v/v). The obtained pigment extract contained large amounts of various chlorophyll forms as well as accessory pigments (carotenoids and xanthophylls). In

order to isolate pure Chl*a* fraction, column chromatography with silica gel as adsorbent (silica gel 60, Merck, 0.063–0.200 mm) and n-hexane/acetone solvent mixture as eluent, was applied [23]. Chl fraction, that contained mostly Chl*a* with 20% of chlorophyll *b*, was eluted at eluent composition of 1:0.1 (n-hexane/acetone, v/v). The purity of isolated Chl*a* was checked by HPLC – High Performance Liquid Chromatography (Agilent 1100 Series, Waldborn, Germany) using a modified Scholz & Ballschmiter method (1981) [24]. Isolated and purified Chl*a* was firstly dissolved in methanol, and then water was added to the final volume ratio of 1/10. The final Chl*a* concentration in the aqueous medium was set to 5×10^{-6} M. The solvents were purchased from Sigma-Aldrich GmbH, Germany.

2.3. PREPARATION OF CHLOROPHYLL *a*-LABELED ARTIFICIAL LIPID MEMBRANES

Liposomes (*artificial lipid membranes*) with incorporated Chls in the lipid bilayers were prepared according to the thin-film hydration method as previously reported [12, 16]. The lipids were dissolved in chloroform (J.T. Baker) together with Chl*a* in a lipid/pigment molar ratio of 1/100. The Chl*a* concentration was 5×10^{-6} M, while lipid concentration was 5×10^{-4} M, considering the final PB buffer volume of liposome suspension. All the operations have been performed above the critical temperature of lipid transition in order to avoid membrane defects. The liposome suspensions were mostly composed of multilamellar vesicles (MLV). In order to obtain small unilamellar vesicles (SUV), the suspensions were subjected to mechanical stirring on shaker (HI 190M, Hanna instruments, Italy) at a speed of 500 rpm, and then to extrusion (LiposoFast-Basic Extruder, Avestin, Inc. Canada) with filters of 100 nm diameter. After preparation, the resulted liposomes: PL-90-SUVs and DPPC-SUVs were stored at 4°C.

2.4. IRRADIATION OF THE SAMPLES

UVB irradiation. Continuous irradiation of Chl*a* in water and Chl*a* incorporated in liposomes was performed in a cylindrical photochemical reactor "Rayonnet", with 8 symmetrically placed lamps having emission maxima at 300 nm. The samples were irradiated in quartz cells ($1 \times 1 \times 4.5$ cm^3) placed on the rotating circular holder. The total measured energy flux (hitting the samples) was about 15.0 Wm^{-2}. Measurements of the energy flux are performed using a UV meter SONARMETER SM 8.0, "Solartech" Inc.

Visible irradiation. The irradiation with visible light was carried out continuously in a cylindrical photochemical reactor with LED lamps (Light Emitted Diodes – light color "Pure White"), which are symmetrically arranged relative to the center of the reactor in which is placed the sample cuvette. The total measured energy flux to the samples received in this way was about 14.0 Wm^{-2}.

Measurements of the energy flux are performed using a VICTOR 1010A – Digital Lux Meter.

2.5. CHARACTERIZATION METHODS

The absorption spectra of the samples were recorded on a double beam Varian Cary-100 spectrophotometer (equipped with 1.0 cm quartz cells), in the wavelength range of 200–800 nm with 1.0 bandwidth. All spectral data have been processed by using Origin 8.0. Software.

Determination of chlorophyll degradation after irradiation exposure. Photo-degradation of Chl*a* was estimated as a percentage *bleaching* or *degradation value* (DV_{Chl}%) of this photopigment, by using the mathematical expression:

$$DV_{Chl} = (A_i - A_0) / A_0 \times 100,$$

where: A_0 is the absorbance at the main red peak of Chl*a* in nonirradiated samples, and A_i is the absorbance at the main red peak for Chl*a* in irradiated liposomes at different exposure times. The photo-degradation of Chl*a*, incorporated into artificial lipid membranes will conduct to an increase in *bleaching* values [12].

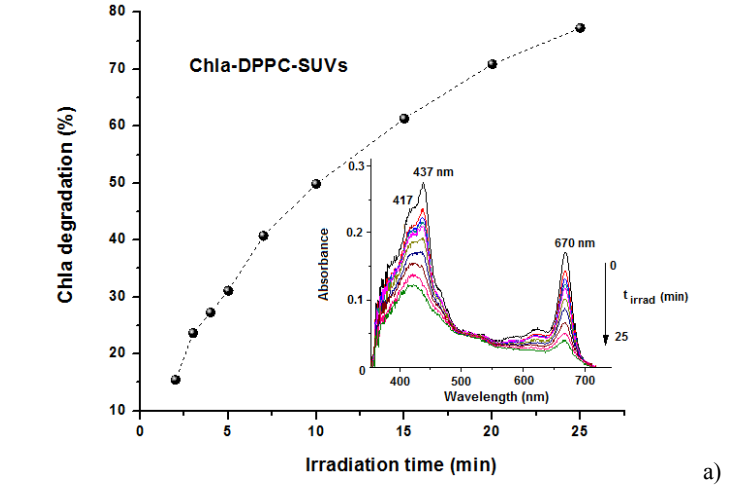
In order to detect the lipid oxidation in lipid vesicles, two methods were performed:

1) *Conjugated Diene test*. The conjugated dienes are monitored by absorbance measuring at 234 nm; the absorbance values were corrected for the contribution of light scattering [25].

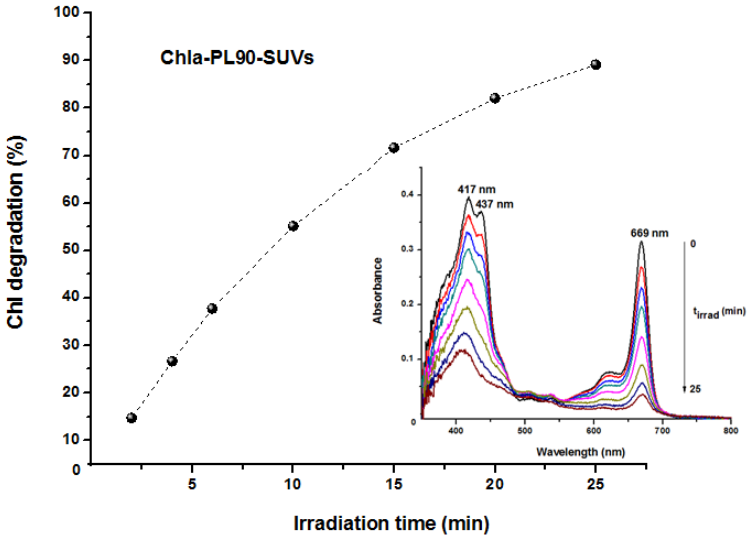
2) *TBA–MDA assay.* This method is based on the reaction of malondialdehyde (MDA – a secondary product of lipid peroxidation) with thiobarbituric acid (TBA), whereby a red-colored complex with a maximum absorption at 532 nm is obtained. Parallel to the prepared liposomes with incorporated Chl*a* (sample), are prepared empty (control) liposomes by the same method. TBA-MDA test is carried out in three cuvettes simultaneously in the following way: cuvette "sample" and "control" contained 0.5 mL of liposomes with incorporated Chl*a*. The cuvette "blank" contained 0.5 mL "empty" liposomes. Peroxidation was initiated by continuous UVB-irradiation and visible light irradiation during increasing times. Only the cuvette "sample" was exposed to irradiation. Thereafter, to the cuvettes "sample", "blank" and "control" was added 1 mL of an aqueous solution of TCA (5.5%) and 0.5 mL of BHT methanol solution to stop further peroxidation. Then, 0.5 mL of TBA solution (4.2×10^{-2} M solution in 5×10^{-2} M NaOH) was added to all three cuvettes. The mixture was incubated for 10 min at 50 °C in dark and then centrifuged (Eppendorf AG 22331 Hamburg, Germany) for 5 min at 13000 rpm. The Vis spectrum of the TBA–MDA complex was subsequently recorded from 400 to 800 nm. The absorbance of the complex in the supernatant was read at 532 nm. The chemicals: TBA, TCA, BHT, NaOH were supplied by Sigma-Aldrich, GmbH, Germany.

3. RESULTS AND DISCUSSIONS

In this work, Chl*a* embedded into liposomes was used as a spectral marker to monitor the photo-oxidative damage of artificial membranes. Degradation of Chl*a* (inserted in DPPC and in PL-90 liposomes), under UVB and Vis light irradiation, at different exposure times, is depicted in Fig. 1, and Fig. 2, respectively. The figure insets display the changes in the absorption spectra of Chl*a* incorporated in artificial lipid bilayers, during irradiation.



a)



b)

Fig. 1 – Degradation of Chl*a* in DPPC-SUVs (a), PL-90-SUVs (b), under UVB irradiation at different exposure times.

It could be observed the Chl*a* spectral fingerprint consisting of two main absorption bands: the Soret band (in the blue region of the electromagnetic spectrum) and a sharp peak in the red region. Both irradiation types resulted in strong reduction in these Chl*a* characteristic red and blue absorption bands, but this decrease was more pronounced in UVB-stressed samples.

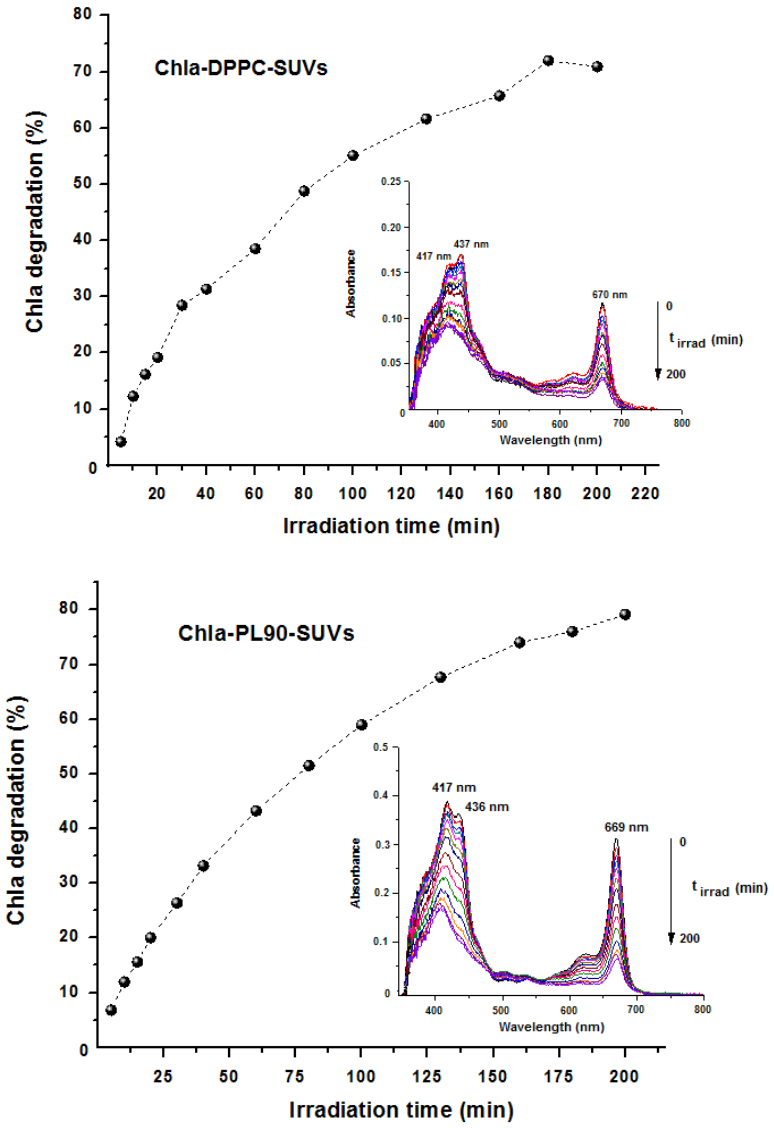


Fig. 2 – Degradation rate of Chl*a* -SUV DPPC (a) and PL-90 (b) liposomes under visible light irradiation at different exposure times.

After 25 minutes of UVB exposure (Fig. 1), the level of Chl*a* degradation was higher in PL-90-SUVs (DV_{Chla} = 89%) than in DPPC-SUVs (DV_{Chla} = 76%).

It is required longer exposure time (200 minutes) to Vis light (Fig. 2) to get the approximately the same levels of Chl*a* photodegradation (DV_{Chla} reached the value of 71% for DPPC-SUVs and 80% for PL-90 SUVs after Vis irradiation) as in the case of UVB irradiation, which demonstrating the most potent harmful effects of UVB rays. These irradiation periods were maintained the same in our further experiments.

In both kinds of irradiation, the bleaching of this porphyrin, and also the changes in the relationship of the Soret band absorbance values ($A^{437nm}/A^{417\ nm}$) were noticeably smaller in the case of DPPC liposomes compared to PL-90 liposomes, indicating that the lipid oxidation process was more significant in the case of biomimetic membranes made of unsaturated lipids (PL-90 SUVs).

Different behavior exhibited by the two types of Chl*a*-liposomes to photo-oxidative stress is due to the differences in the chemical structure of the lipid molecules, the unsaturated one (PL90) being more vulnerable to OS.

These findings were further confirmed by lipid oxidation measurements. Lipid peroxidation (LP) is a key chemical process occurred in the oxidative stress leading to many pathological conditions [26]. As lipid peroxidation biomarkers, *conjugated dienes* and *malonyldialdehyde* (MDA) were monitored to evaluate the photo-oxidative stress damage of biomimetic membranes. As known, the conjugated double bonds (conjugated dienes) have a strong absorption at 234 nm. It could be observed that the value of the absorbance at 234 nm ($A^{234\ nm}$) increased with irradiation time (Fig. 3), especially in the case of unsaturated PL90 liposomes because of the presence of double bonds which are vulnerable to free radical attack. For Chl*a*-PL90-SUVs, the increase in $A^{234\ nm}$ was exponential when UVB-stressed, and linear under Vis irradiation, indicating the high level of degradation in the first case.

Photo-oxidative stress gave rise to different shapes for the curves of accumulation of the resultant conjugated dienes (Fig. 3). So, UVB-stress conditions lead to exponential curves (absorbance at 234 nm, A^{234}, *vs.* irradiation time, *t*) following equations: $A^{234}(t) = 157.4 \cdot \exp(-0.5 \cdot ((t - 196.1)/56)^2) - 0.35$ for Chl*a*-PL90-SUVs, and $A^{234}(t) = 1988 \cdot \exp(-0.5 \cdot ((t - 19.4)/1240)^2) - 1987.8$ for Chl*a*-DPPC-SUVs (Fig. 3a). On the contrary, under Vis light radiation, the samples exhibited linear increase (Fig. 3b) with the equations: $A^{234}(t) = 8.3 \cdot 10^{-3} \cdot t +$ + 0.024 (Chl*a*-PL90-SUVs) and $A^{234}(t) = 1.35 \cdot 10^{-4} \cdot t + 0.01$ (Chl*a*-DPPC-SUVs), the last systems exhibiting lower slope, so they are more stable to photo-OS due to their saturated lipid components.

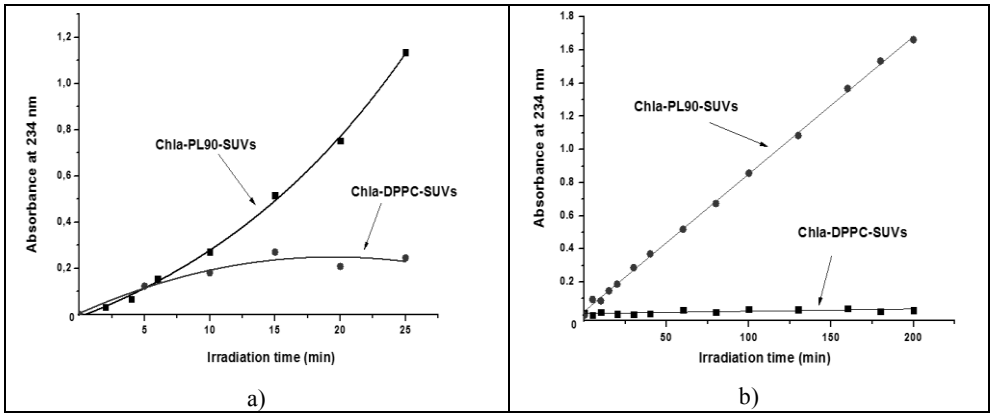


Fig. 3 – Conjugated diene test for Chl*a*-DPPC-SUVs and Chl*a*-PL90-SUVs under UVB (a) and Vis (b) irradiation.

The second marker of free radicals, TBA-MDA test (Fig. 4), consists of the reaction of thiobarbituric acid with malondialdehyde ($C_3H_4O_2$) through an aldol condensation:

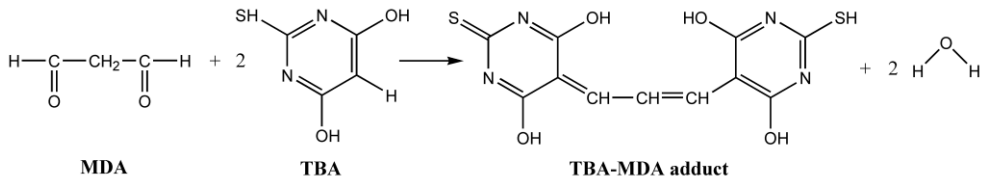


Fig. 4 – Reaction between MDA and TBA.

The resulted TBA-MDA adduct can be easily quantified spectrophotometrically by monitoring the absorbance at 532 nm. A change in the amount of the generated TBA-MDA complex is a direct result of alteration in structure of lipids used for the preparation of liposomes. The absorbance at 532 nm is directly proportional to the concentration of MDA, a secondary product resulted in LP of artificial lipid membranes. It has been demonstrated that increasing the number of double bonds in the fatty acid chains of lipids leads to a proportional increase in lipid radicals when a photosensitizer is chosen for LP initiation [27–30]. Due to the fact that the oxidation process acts on the Chl*a* molecules as well as on lipids, the absorbance at 532 nm was simultaneously measured for Chl*a*-based liposomes that have not been subjected to irradiation (Control) and those which are Vis/UVB irradiated, (Sample). Initiation of lipid peroxidation was done by continuous UVB and Vis irradiation of Chl*a*-DPPC-SUVs and Chl*a*-PL90-SUVs, for 25 and 200

minutes respectively, in order to reach approximately the same level of DV_{Chla} in both cases. For propagation of lipid peroxidation, a key factor is long hydrocarbon chain in lipid molecules, while their polar "heads" are completely irrelevant. Because its unsaturated structure, the liposomal PL90 undergone strong oxidation after subjecting to photo-OS. Thus, the MDA level in Chl*a*-PL90-SUVs was 2.8 times greater under UVB stress, and 3.4 times higher under Vis irradiation, than in Chl*a*-DPPC-SUVs (Fig. 5). The powerful destructive effect of UVB rays on artificial cell membranes was observed. Despite short time of UVB exposure, the MDA level for both liposomes was about 2 times greater in the case of UVB as compared to Vis irradiation.

Based on our findings and starting from previous reports [31–34], we proposed the following possible oxidation mechanism when irradiating the Chl*a*-liposomes:

$$\mathrm{Chl} \xrightarrow{h\nu} {}^{1}\mathrm{Chl}^{*} \quad (1)$$

$${}^{1}\mathrm{Chl}^{*} \rightarrow {}^{3}\mathrm{Chl} \quad (2)$$

$${}^{3}\mathrm{Chl} + {}^{3}\mathrm{O_2} \rightarrow \mathrm{Chl} + {}^{1}\mathrm{O_2} \quad (3)$$

$$\mathrm{H_2O} \xrightarrow{h\nu} \tfrac{1}{2}\,\mathrm{H_2} + \mathrm{HO}\cdot \quad (4)$$

$$\mathrm{LH} + \mathrm{HO}\cdot \rightarrow \mathrm{L}\cdot + \mathrm{H_2O} \quad (5)$$

$${}^{1}\mathrm{O_2} + \mathrm{L}\cdot \rightarrow \mathrm{LOO}\cdot \quad (6)$$

$$\mathrm{LOO}\cdot + \mathrm{LH} \rightarrow \mathrm{LOOH} + \mathrm{L}\cdot \quad (7)$$

$$\mathrm{LOOH} \rightarrow \ldots \rightarrow \mathrm{MDA} \quad (8)$$

Chl*a* is absorbing light and forms a singlet excited state ($^{1}Chl^{*}$), then through an inner-system crossing from the initially formed $^{1}Chl^{*}$ resulted the triplet state ^{3}Chl which further quenched by ground state oxygen ($^{3}O_2$) generating singlet oxygen ($^{1}O_2$) (pathways 1–3). The irradiation events in aqueous media give rise to a large variety of free radicals including OH• (hydroxyl radicals) which are the most dangerous (pathways 4). Being highly reactive, these free radicals attack the liposomal phospholipid molecules (especially at carbon-carbon double bond(s)), by hydrogen abstraction, resulting in phospholipid radicals (L·) that by molecular rearrangement and by $^{1}O_2$ uptake give rise to conjugated dienic lipid hydroperoxide (LOOH) (pathways 5–6). The LP propagation generates damage to many lipid molecules (pathway 7). Because LOOH are unstable, they easily decomposed with formation of shortened reactive products, like aldehydes including malondialdehyde (pathway 8).

All the lipid oxidation products are very reactive and may indirectly contribute to Chl*a* degradation. Thus, through this complex mechanism Chl*a* detected not only deterioration of the biomimetic membranes, but also its own destruction.

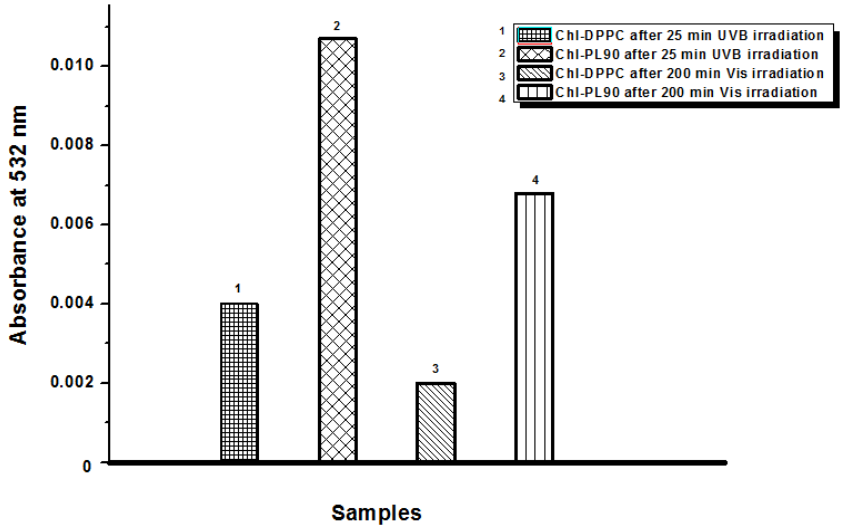


Fig. 5 – Comparatively results of TBA-MDA test for Chl*a*-DPPC-SUVs and Chl*a*-PL90-SUVs under UVB or Vis irradiation.

4. CONCLUSIONS

In this study, Chl*a* inserted in two types of liposomes (made of saturated and unsaturated lipids) was used as a spectral sensor to detect any change in artificial lipid bilayers under photo-oxidative stress. For all liposomes, a strong hypochromic effect of the red and blue bands characteristic for Chl*a* occurred under UVB and Vis light irradiation. The oxidation process was noticeably higher in the case of unsaturated PL90 liposomes due to the presence of double bonds in the lipids structure, which are the targets to free radical attack, resulting in lipid radical formation which initiate the chain propagation of lipid peroxidation. Therefore, Chl*a* incorporated into PL90-liposomes showed a much higher degree of photodegradation. Chl*a*-DPPC-SUVs, which are made of saturated lipids, are more stable to photo-oxidative stress, so these liposomes undergone low level of degradation process, especially when Vis light irradiated. Accumulation of the resultant conjugated dienes lead to different shapes for the photodegradation curves of both liposomal systems.

The powerful deteriorative effect of UVB rays as compared to Vis light irradiation was highlighted by the long exposure time of samples to Vis light to reach the approximately the same values of Chl*a* bleaching under UVB-stressed. A strong correlation between lipid oxidation and degradation of chlorophyll has been observed. The data provided from Chl*a* absorption spectra are in good agreement with the results obtained by monitoring the level of lipid peroxidation biomarkers.

Taking into account all these findings, we can conclude that Chl*a* is sensing not only the deterioration of lipid molecules, but also its own degradation. From the results presented in this study it can be concluded that a more accurate knowledge of chlorophyll behavior mechanism under the influence of various oxidative stress, as well as its stability in the potential formulations, is crucial for its further use.

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