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Spectral Characterization of Model Systems Containing Lipids and Chlorophyll

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The objective of the present work was to prove that the spectral features of chlorophyll a *allow its use as a sensor for physical properties of liposomes. For this purpose, UV-VIS absorption, fluorescence emission and dynamic light scattering measurements were recorded and analyzed comparatively. Multilamellar and unilamellar vesicles with Chl*a *have been prepared by a mechanical dispersion method and a further sonication treatment for obtaining unilamellar vesicles. The Chl*a *– liposomes were prepared using an appropriate molar ratio and three types of lipids. It was confirmed that Chl*a *– based model system can be used to assess specific molecular interactions in the liposome bilayer.*

Keywords Absorption and emission spectroscopy; Chl*a*; liposomes; thin-film hydration method

1. Introduction

Liposomes are spherical vesicles formed by a membrane bilayer (concentric amphiphile lipidic layers) that alternate with aqueous layers [1]. They can range from nanometer to micrometer size. The main categories of liposomes according to the size are [1–3]: small unilamellar vesicles (SUVs) – liposomes with diameters in the range 25–100 nm; intermediate unilamellar vesicles (IUVs) – ranging from 100 to 250 nm; multilamellar vesicles (MLVs) – with a population of vesicles usually composed of five or many concentric membrane lamellae and can range in size from 100 to 1000 nm in diameter; large unilamellar vesicles (LUVs) – liposomes with diameters of the order of 1000 nm (2–5 μm).

The value of liposomes as membrane model systems derives from the fact that they can be prepared by using natural constituents and the liposome membrane forms a bilayer structure which is almost identical to the lipid fraction of the natural cell membranes [4–5]. The similarity between liposomes and natural membranes can

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be increased by chemical modification of the liposome membrane, by inserting different components naturally occurring in biomembranes. Moreover, the liposomes can be exploited in different applications, both *in vivo* and *in vitro*, mimicking successfully the behaviour of natural membranes. Apart from their chemical constituents, which determine such properties as membrane fluidity, charge density and permeability, liposomes can be characterized by their size and shape. The liposomes that can range in a size between 25 nm and 1000 nm equals the dimensions of a living cell. Alternatively, liposomes can be prepared from entirely artificial components, chosen for their improved chemical properties. Liposomes have been under extensive investigation as carriers for the improved delivery of a broad spectrum of agents such as chemotherapeutic agents, imaging agents, antigens, chelating compounds, hemoglobin and cofactors, lipids and genetic materials [1]. Liposomes can be prepared so that they can entrap certain quantities of materials both within their aqueous compartment and within the membrane. In the present study the chlorophyll a was loaded into liposome vesicles, prepared with three phosphatidylcholines with different hydrophobic tails as lipid component, by using thin-film hydration method.

Liposomes with chlorophyll *a* (Chl*a*) have been used successfully as models for photosynthetic membranes, the lipid bilayer providing a hydrophobic environment for Chl*a*. The liposomes with Chl*a* are excellent models for biomembranes, specifically for photosynthetic membranes and are successfully used to study the influence of different agents on the bilayer structure at molecular level [2–6]. It has been shown that Chl*a* has also radioprotective, antimutagenic and anticarcinogenic activities. Chlorophyll's antioxidant properties could be partly responsible for its protective effects in natural and artificial structures. Thus, chlorophyll *a* is a valuable molecule, but insufficiently exploited in biotechnological applications. Therefore, in this paper is demonstred how intensive visible absorption and fluorescence emission of Chl*a* may allow its use as a sensor for interactions at molecular level and as a fluorescence marker.

Dynamic light scattering (DLS) also known as photonic correlation spectroscopy (PCS) is a well-established technique for measuring the size of molecules and particles typically in the submicron region. This analysis, largely used in monitoring of the dimensions of particles, was used in this research as a valuable tool for characterizing vesicle suspensions of Lipid-Chl*a*-MLVs/SUVs in order to validate the information provided by UV-VIS absorption and Fluorescence emission spectra, using Chl*a* as marker. It is well known that liposomes of various size require completely different methods of manufacture. Due to the different applications that demand the use of liposomes with particular size, the main objective of the present work was to prove that the spectral features of Chl*a* allow its use as a sensor for physical properties of liposomes (size, shape, molecular dynamics).

2. Materials and Methods

2.1. *Chl*a – *Liposomes Preparation Procedure*

Chl*a* – liposomes were prepared using the thin-film hydration method. The lipid and Chl*a* were dissolved in chloroform, with Chl*a*/lipid molar ratio 1/100. Different types of thin films were obtained, by vacuum evaporation, using three lipids (dioleoyl-phosphatidylcholine – DOPC, dimyristoyl phosphatidylcholine – DMPC,

dipalmitoyl phosphatidylcholine – DPPC). Chl*a* was prepared from fresh spinach leaves by chromatography using the Strain & Svec method [7] and checked for purity (absorption and fluorescence in VIS). The hydration (2 hours) of the lipid-Chl*a* film was done by adding phosphate buffer (pH in the range of $6.2 \div 7.6$), in a rotary evaporator (BIOBLOCK SCIENTIFIC – Heildolph 94200, 60–90 rpm). Multilamellar vesicles (MLVs) were obtained by mechanical dispersion (VIBRAX stirrer, 200 rpm, 30 min) of the hydrated films. Intermediate unilamellar vesicles (IUVs) or small unilamellar vesicles (SUVs) were obtained from the multilamellar liposomes by using the exposure to ultrasound treatment in a bath (Branson 1210), for variable periods, above the critical temperature (T_c) of phase transition of lipids. For obtaining a homogenous population of liposomes, the suspension was collected after a centrifugation at 20000 rpm for 30 min. After centrifugation, 3 mL suspension from upper phase were sampled (supernatant 1). A further sampling was realized after 2 min. from the same upper phase (supernatant 2). The bottom phase was considered as the sediment.

2.2. *Characterization Methods*

The optical absorption spectra were recorded on a double beam UV-VIS spectrophotometer Lambda 2S Perkin Elmer & PECSS software. Fluorescence measurements of Chla-liposomes were performed on a PERKIN-ELMER LS55 spectrophotofluorimeter, equipped with a computer for data recording. The samples were illuminated with a 430 nm excitation light and the fluorescence emission was collected at 620–730 nm, respectively.

The hydrodynamic diameter of liposomes, suspended in phosphate buffer, were measured by dynamic light scattering (DLS) by using a Zetasizer Nano ZS from Malvern Instruments. Light scattering measurements have been performed at 25°C. Hydrodynamic measurements were carried out at 90° angle, without dilution of samples. The particle size analysis data were evaluated using intensity distribution. The average size and the size distribution were calculated with the regularization algorithm provided by Malvern software.

3. Results and Discussion

*Characterization of Chl*a*-liposomes by UV-VIS Spectroscopy.* The liposomes scatter light in the visible range of the electromagnetic spectrum, if their dimension is in the range of the respective wavelengths. In order to do a rapid estimation of the size of the lipid vesicles, the absorption changes in the VIS spectra of Chl*a* (inserted into lipid bilayers) were monitored and some spectral criteria were established. Inspecting the absorption spectrum of Chl*a* in different liposomes, presented in Figure 1, major differences can be observed regarding the baseline (background) of the spectra. The main absorption bands of Chl*a* (Soret band and red band) are present in the spectrum and confirm the localization of the porphyrin ring of Chl*a* at the lipid-water interface in the vicinity of polar lipid heads (the red band is shifted to longer wavelengths as comparing with Chl*a* spectrum in solution).

A $1/\lambda^4$ dependence of the baseline was observed in VIS absorption spectra of the Chl*a*-unilamellar vesicles (SUV or IUV), suggesting that the size of the vesicles is small enough to act as point sources of scattered light (Fig. 2).

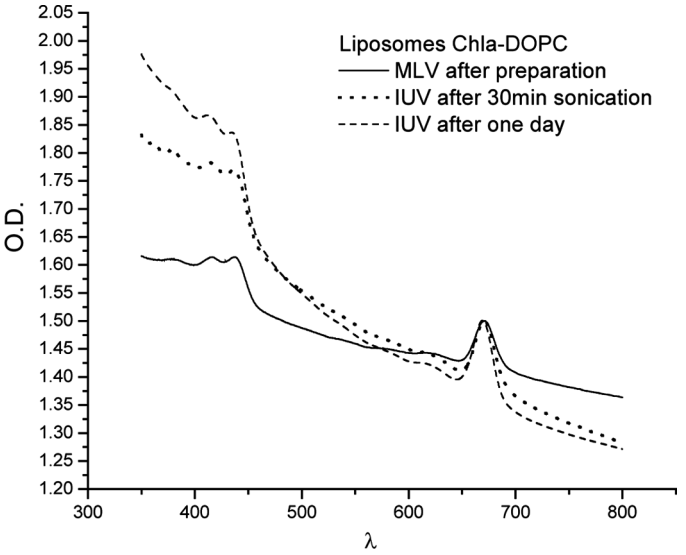


Figure 1. Absorption spectra of DOPC-Chl*a*-liposomes.

It was observed that smaller liposomes scatter shorter wavelengths (blue) more intensively, while larger liposomes scatter long (red) wavelengths. It is the case of Rayleigh scattering, applying to particles with a radius (R) shorter than $\lambda/20$ ($R < \lambda/20$) [8]. The increase of the background scattering observed in the stability studies can be explained by the aggregation or fusion of the vesicles. The larger vesicles formed by aggregation strongly scatter light in comparison with the non-aggregated vesicles obtained after sonication.

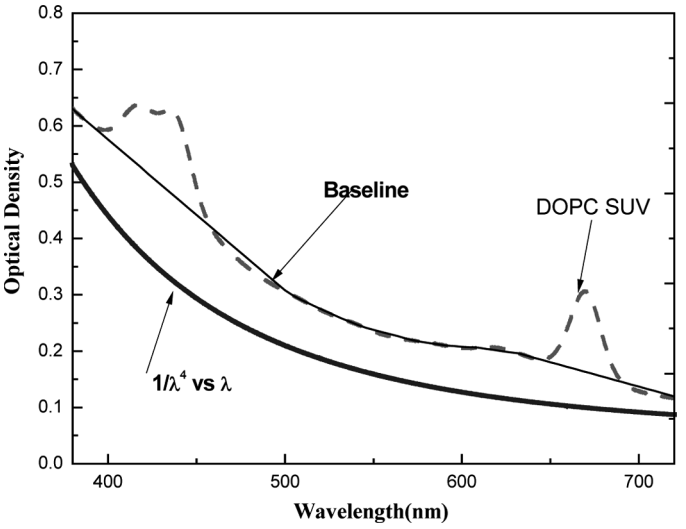


Figure 2. The $1/\lambda^4$ dependence of the baseline in VIS absorption spectra of the DOPC-Chl*a*-SUVs.

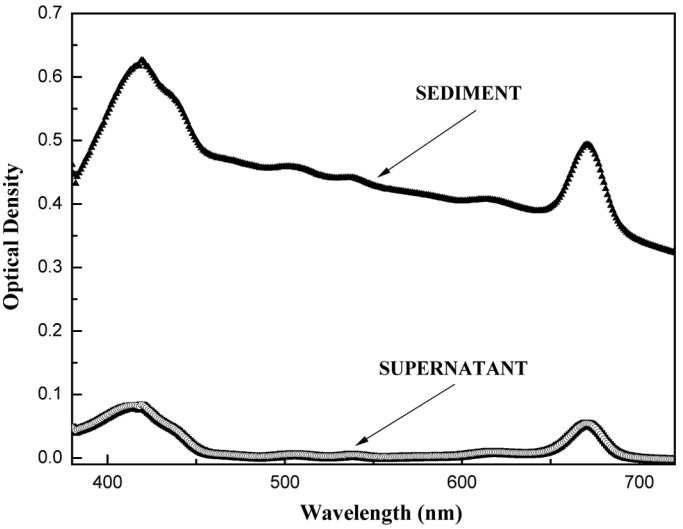


Figure 3. The corrected VIS absorption spectra of the DPPC-Chl*a*-SUVs after centrifugation (sediment and supernatant).

Assuming a Rayleigh scattering, the over-estimated absorbances values can be corrected for the contribution of light scattering on the basis of the optical density (OD) measured at 325 nm, where the scattering is the sole contributor to the OD [9]:

$$(OD_\lambda)_{\text{corrected}} = (OD_\lambda)_{\text{measured}} - (OD_{325})_{\text{measured}}\ x(325/\lambda)^4$$

In Figures 3 and 4, the corrected spectra are presented for two cases: supernatant and sediment obtained after the centrifugation of Chl*a*-liposomes. The suspension of

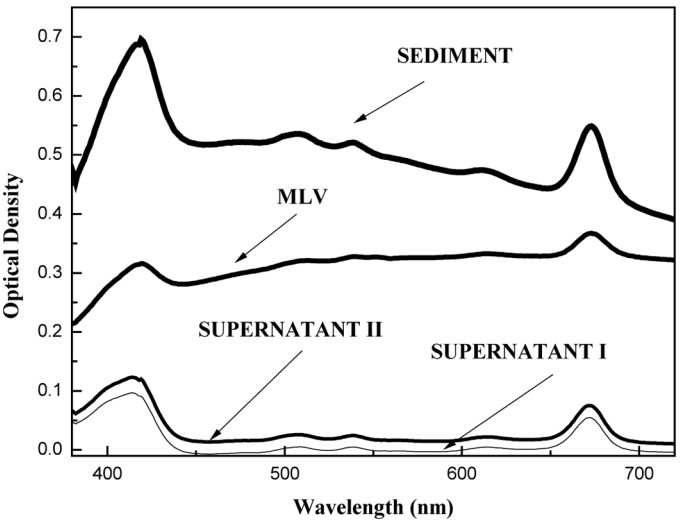


Figure 4. The corrected VIS absorption spectra of the DMPC-Chl*a*-SUVs + MLVs after centrifugation (sediment and supernatant).

Chl*a*-liposomes (SUVs) was manufactured as described above, by sonicating the MLVs. By ultracentrifugation, the remained large vesicles are deposited on the bottom of the tube and a homogenous population of liposomes remains in the supernatant. The correction by the above formula of the absorption spectra works very well in the case of the supernatant, but does not correct the baseline in the case of the sediment. The supernatant contains the unilamellar liposomes, more homogenous in dimension – with a smaller size than of liposomes in the sediment. An estimation of the size of the liposomes present in the supernatant is possible, considering the relation: $R < \lambda/20$. The spectrum is in the visible range, therefore the average λ is 600 nm and $\lambda/20 = 30$ nm. Therefore, an average diameter of 60 nm of the liposomes (SUV) in the homogenous population is estimated from the spectral absorption measurements.

It followes that the spectral analysis of Chl*a*-liposomes, using the spectral properties of Chl*a*, results to a rapid monitoring of the liposome preparation. The Chl*a*-liposomes prepared as described above are unilamellar and have diameters in the range 40–100 nm, as proved by the DLS measurements (see below).

Stability studies on liposomes with Chl*a* were performed by monitoring the changes in the Chl*a* spectra for different factors. The nature of lipids and pH value of the buffer influences the dimension of liposomes. The neutral pH values proved to be the best as concerning the stability of liposomes [10]. In Figure 5 the time-dependent behaviour of liposomes is presented. An increase of the baseline is observed during ageing, since the liposomes have the tendency to fusion.

The influence of the lipid nature on Chl*a*-liposomes was also investigated. Figure 6 presents the normalized absorption spectra (corrected for the baseline) of Chl*a*-IUVs, obtained by using different lipids. The background absorption is depending on the lipid nature. The difference between DMPC and DOPC consists

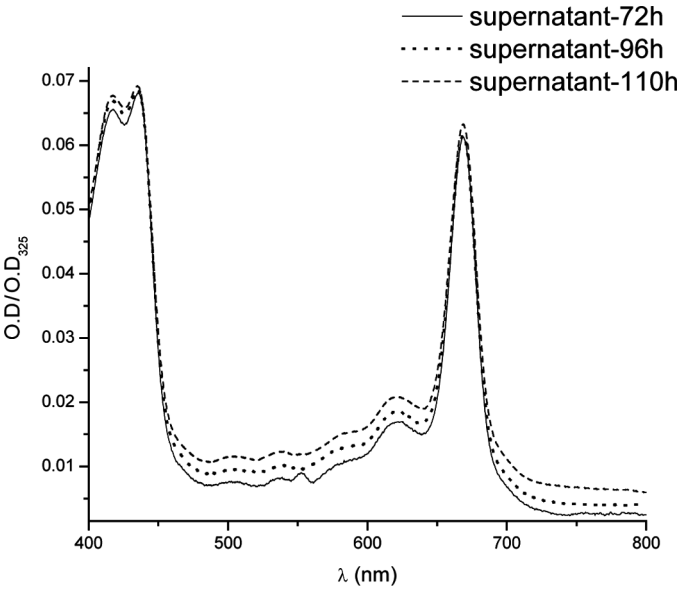


Figure 5. Time course of VIS corrected absorption spectra of DMPC-Chl*a*-SUVs in suspension, after centrifugation.

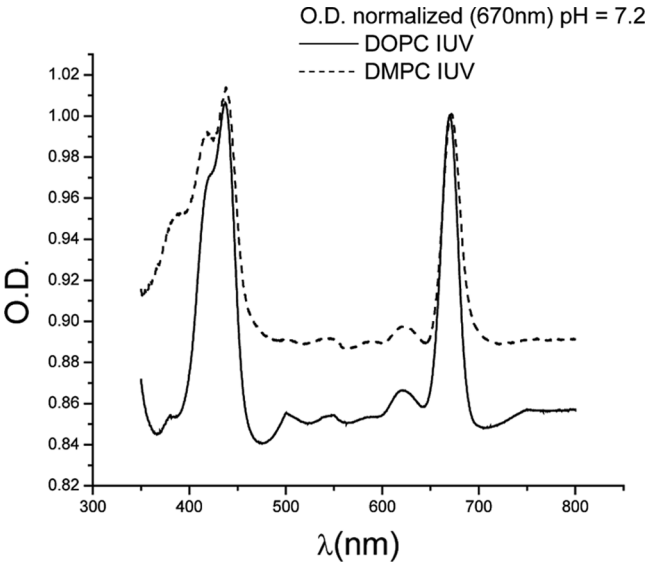


Figure 6. Absorption spectra of Chl*a*-IUVs.

in the presence of unsaturated acyl chains in the case of DOPC, thus providing a different environment for Chl*a* in the lipid bilayer. This explains the lower baseline position and therefore the smaller dimension of the DOPC-liposomes.

The photooxidation of Chl*a* was monitored by comparing the absorbance ratio in the Soret band $O.D._{438}/O.D._{416}$, the Chl*a* degradation (and also liposome degradation) leading to a decrease of this ratio [3–6]. A correlation between Chl*a* oxidation and lipid oxidation was also observed by performing VIS and UV spectra on the lipids (Fig. 7).

*Characterization of Chl*a*-liposomes by Fluorescence Spectroscopy*. Fluorescence spectra of Chl*a* in liposomes have been performed using Chl*a* as a fluorescent marker. Figure 8 presents the emission spectra of Chl*a* in MLV and SUV, prepared by using DMPC as lipid. The emission intensities are normalized using the relative intensity of the emission at corresponding emission maximum. A red

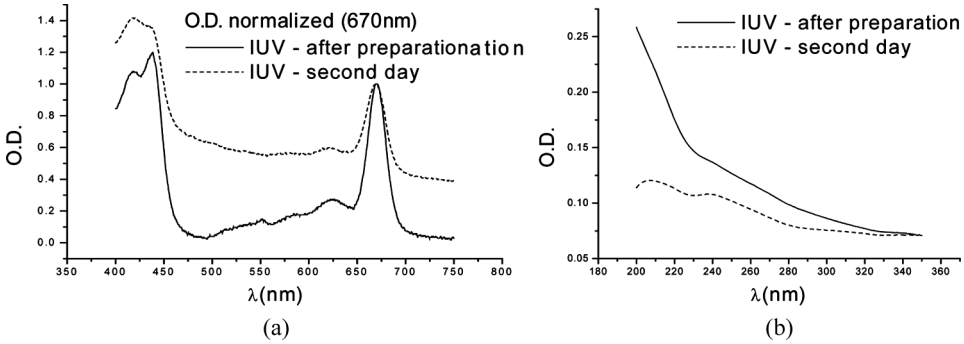


Figure 7. Absorption spectra of DMPC-Chl*a*-IUVs in VIS (a) and UV (b).

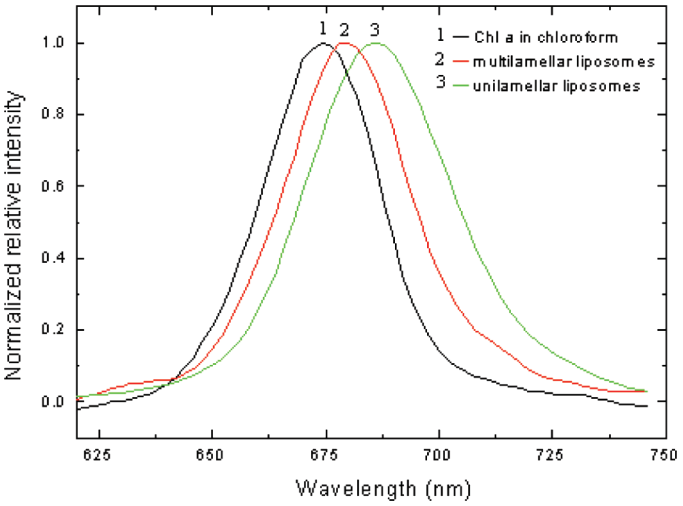


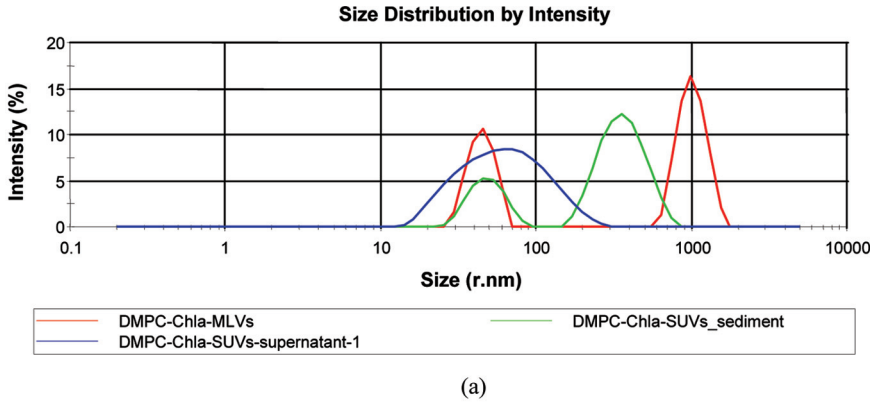
Figure 8. Emission spectra of Chl*a* in DMPC-liposomes (SUV and MLV suspensions), against Chl*a* spectrum in chloroform.

Table 1. Average sizes and polydispersity indices of lipid-Chl*a*-liposomes

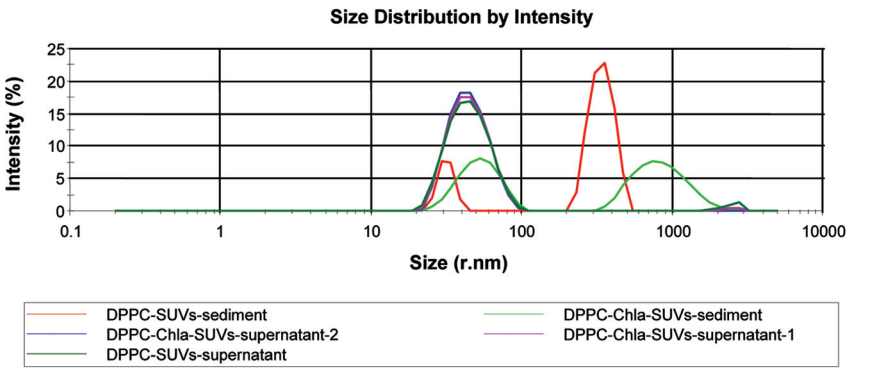
Sample name	Average diameter (nm)	Peak1 (nm)	Peak2 (nm)	Peak3 (nm)	PDI index
DPPC-SUVs supernatant	88.8	92.1 (97.2%)	4920 (2.8%)	–	0.200
DPPC-SUVs sediment	476	690 (81%)	63.5 (19%)	–	0.670
DPPC-Chl*a*-SUVs sediment	218	1770 (54.9%)	108 (44.7%)	5190 (0.5%)	0.852
DPPC-Chl*a*-SUVs supernatant 1	86.2	91.8 (100%)	–	–	0.128
DPPC-Chl*a*-SUVs supernatant 2	86.7	92.3 (98.9)	5050 (1.1%)	–	0.140
DMPC-MLVs	3110	3330 (100%)	–	–	0.123
DMPC-SUVs supernatant 1	105	136 (100%)	–	–	0.242
DMPC-SUVs supernatant 2	112	160 (%)	–	–	0.275
DMPC-SUVs sediment	243	386 (72.2%)	84 (27.8%)	–	0.572
DMPC-Chl*a*-MLVs	616	2050 (61.4%)	89.8 (38.6%)	–	0.776
DMPC-Chl*a*-SUVs supernatant 1	103	–	–	–	0.242
DMPC-Chl*a*-SUVs supernatant 2	123	166 (97%)	4500 (3%)	–	0.322
DMPC-Chl*a*-SUVs sediment	329	748 (74.7%)	98.7 (25.3%)	–	0.554

shift of the maximum position is observed when comparing the spectrum of Chl*a* incorporated in liposomes against its spectrum in solution (in chloroform, at a concentration of 10^{-6} M). A red shift is further observed in the case of unilamellar liposomes as compared with multilamellar liposomes. The red shift of emission is usually explained by a more polar environment sensed by the fluorophore. The fluorophore in the case of Chl*a* is represented by the porphyrin macrocycle. It is known that Chl*a* is located in the liposome lipid bilayer with the macrocycle at the interface with the water phase, in the vicinity of the lipid polar heads and the phytol in the hydrophobic lipid chains region [3]. Therefore, Chl*a* is penetrating more deeply with its macrocycle within the polar region of the lipid bilayer during formation of small unilamellar vesicles. Thus the emission spectra of Chl*a* in liposomes could be used for a rapid monitoring of the presence of unilamellar liposomes during the experiments with liposomes.

*Size Characterization of Chl*a*-liposomes by DLS Measurements.* DLS analysis is largely used in monitoring the dimensions of particles from various dispersions. This analysis was used in the present study in order to validate the information provided by UV-VIS absorption and emission spectra, using Chl*a* as marker. DLS measurements data are reported in Table 1. These results shown that the thin-film



(a)



(b)

Figure 9. Particle size distribution of DMPC-Chl*a*-MLVs/SUVs (a) and DPPC-Chl*a*-SUVs (b), by dynamic light scattering.

hydration method combined with a ultrasound treatment used for Chl*a*-liposomes synthesis, typically lead to relatively homogeneous population of liposomes (unilamellar vesicles) with average diameters ranging from 86 nm to about 330 nm.

Figures 9a and b illustrate how the size distribution of the lipid vesicles changes from large multilamellar vesicles to small unilamellar vesicles during the sonication process. For instance, the size distribution of DMPC-Chl*a*-SUVs shows a single peak after ultrasound treatment of MLVs. From Figure 9b it may be observed the unilamellar liposomes loaded with Chl*a* which comprises a population between $20 \div 200$ nm with a good polidispersity index of 0.242. Moreover, by using DPPC as lipid for liposomes synthesis (Fig. 9b), the best results are obtained by means of a nanometer range also for sediment (476 nm), even the polydispersity index had a high value, thus indicating a broader distribution of particles. In this case, after ultrasound treatment an uniform unilamellar population with average size around 86 nm and low polydispersity indices (PDI < 0.14) were obtained (Table 1 and Figure 9).

4. Conclusion

Multilamellar and unilamellar vesicles with Chl*a* have been prepared by a mechanical dispersion technique (thin-film hydration method) that involved the obtaining of different types of thin films and a further sonication treatment for obtaining unilamellar vesicles. Chlorophyll *a* incorporated into lipid bilayers of the liposomes formed by three phosphatidylcholine with different hydrophobic tails was used to monitor the formation of Chl*a*-liposomes. Several spectral criteria have been applied on developed lipid-Chl*a*-SUVs model systems (UV-VIS absorption, fluorescence emission and dynamic light scattering measurements) and analyzed comparatively in order to monitor and validate the size and shape of Chl*a*-liposomes and the oxidation state of the components. The spectral features obtained from visible absorption and fluorescence emission spectra of chlorophyll sustain its use as a sensor for specific molecular interactions in the liposome bilayer and as a fluorescence marker.

Acknowledgments

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References

[1] New, R. R. C. (ed.) (1990). *Liposomes: A Practical Approach*, IRL press: Oxford University.
[2] Nadtochenko, V. A., Lavrentyev, A. I., Rubtsov, I. V., Nikandrov, V. V., & Barannikova, Ya. V. J. (1993). *Photochem. Photobiol. B: Biology*, *18*, 221.
[3] Daniels, R. Galenic principles of modern skin care products, http://www.scf-online.com.
[4] Barbinta-Patrascu, M. E., Tugulea, L., Meghea, A., & Popescu, A. (2008). *Optoelectronics and Advanced Materials – Rapid Communications*, *2*(2), 113.
[5] Barbinta-Patrascu, M. E., Badea, N., Tugulea, L., Giurginca, M., & Meghea, A. (2008). *Revista de Chimie*, *59*(8), 834.

[6] Barbinta-Patrascu, M. E., Voinea, M., & Tugulea, L. (2003). *Bucharest University Annals, Physics*, *LII*, 35.
[7] Strain, H. H. & Svec, W. A. (1966). *The Chlorophyll* (Vernon, L. P. & Seely, G. R., eds.), Academic Press: New York, USA, 21.
[8] Moore, W. J. (1962). *Physical Chemistry*, Longmans Green and Co. Ltd.: London, 761.
[9] Bittner, O., Gal, S., Pinchuk, I., Danino, D., Shinar, H., & Lichtenberg, D. (2002). *Chemistry and Physics of Lipids*, *114*, 81.
[10] Tugulea, L. & Iacovache, I. M. (2002). *Cell Mol. Biol. Lett.*, *7*(2), 302.