Photostability of bacteriochlorophyll *a* and bacteriopheophytin *a* against UV-A, UV-B and visible light treatments in methanol solutions

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ABSTRACT

Bacteriochlorins as the porphyrins derivatives are well known photosensitizers with great potential for use in various fields of pharmacy and medicine. Photostability of selected bacteriochlorins, bacteriochlorophyll *a* and bacteriopheophytin *a*, in different methanol solutions (with and without lipids) during continual UV-A, UV-B and visible light treatments were studied using absorption UV-VIS spectroscopy providing kinetic analysis. Applied irradiation treatments resulted in irreversible degradation of both selected bacteriochlorins obeying the first order of kinetics. Bacteriopheophytin *a* showed significantly higher photostability in comparison to bacteriochlorophyll *a* for all applied irradiation treatments, for about one to three orders of magnitude. Photochemical degradation of bacteriochlorins is energy dependant process, governed by photons energy input.Lipid environment play stability role for both bacteriochlorins against all, UV-A, UV-B and visible light treatments. Bacteriopheophytin *a* induced lipid peroxidation processduring UV-A irradiation treatment.

Keywords: photostability, bacteriopheophytin a, bacteriochlorophyll a, irradiation, lipids, lipid peroxidation

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Introduction

Bacteriochlorophyll *a* (BChl*a*) is a bacteriochlorin type chlorophyll which is the most widely distributed. It is present in the reaction center (RC) and the core-antennas of most anoxygenic bacteria, as well as in the peripheral antennas of the purple bacteria (Grimm et al., 2007, Permentier et al., 2001). A major bacteria pigment bacteriochlorophyll*a* (BChl*a*), with an isocyclic cyclopentanone ring fused to a C-pyrrole ring of the porphyrin core between the C-13 and C-15 positions (Figure 1A) contains one magnesium in the center which coordinates four pyrrole rings by two covalent and two coordination bonds (Kay and Gräcel, 1993). Bacteriopheophytin *a* (BPheo*a*) is a derivative of BChl*a* with only difference in the central position of macrocycle, the absence of Mg (Figure 1B).

In recent decades, there has been a growing interest in the use of porphyrins and their derivates as well as BChls and their derivates in medical field by giving consideration for photophysical and photochemical properties as efficient and promising sensitizers in photodynamic therapy known as Photodynamic Therapy (PDT) (Brandis et al., 2006; Henderson et al., 1990; Pandey and Zheng, 2000). Photodynamic Therapy is a successful treatment method for cancer and premalignant conditions that leads to the selective destruction of tumor through photodynamic process and it is based on the induction of tissue and cellular damage by the combined effects of three components including a photosensitizer (PS), light and oxygen (Kübler, 2005). Porphyrins and their derivates have interesting and good photochemical characteristics and exhibit desirable properties for drug candidates in PDT (Pandey and Zheng, 2000; Rinco et al., 2009). They also have some drawbacks (Yano et al., 2011), but in general, porphyrins and their derivates, as well as chlorins and bacteriochlorins, have en excellent PDT efficacy (Sternberg et al., 1998) and some of them have been approved for clinical use (Henderson and Dougherty, 1992).

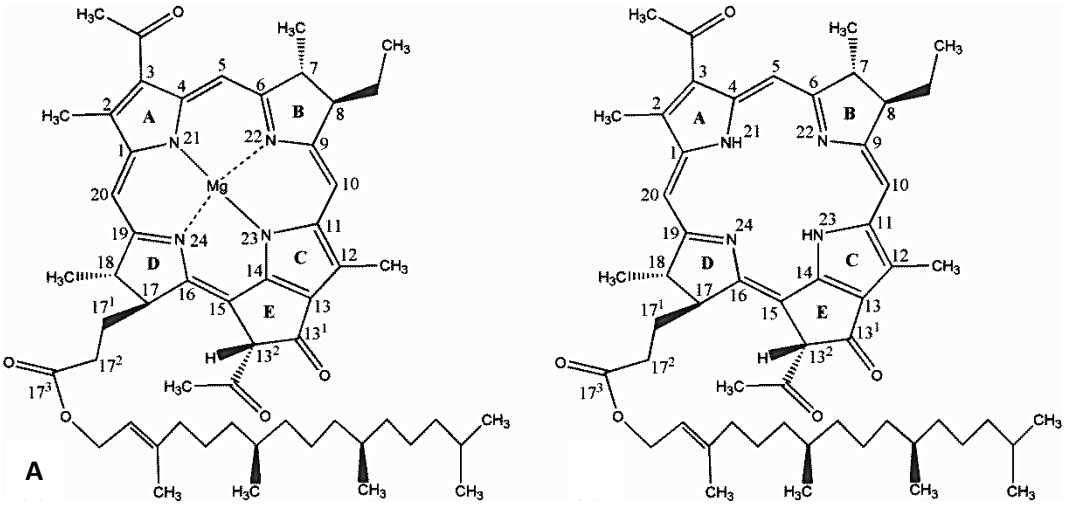


Figure 1. Structure of BChl*a* (A) and BPheo*a* (B).

It is important to examine the connection between photosensitizers and lipid peroxidation process because lipid peroxidation is considered as the main molecular mechanism involved in the oxidative damage to cell structures and in the destruction and

cell death in PDT and generally in biological processes (Repetto et al., 2012; Yin et al., 2011). In earlier experiments with bacteriochlorophyll analogues chlorophylls, it was found that UV-irradiation induces degradation of these compounds. Chlorophyll degradation obeys a first-order law and it is highly dependant on the used energy input and also dependant on the used solvents (Petrovic et al., 2017; Zvezdanović and Marković, 2008; Zvezdanović et al., 2009). The interaction of bacteriochlorin derivatives with oxygen under UV-VIS light (to produce harmful reactive oxygen species) and their photostability against different irradiation treatments could play important role in various fields of research and their applications. The objective of this study was to determine photostability of two chosen bacteriochlorins, BChl*a* and BPheo*a*, during continual UV-A, UV-B and VIS irradiation treatments in methanol solutions - without and with phospholipid mixture, PL90, in order to determine the corresponding influence of lipid medium, and possibly, their photosensitizing potential for the induction of lipid peroxidation process under the given conditions.

Experimental

All experiments were performed under dim light and equipment covered with aluminium foil, preventing possible BChl*a* and BPheo*a* photooxidation during sample preparation experiments.

The samples of BChl*a* and BPheo*a* used in this study are a gift from professor Fiedor Leszek, Faculty of Biochemistry, Biophysics and Biotechnology, Jagellonian University of Krakow, Poland. Phospholipid mixture, Phospholipon®90,PL90[†] is a gift from Phospholipid, GMBH, Cologne, Germany. All solvents used in the experiments were HPLC or LC/MS grade purity.

Samples preparation

For the purpose of this study, four different solutions of BChl*a* and BPheo*a* in methanol were made: BChl*a*,BPheo*a*,BChl*a*+PL90 and BPheo*a*+PL90 solutions.

Bacteriochlorophyll *a* and BPheo*a* were dissolved in methanol to concentration $2.5x10^{-5}$ M in all solutions; BChl*a* and BPheo*a* concentration was adjusted to the chosen by Beer's law in UV-VIS spectrophotometric method, using extinction coefficients values in methanol at 772 and 530 nm, respectively (Kobayashi et al., 2006; Pandey and Zheng, 2000). In addition, lipids concentration for BChl*a*+PL90 and BPheo*a*+PL90 solutions were 1.0×10^{-4} M. Additionally, the solution of lipids, PL90 in methanol without bacteriochlorins (the same concentration 1.0×10^{-4} M) was made for the control experiments. Control experiments with PL90 methanol solutions were obtained to give better insight to possible changes of lipids itself under continual treatment with UV-B, UV-A and visible light, especially in the area of lipid peroxides formation (at 234 nm in the absorbance spectra).

[†]Declaration: phosphatidylcholine 94.7%, lysophosphatidylcholine 0.9%, tocopherol 0.21% andfatty acids: palmitoleic 12.0±2%; stearic 3±1%; oleic 3±3%; linoleic 66±5% and linolenic 5±2%. Peroxide value of mixture is 1.4 (max. 5.0), acid value is 0.2 (max. 0.5) the content of ethanol and water 0.0% (max. 0.2%) and 0.3% (max. 1.5%), respectively.

UV irradiation treatments

Continuous irradiation of the samples(BChl*a*, BPheo*a*, BChl*a*+PL90 and BPheo*a*+PL90 and the controls) in methanol were performed in a cylindrical photochemical reactor "Rayonet", with 8 symmetrically placed lamps having emission maxima at 350 nm (UV-A) and 300 nm (UV-B). The samples were irradiated in quartz cells (1×1×4.5 cm^3) placed on the rotating circular holder. The total measured energy flux (hitting the samples) was about 10.3 W m^{-2} for 350 nm and 12.0 W m^{-2} for 300 nm.

Visible light treatment

Continuous illumination of bacteriochlorins solutions with visible light in the visible range (200-800 nm), was performed in hand-made cylindrical photochemical reactor equipped with symmetrically placed LED lamps at 10 cm distance from the samples (number of LED lamps was 60 com./m, distance between lamps 16 mm, light color" Pure White", emitting angle 120° spherical). The total measured energy flux received by the samples was 14 W m^{-2}.

UV-VIS spectrophotometry

Absorption UV-VIS spectra of all samples in the methanol were recorded on VARIAN Cary-100 Spectrophotometer equipped with 1.0 cm quartz cells. All spectra, before and after illumination/irradiation treatments (visible light / UV-A and UV-B) were recorded in the range from 200 to 800 nm, with 1.0 bandwidth. Degradation of BChl*a* and BPheo*a* in different methanol solutions induced by visible light, UV-A and UV-B irradiation treatments has been studied by using Q_y absorption band of these compounds as a sensible indicator of detected changes and providing kinetic analysis.

Data analysis was performed by the OriginPro 8 software. For the presentation of selected bacteriochlorins degradation, the dynamic plots based on the data from the corresponding UV-VIS spectra were used, *i.e*. absorbance values at Q_y-bands maximums (A_{772nm} for BChl*a* and A_{754nm} for BPheo*a*). Percent of BChl*a*and BPheo*a* retained in the treated samples were calculated by using equation: *Content of bacteriochlorins* (%) = $(A_t \times 100)/A_0$, where A_0 and A_t were the absorbance maximum values of BChl*a* and BPheo*a* for different t_{irr} periods and for t_{irr}= 0 min, respectively (for*y*-axis); $t_{irr.}$ given in min representing *x*-axis. For kinetic analysis,calculated rate constants were obtained from linear dependence of the corresponding $\ln A_{772nm}$ and $\ln A_{754nm}$ values during time of treatment, t_{irr} (min).

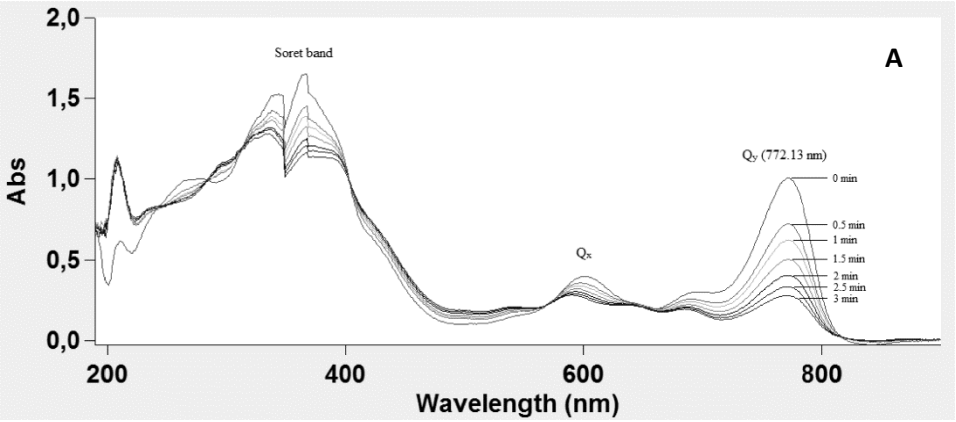
Results and Discussion

Absorption spectra of bacteriochlorins show the electronic transitions along the x-axis of the molecule running through two nitrogen (N) atoms of rings B and D, and along the *y*-axis through the N atoms of rings A and C (Grimm et al., 2007). The two pairs of absorption bands in the blue and red spectral regions of bacteriochlorins are called *B* (or Soret) and Q bands, respectively, and arise from $\pi \rightarrow \pi^*$ transitions of the four frontier orbitals (Weiss, 1978). One band of each pair is polarized along the *x*-axis (B_x, Q_x), the

other along the *y*-axis (B_y, Q_y) (Grimm et al., 2007). Rings B and D in bacteriochlorins are reduced by two hydrogens (Figure1); the conjugated system is longer along the *y*-axis than the *x*-axis (Ke, 2001; Weiss, 1978)and the spectroscopic consequence of this hydrogenation of rings B and D (Figure 1) is a considerably increased gap among the absorption bands (Grimm et al., 2007). The Q_y-absorption band is red-shifted to 750-800 nm in mono-disperse solutions, and even more (800-1020 nm) *in situ*, while the Soret-band is a blue-shifted (<400 nm) and split (Drews and Giesbrecht, 1966). Bacteriopheophytin *a* can be easily distinguished from BChl*a* by the shape of its *B* bands, by its higher B/Q_y band ratio (ca. 1.6 in BPheo*a* and ca. 0.75 in BChl*a*) and by its blue shifted Q_x maximum (520 nm in BPheo*a* and 570 nm in BChl*a*), as shown in Figure 2 (Grimm et al., 2007).

Photostability of selected bacteriochlorins were monitored on the basis of the change of the maxima of absorption Q_y band ($Q_{y,max}$) during the UV-A, UV-B and visible light treatment.

Absorption spectra of BChl*a* and BPheo*a* solutions treated with UV-B and UV-A irradiations, and BChl*a* and BPheo*a* in mixture with lipids treated with UV-A and visible light,were shown in Figures 2A,B, C and D, respectively. The rest of UV-A, UV-B and visible light treated solutions of BChl*a* and BPheo*a* as well as BChl*a*+PL90 and BPheo*a*+PL90 showed similar spectral behaviour (spectra not shown). The corresponding time dynamic of bacteriochlorins (photo)degradation during the treatments were shown in Figures 3A, B and C, for all solutionsunder UV-B, UV-A and VIS irradiation regime, respectively, by using absorbance values recorded at Q_y-band maximums - 772 nm for BChl*a* and 754 nm for BPheo*a*, A_{752nm} and A_{754nm}. The corresponding ln-plots of A_{752nm} and A_{754nm} valuesfor increasing UV-A, UV-B and VIS treatment periods ($t_{irr.}$), with linear fitting (mainly, R^2 values > 0.94) were used for kinetic analysis, and the corresponding calculated (photo)degradation rate constants (in min^{-1}), determined as the slopes of linear plots, were listed in Table 1.



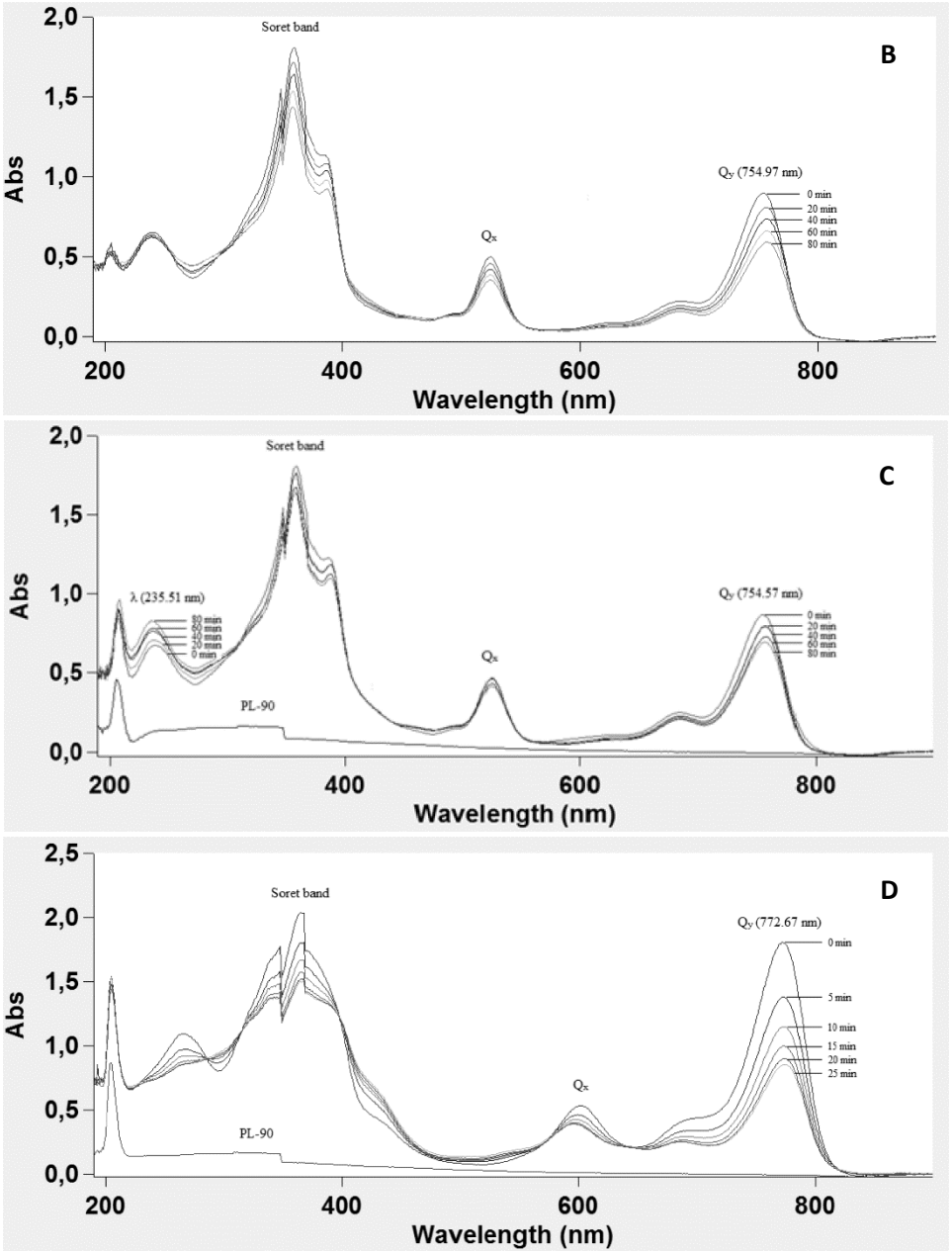
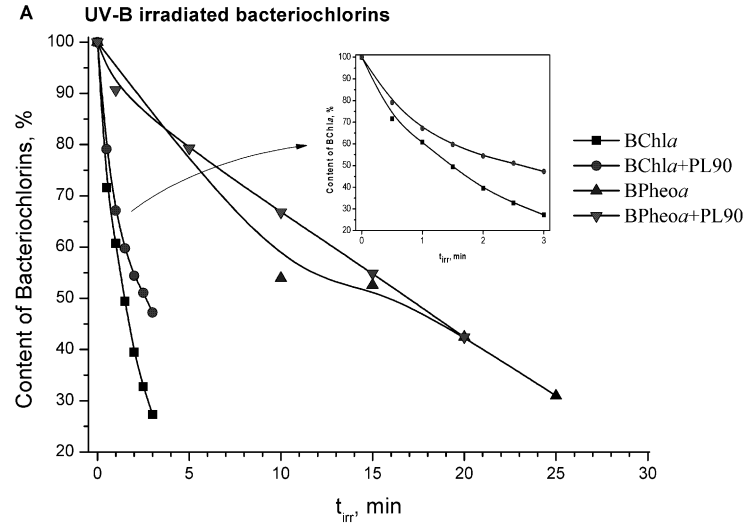
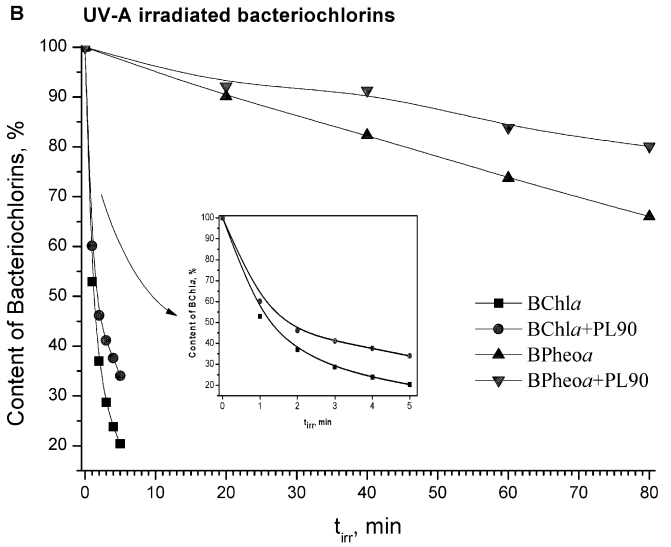


Figure 2.Absorption spectra of BChl*a* and BPheo*a* solutions treated with continual UV-B and UV-A irradiations (A and B, respectively) and BPheo*a*+PL90 and BChl*a*+PL90 solutions treated with UV-A and visible light (C and D, respectively). The time periods of treatments were shown on the figure. From the control experiments, UV-A and visible light treated PL-90 in methanol, the corresponding UV-VIS spectra were shown and marked on the figures (C and D, respectively).


A
UV-B irradiated bacteriochlorins
Content of Bacteriochlorins, %
t_irr, min
BChla
BChla+PL90
BPheoa
BPheoa+PL90


B
UV-A irradiated bacteriochlorins
Content of Bacteriochlorins, %
t_irr, min
BChla
BChla+PL90
BPheoa
BPheoa+PL90

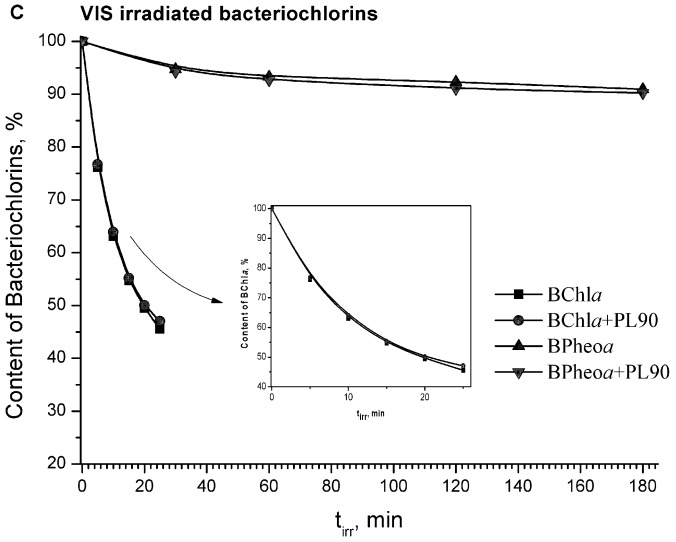

C
VIS irradiated bacteriochlorins
Content of Bacteriochlorins, %
t_irr, min
BChla
BChla+PL90
BPheoa
BPheoa+PL90

Figure 3. The dynamic plots of bacteriochlorophyll *a* and bacteriopheophytin *a* degradation in both mixtures (with and without lipidsin methanol solutions) under continuous UV-B, UV-A and visible light irradiation regime (A, B and C, respectively), expressed as the changes in percent of BChl*a* and BPheo*a* (compared to not-irradiated) for different irradiation time periods (t_{irr}).

Table 1. List of calculated photodegradation rate constants for selected bacteriochlorins

	k_{BChla} (min^{-1})	R^2	$k_{BChla+PL90}$(min^{-1})	R^2	k_{BPheoa} (min^{-1})	R^2	$k_{BPheoa+PL90}$ (min^{-1})	R^2
UV-B	0.421	0.99	0.263	0.94	0.044	0.95	0.040	0.99
UV-A	0.348	0.92	0.203	0.88	0.005	0.99	0.003	0.95
White light	0.035	0.95	0.034	0.96	$^{*}\approx 10^{-4}$	0.70	$^{*}\approx 10^{-4}$	0.70

*Due to small R^2 values, the corresponding rate constants could only considerate as roughly approximate values.

According to obtained results, the continual UV-A, UV-B and visible light treatment of BChl*a* and BPheo*a* in methanol solutions result in their irreversible degradation. The corresponding absorption spectra were changed, showing continuous decrease in whole measured spectral range (190-900 nm) during the treatments (Figure 2). Mixtures of lipids as the controls in methanol are not considerably changed during irradiation (Figures 2C and D).

Degradation of BChl*a* and BChl*a*+PL90 with UV-A irradiation treatment is significant even in the first minute of irradiation (only 50% and 60 % of BChl*a* is retained in the samples, respectively, Figure 3B). On the other hand, BPheo*a* and BPheo*a*+PL90 solutions showed much slower degradation during continuous UV-A irradiation (even 70% and 85% of BPheo*a* are retained after 80 min of treatment, respectively, Figure 3B). Similarly, BChl*a* in all solutions (with and without PL90), treated with UV-B and visible light,has shown degradation trend during time of irradiation, more intensively in solutions without PL90 (Figures 3A,C, respectively). Calculated degradation rate constants for UV-B, UV-A and visible light treated BChl*a* are for about one, two and three orders of magnitude higher than the same for treated BPheo*a* in methanol (Table 1). Similar relation can be established for the treated BChl*a* and BPheo*a* in the mixture with PL90 in methanol. Even more, chosen bacteriochlorins are more stable in the mixtures with lipids, PL90. This is in accordance with calculated degradation rate constants (Table 1) confirming the stability order: BChl*a*<BChl*a*+PL90<BPheo*a*<BPheo*a*+PL90, for all three used treatments. And finally, photostability of chosen bacteriochlorins is energy dependent. Starting from the visible light (the lowest photons energy input), through UV-A to UV-B (the highest photons energy input) irradiation treatments, degradation of both bacteriochlorins rising in all mixtures - their stability decreasing (Table 1). Detected differences are more noticeable for BPheo*a* in comparison to the corresponding ones for BChl*a* in both solutions (with PL90 and without PL90). For example, calculated rate constant values for UV-B, UV-A and visible light treated BPheo*a* in methanol are 0.044 min^{-1}, 0.005 min^{-1} and $\approx 10^{-4}$ min^{-1}, respectively. So,the corresponding UV-B induced degradation rate is almost one order of magnitude faster than UV-A, and two orders of magnitude faster than visible light induced degradation of BPheo*a* (Table 1). On the other hand, the corresponding values calculated for BChl*a* are 0.421 min^{-1}, 0.348 min^{-1} and 0.035 min^{-1} for UV-B, UV-A and visible light induced degradation, respectively, meaning

only one order magnitude faster UV-B and also, UV-A induced degradation in comparison to visible light, and almost equal UV-A and UV-B degradation rates (Table 1).

The answer to arising question "why?" is a more complex and out of the experimental data used in this study. But we will try to give some directions in better understanding of the obtained results for chosen two (bacterio)chlorins, BChl*a* and BPheo*a* treated by light. It is known that chlorophylls (bacteriochlorophylls as well) in solutions - *in vitro* in general are very unstable, much more in comparison to the corresponding their pheophytin derivatives (bacteriopheophytins), not only when exposed to the light, UV-irradiation, room and higher temperature values, oxygen in the dark and light, different solvents, acids *etc.* (Grimm et al., 2007). The stability of chlorophyll *a*wealready studied and the results were published (Petrović et al., 2017; Zvezdanović and Marković, 2008;Zvezdanović et al., 2009). The main explanation for the detected changes can be found in the structural differences between (B)Chl*a* and (B)Pheo*a*. (Bacterio)chlorophyll *a* has a central, labile bonded metal Mg; (B)Pheo*a* is without Mg (Figure 1), and this can be very important explanation for the photostability differences between selected compounds. Based on the previous research (Katz et al., 1978; Scheer, 1991), it can be supposed that central metal Mg in BChl*a* extends the half-life of a molecule excited triplet state after interaction with the irradiation (Melkozernov and Blankenship, 2006) resulted in a larger production of reactive oxygen species. In turn, formed reactive oxygen species could directly participate in the degradation of selected bacteriochlorins. Another important factor is the role of solvent. Methanol is a polar protic solvent and can be an electron donor. In that environment (solvents molecules) central metal Mg in BChl*a* can be an electron-acceptor, surrounded with molecules of solvent above and below bacteriochlorin tetrapyrrole macrocycle (Cotton and Van Duyne, 1979) somehow acting as an instability factor.

On the other hand, lipid environment playsa stability role to BChl*a* and BPheo*a*. Lipids used in this study in general could play a stability role to irradiated molecules (Stanojevic et al., 2013; Zvezdanovic et al., 2012). In the treated mixtures of bacteriochlorins, they can be "target" molecules for both, action ofdirect irradiation and also indirect irradiation treatments. Indirectly, the lipids can interact with molecules of singlet oxygen formed in photosensitizer (such as bacteriochlorin) - ground-state oxygen interactions under photon energy inputs (from UV-B, UV-A and visible light) and form lipid peroxides which can be detected in the UV-VIS spectra as rising absorbance at 234-235 nm. Namely, lipid peroxides, peroxidative dienes structures are lipid peroxidation products of lipids under many oxidative treatments (Cvetković and Marković, 2011; Repetto, 2010), including photooxidative were used in this study. In case of UV-A irradiated mixture BPheo*a*+PL90 in methanol, its clearly observed lipid peroxidation process (Figure 2C), implicated BPheo*a* photosensitizing properties under the given conditions. It is in accordance with known fact that porphyrins in general are well known photosensitizing molecules when they are exposed to light, especially visible, in the Q_y-band area of absorption (DeRosa, 2002). Also, from the absorption spectra of BChl*a* and BPheo*a* (Figure 2), it can be observed significant absorption in the area of UV-A radiation (320-380 nm) implying a possibility of photosensitization reaction upon UV-A absorption and as a consequence, lipid peroxides formation (Figure 2C).

Conclusion

The chosen bacteriochlorophylls, BChl*a* and BPheo*a* in methanol and methanol-lipid solutions undergo light/irradiation induced photochemical degradation. Photochemical degradation of bacteriochlorins is energy dependant process, governed by photons energy input. On the other hand, bacteriochlorophyll *a* and bacteriopheophytin *a* showed different photostability: BPheo*a* is more stable against all applied treatments in comparison to BChl*a* due to the structural differences. The impact of the environment on photostability is also an important factor. Experiments showed that BPheo*a* in mixture with lipid induce lipid peroxidation process, but only with UV-A irradiation is clear observed. This is probably because BPheo*a* absorption is the strongest in UV-A irradiation area (350 nm).Studied bacteriochlorins BChl*a* and BPheo*a* are photosensitive compounds and they show potential for future investigations in different areas.

Acknowledgement

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Conflict-of-Interest Statement

Declarations of interest: none.

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