Acta Physiologiae Plantarum (2023) 45:133
https://doi.org/10.1007/s11738-023-03614-8

SHORT COMMUNICATION

Foliar application of Pluronic P85-grafted single-walled carbon nanotubes induces thylakoid membrane structural remodeling

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Received: 12 August 2022 / Revised: 31 January 2023 / Accepted: 16 October 2023 / Published online: 3 November 2023

Abstract

Moderation and optimization of the photosynthetic function of higher plants by nanomaterials is under intensive investigation, but remain still far from practical utilization. We have previously demonstrated that foliar spraying of Pluronic P85-grafted single-walled carbon nanotubes (P85-SWCNT) affects the functionality and structural organization of the photosynthetic thylakoid membranes in pea plants. In the present work, we further study in more details the structural changes in the photosynthetic machinery induced by P85-SWCNT treatment. Evidences are provided that P85-SWCNT induces thylakoid membrane remodeling, namely—partial membrane unstacking, thermal stabilization of the major light-harvesting complex of photosystem II and its migration toward the stroma lamellae. The observed effects are most pronounced for the highest used concentration of 300 mg/L P85-SWCNT. Our results reveal that P85-SWCNT in concentrations below 300 mg/L is an interesting object for further investigation of the potential application of nanomaterials in plant science, e.g., as nanocarriers of beneficial substances reaching the photosynthetic apparatus.

Keywords Photosynthetic apparatus · Major light-harvesting complex of photosystem II · Thermal stability · Grana membranes

Introduction

The development of novel technologies for targeted transportation of nanomaterials to the photosynthetic apparatus in vivo is a hot topic in plant bio- and nanotechnology but still far from practical realization. Based on in vitro and *in silico* studies revealing the possibility for direct interaction (electron and exciton transfer, formation of chemical bonds) between photosynthetic reaction centers and single-walled carbon nanotubes (SWCNT), demonstrated in several studies (Dorogi et al. 2006; Mackowski et al. 2010; Hajdu et al. 2011; Nagy et al. 2014; Wiwatowski et al. 2016; Ghasemi-Kooch et al. 2018; Orlanducci et al. 2020), we can envisage protocols for manufacturing nanocarriers loaded with antioxidants, essential microelements or other beneficial substances that are delivered to the chloroplasts. This would help the photosynthetic apparatus, and thus the whole plant, to cope with extreme environmental events, ensure survival and improve yields.

To obtain fundamental knowledge on the effect of foliar application of SWCNT on plant development (with emphasis on photosynthetic performance) in our previous work, we exploited poly(ethylene oxide)$_{26}$-block-poly(propylene oxide)$_{40}$-block-poly(ethylene oxide)$_{26}$ triblock copolymer (Pluronic P85)-grafted SWCNT, denoted hereon P85-SWCNT. We revealed that the application of 300 mg/L P85-SWCNT induced changes in the functionality of the photosynthetic apparatus, as well as an alteration in the thylakoid membrane structure manifested as increased lumenal space throughout the grana and stroma regions of the thylakoid system (Velikova et al. 2021). On the contrary, application

Communicated by G. Bartosz.

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solely of Pluronic P85 led to features that were very similar to the control untreated variants (Velikova et al. 2021). Therefore, to further explore the P85-SWCNT-induced structural changes, here we investigate thylakoid membranes isolated from pea plants treated with those nanomaterials in the same manner as reported in Velikova et al. (2021).

The ultrastructure of thylakoid membranes is largely determined by the extent of stacking of confined membrane domains into grana structures, highly enriched in photosystem II (PSII) and its major and minor antennae complexes. The study of thylakoid membrane ordering in lateral (macroorganization) and vertical (stacking) direction is of high interest, since it is not static but dynamically responds to a variety of environmental and stress factors, and hence is involved in plants' adaptation and response mechanisms (Lambrev and Akhtar 2019; Rantala et al. 2020; Johnson and Wientjes 2020; Gu et al. 2022). The major light-harvesting complex of photosystem II (LHCII) is known to be a key factor in those processes (Kouřil et al. 2012; Albanese et al. 2020; Rantala et al. 2020; Mazur et al. 2021).

Materials and methods

Thylakoid membrane preparation

Thylakoid membranes were prepared from 14-day-old pea plants (*Pisum sativum* cv. RAN 1) sprayed with H_2O (control) or 10, 100 or 300 mg/L of P85-SWCNT (polymer:SWCNT ratio of 100:1 w/w), as described in Velikova et al. (2021). For the isolation procedure, the protocol described in Petrova et al. (2018) was utilized and the samples were stored at − 20 °C until further use. Before each experiment, the membranes were washed twice with buffer containing 20 mM tricine, 250 mM sorbitol, 5 mM $MgCl_2$ (pH 7.6) and adjusted to the respective chlorophyll (Chl) concentration required for the analyses carried out.

Digitonin fractionation

The extent of grana stacking was determined by the method of membrane fractionation with digitonin (Chow et al. 1980). Thylakoid suspension (with concentration of 100 µg Chl/ml) was treated with 0.5% digitonin and incubated for 15 min. The % of grana in the thylakoid membranes was estimated by determining the Chl concentration of the digitonin-solubilized pellet, relative to the total Chl content of the thylakoid suspension before the digitonin treatment.

Differential scanning calorimetry

Thylakoid membranes (with a concentration of 700 µg Chl/ml) were linearly heated at a scan rate of 0.5 °C/min and the change in their excess heat capacity as a function of temperature was recorded by DASM 4 calorimeter (Puschino, Russia). The original differential scanning calorimetry (DSC) scans were smoothed, corrected for buffer–buffer baseline and normalized to the Chl content.

Malondialdehide concentration determination

The extent of peroxidation of the thylakoid lipids was determined on the basis of malondialdehide (MDA) level in isolated thylakoid membranes. The MDA content of thylakoid suspensions with Chl concentration of 50 µg Chl/ml was determined according to a protocol described in Mishra and Singhal (1992).

Statistical evaluation

The obtained data are presented as mean values and standard deviation (SD). Student's *t* test was applied to define the statistically significant differences between control (H_2O) and P85-SWCNT-treated plants.

Results and discussion

To precisely quantify the changes in the extent of grana stacking in thylakoids derived from leaves treated with P85-SWCNT, we utilized the mild detergent digitonin, which splits the thylakoid membrane at the grana/stroma margin (Chow et al. 1980). The data presented in Fig. 1a show that there are no statistically significant changes in grana stacking in the studied treatments as compared to the control. These results confirm the hypothesis stated in Velikova et al. (2021) that the observed changes in the chloroplast ultrastructure of 100 and 300 mg/L P85-SWCNT-treated variants are not associated with alteration of the relative abundance of grana and stroma lamellae but most probably are due to changes in the macroorganization of the photosynthetic complexes.

To explore further this possibility, as a next step we measured the Chl *a/b* ratio in intact thylakoids as well as in isolated grana membranes. This parameter is a realistic measure of the abundance of LHCII relative to the photosynthetic core complexes since LHCII is by far the most abundant membrane protein that binds the vast majority of Chl *b* in the thylakoid membranes of higher plants, while the photosynthetic core complexes bind only Chl *a* (Peter and Thornber 1991; Kirchhoff et al. 2002). We found out that the applied P85-SWCNT treatments did not induce change in Chl *a/b* ratio in intact thylakoids (it remained in the range of 2.5–2.7 for all samples). However, we detected significantly higher Chl *a/b* in the grana membranes of P85-SWCNT-treated leaves than of control ones, and the observed effects were

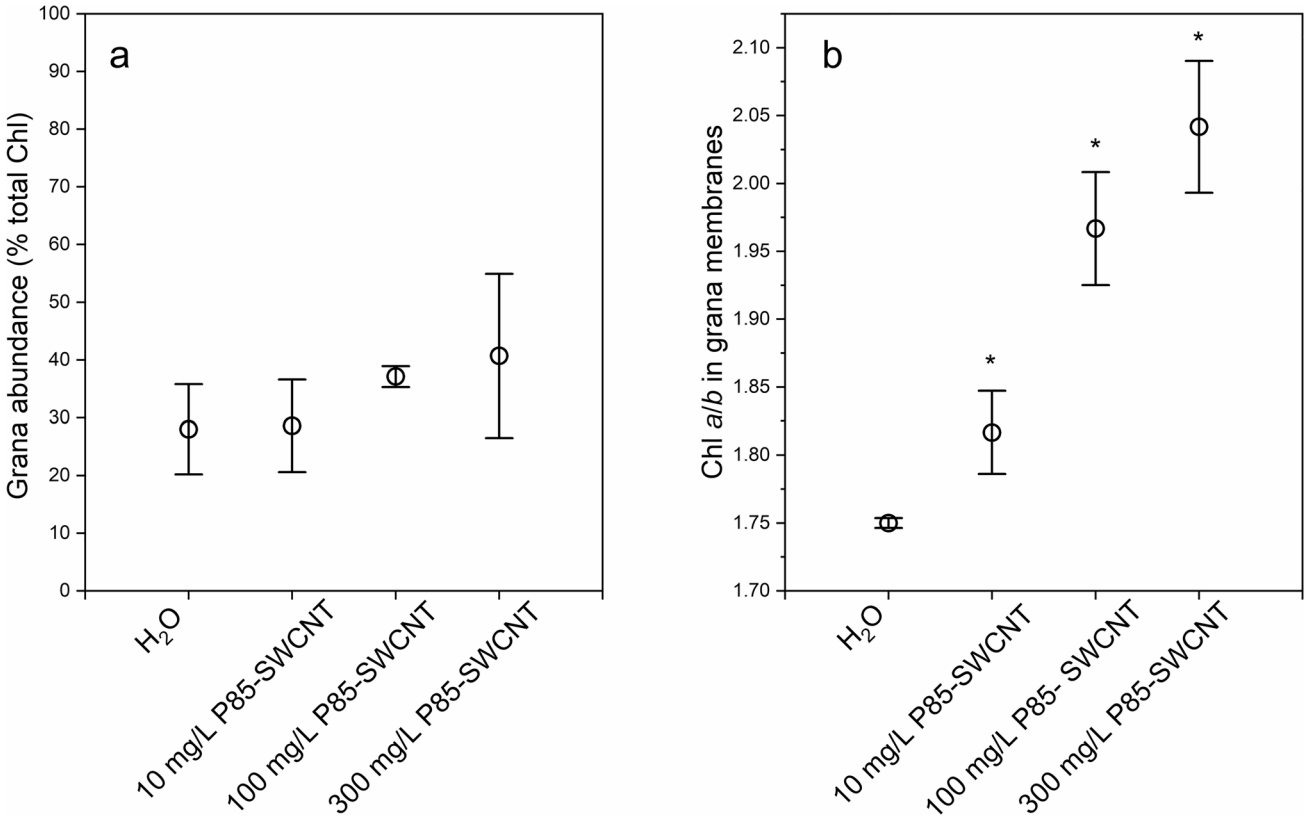

Fig. 1 Abundance of grana membranes, as determined by the relative Chl concentration of digitonin-fractionated thylakoids obtained from control (H_2O) plants and plants treated with different concentrations of P85-SWCNT (**a**). Chl *a*/*b* ratio determined for the isolated grana fragments (**b**). Data are presented as mean ± SD. *Statistically significant difference from the control at $P < 0.02$ according to Student's *t* test

proportional to the applied P85-SWCNT concentrations (Fig. 1b). Thus, we assume that although the overall LHCII content of the thylakoid membranes remains unchanged, P85-SWCNT treatment leads to reorganization of the PSII-LHCII supercomplexes, i.e. application of P85-SWCNT concentrations in the range 10–300 mg/L induce migration of LHCII outside the grana regions and subsequent location in the stroma lamellae. This leaves a portion of LHCII-depleted PSII complexes in the grana membranes. This process would require at least partial membrane unstacking that would facilitate LHCII diffusion. It is apparently different from the migration of LHCII toward stroma associated with the transition from state I (PSII bound LHCII) to state II (PSI bound LHCII) that results in gross swelling of the thylakoid lumen and the whole thylakoid membrane system (Chuartzman et al. 2008).

Next, we explored the effect of P85-SWCNT treatment on the thermotropic properties of the thylakoid membranes, which also reflect the membrane structural features. For each of the studied variants, six thermally induced transitions (denoted as T1–T6) were clearly observed (Fig. 2a). The first calorimetric transition was detected at ca. 47 °C in the control and 10 and 100 mg/L P85-SWCNT-treated plans; however, it was absent in the 300 mg/L P85-SWCNT sample. This thermal transition is of special interest, since it is related to membrane stacking and macroorganization, in particular it is due to heat-induced disassembly of chirally organized LHCII-containing macrodomains in stacked thylakoid membranes (Dobrikova et al. 2003). The thermal denaturation transition of LCHII within its native membrane environment was clearly resolved at about 70 °C in similarity to our previous report (Petrova et al. 2018). Its denaturation temperature, however, varied as a function of the applied P85-SWCNT concentrations. The data presented in Fig. 2b reveal that the increase in P85-SWCNT concentration results in statistically significant upshift of the transition temperature of LHCII, from 68.5 ± 0.9 °C for the control to 70.9 ± 0.5 °C for 300 mg/L P85-SWCNT treatment. The observed changes in T1 and T4 calorimetric transitions in 300 mg/L P85-SWCNT-treated plants resembled those obtained in chemically unstacked thylakoid membranes in vitro (Petrova et al. 2018), but are expressed at a lower extent, evidencing that partial unstacking process occurs in vivo in the plants treated with 300 mg/L P85-SWCNT.

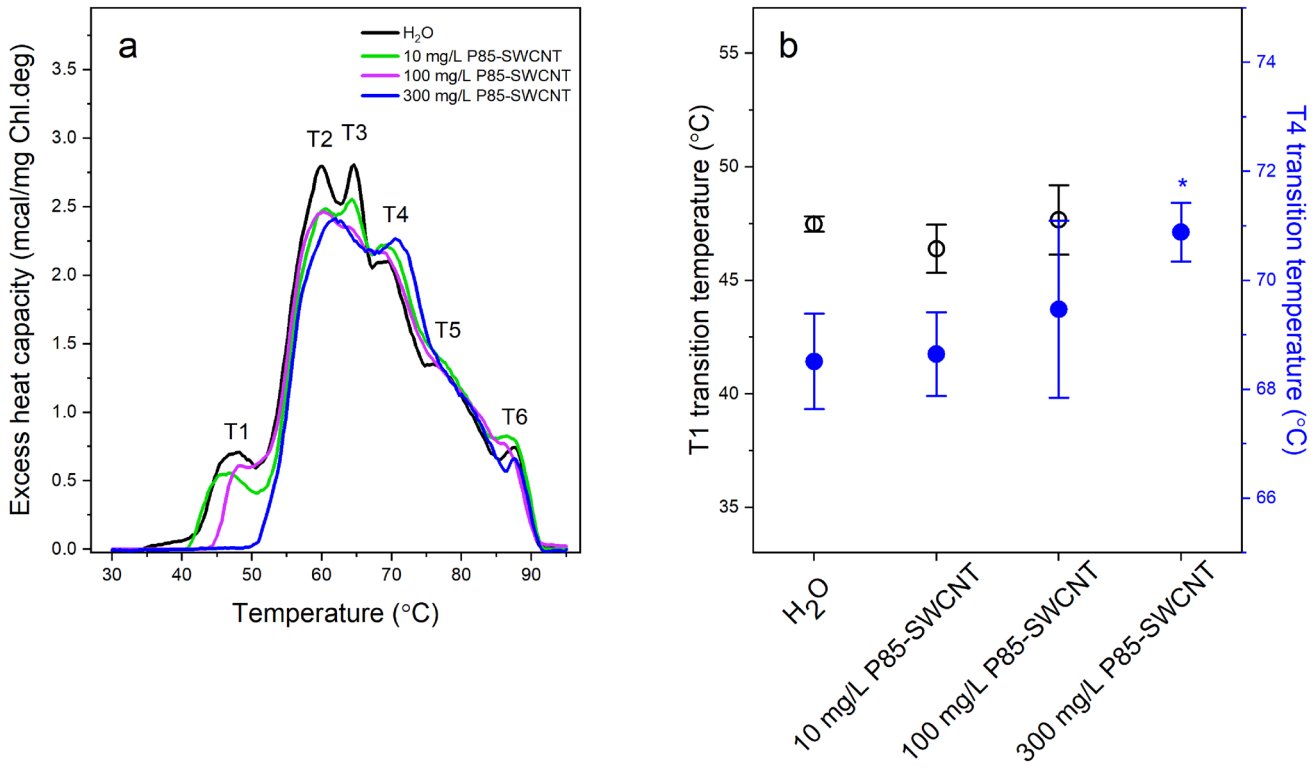

Fig. 2 Series of DSC scans of thylakoids isolated from pea leaves sprayed with different concentrations of P85-SWCNT. For clarity, the individual thermal transitions are indicated by T1–T6 (**a**). The transition temperatures (mean ± SD) of T1 (open black circles) and T4 (full blue circles), as a function of SWCNT concentration, are presented in (**b**). *Statistically significant difference from the control (H_2O treated) at $P < 0.02$ according to Student's *t* test

To detect any plausible harmful effects of P85-SWCNT on the thylakoid lipid matrix, we tested the level of MDA, which is generally considered as an indicator of lipid peroxidation in biological membranes (Mishra and Singhal 1992). In the case of thylakoids isolated from healthy plants, however, this parameter also correlated with the level of polyunsaturated fatty acids (Velikova et al. 2015). We found that MDA was 1.08 ± 0.21 µM in control and remained similar in 10 and 100 mg/L P85-SWCNT sprayed plants (1.16 ± 0.10 and 0.92 ± 0.20 µM, respectively). On the contrary, MDA amount was lower in thylakoids isolated from 300 mg/L P85-SWCNT-treated leaves (0.65 ± 0.06 µM), most probably due to a lower concentration of polyunsaturated fatty acids (and consequently reduced thylakoid membrane fluidity) in these samples. MDA is primarily derived from triunsaturated fatty acids in chloroplasts (Yamauchi et al. 2008; Schmid-Siegert et al. 2012) and serves to adsorb a portion of the reactive oxygen species (Mène-Saffrané et al. 2009) formed during the normal cell life as a part of the cell protection system. Therefore, the lower level of MDA in thylakoids isolated from 300 mg/L P85-SWCNT-treated leaves might in fact be related to decreased amount of polyunsaturated fatty acids rather than to oxidative stress.

Finally, we explored the correlation between the observed effects by means of Pearson's correlation analysis. We established that T4 temperature shift in the different P85-SWCNT treatments correlated very strongly with Chl *a*/*b* concentration in the grana [Pearson's $R = 0.94$, *R*-square (COD) = 0.88] and with MDA concentration [Pearson's $R = -0.98$, *R*-Square (COD) = 0.96].

The obtained results on grana abundance, grana Chl *a*/*b* ratio and thylakoid membrane thermal stability provide clear evidence that foliar application of P85-SWCNT affects strongly the structural organization of the photosynthetic machinery, most pronounced at 300 mg/L. Thylakoid swelling observed in our earlier work on P85-SWCNT-treated plants (Velikova et al. 2021) allows major reorganization of the photosynthetic complexes and thus serves as a crucial prerequisite for the regulation of photosynthesis by state transitions and NPQ (Lambrev and Akhtar 2019; Gu et al. 2022). Indeed, preserved relative grana abundance along with the increased Chl *a*/*b* ratio in grana, as well as the thermally induced changes in P85-SWCNT-treated thylakoids, strongly suggests that partial membrane unstacking and LHCII migration toward the stroma regions take place without full disassembly of the stacked membrane organization. The structural organization of the thylakoid membrane

in the 100 and 300 mg/L P85-SWCNT variants resembles the one observed for high light-adapted *Arabidopsis* leaves, characterized by expanded luminal space that facilitates protein diffusion and thus PSII repair (Kirchhoff et al. 2011; Li et al. 2020). This must also be associated with osmotic water and ionic fluxes (as proposed by Guo et al. 2022), which however are still not thoroughly studied.

Normally thylakoid swelling is reversible in the dark; however in our experiments, the 100 and 300 mg/L P85-SWCNT variants seem to be trapped in the light-adapted state and do not revert to the stacked configuration in the dark. This membrane remodeling might be a consequence of generation of a number of damaged PSII centers that need repair. Indeed, the lower number of active PSII centers, the lower extent of de-epoxidation, and the lack of the fast NPQ development phase, as previously reported in Velikova et al. (2021) could be associated with damaged PSII centers. The fact that a pool of LHCII migrates to the stroma regions (as judged by the change in Chl *a*/*b* ratio of grana) also means that less PSII-associated LHCII complexes are available for efficient NPQ formation and violaxanthin de-epoxidation in the grana (Johnson et al. 2011), which would explain why the fast phase of NPQ development is essentially lacking in the 100 and 300 mg/L P85-SWCNT samples (Velikova et al. 2021). Alternatively, NPQ development might be obstructed due to the diluted concentration of protons and proteins involved in photoprotection (by violaxanthin de-epoxidase) and protein repair (by DEG proteases) in the lumen induced by osmotic water influx as suggested by Li et al. (2020) and Guo et al. (2022).

However, as noted before, the effects induced by P85-SWCNT treatments in concentration lower than 300 mg/L are far from detrimental for the overall plants fitness and might even prove beneficial at certain environmental conditions, which however is yet to be explored. In support of this notion, here we did not find indication for generation of harmful active oxygen species; further detailed fatty acid composition analysis is needed to confirm this finding. Nevertheless, the presented data open up the possibility for future exploration of P85-SWCNT as cargo material for delivery of beneficial substances directly to the photosynthetic apparatus that would enhance its operation and functionality in a variety of stress conditions.

Conclusion

For the first time, we demonstrate that foliar application of P85-SWCNT in the concentration range 10–300 mg/L on intact pea plants induces structural remodeling of the thylakoid membrane system. In particular, the presented data indicate partial membrane unstacking, accompanied by thermal stabilization of LHCII and its migration from the grana toward stroma membrane regions, most pronounced at the highest used P85-SWCNT concentration.

Author contribution statement All authors contributed to the study conception, design, material preparation, data collection and analysis. The first draft of the manuscript was written by SK and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgements The work was supported by the Bulgarian National Science Fund, grant number KP-06-H36/8/13.12.2019.

Data availability The data are contained within the article and are available upon request.

Declarations

Conflict of interest The research leading to these results received funding from the Bulgarian National Science Fund under Grant Agreement No KP-06-H36/8/13.12.2019. The authors declare no conflict of non-financial interests.

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