A water-soluble [60]fullerene-derivative stimulates chlorophyll accumulation and has no toxic effect on *Chlamydomonas reinhardtii*

Jakub Lang¹, Mariia Melnykova¹, Michele Catania¹, Alicja Inglot¹, Aleksandra Zyss¹, Katarzyna Mikruta¹, Daria Firgolska¹, Agata Wieremiejczuk¹, Izabela Książek¹, Maciej Serda²,³, Paweł Nalepa², Bartosz Pluciński¹, Aleksandra Giza³ and Paweł Jedynak¹

¹Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Kraków, Poland; ²Institute of Chemistry, Faculty of Mathematics, Physics and Chemistry, University of Silesia in Katowice, Katowice, Poland; ³De-
glycine residues; Fv/Fm, maximal quantum efficiency of Photosystem II; C₆₀, water-soluble fullerene (GF, water-soluble [60]fullerene derivative with 12 glycine residues (GF) has been synthesized and tested for acute toxicity (up to 50 µg/ml) and as a potential biostimulant of algal growth. The effects of GF on pigment composition and growth rate of *Chlamydomonas reinhardtii* were systematically investigated. Our results suggest that GF was not toxic, and no negative change in the pigment content and no stress symptoms were observed. No changes in the photosynthetic parameters based on the fluorescence of chlorophyll a in Photosystem II (NPQ, Fₚ/Fₚ′, Fₚ/Φₚ, PI and RC/ABS) were observed. The GF had no effect on cell size and growth rate. At a concentration of 20 µg/ml, GF stimulated chlorophyll accumulation in 3-day-old cultures.

Key words: *Chlamydomonas reinhardtii*, algae, [60]fullerene-derivative, chlorophyll, toxicity

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INTRODUCTION

Engineered carbon nanomaterials have become more and more prevalent in industry, medicine and in precision agriculture. Nanoparticles (Rizvi et al., 2017), and the fullerenes among them, arouse potential interests for biotechnology. Pristine fullerenes are water-insoluble carbon spheres, typically containing about 60 atoms of carbon and fullerene C₆₀ has been the best studied one so far. Fullerenes became quickly recognized as generally detrimental or toxic to microbes, cyanobacteria, algae, plants and animals (Lin et al., 2009; Landa et al., 2012; Chen et al., 2018; 2019). Thus, their usage as biostimulants is limited, but on the other hand they can act as potential cytotoxic agents (Lucato et al., 2013; Frankevych et al., 2017), and as carriers for targeted drug-delivery in cancer therapies (Prylutksa et al., 2015; Lapin et al., 2017). In addition, fullerene supplementation may enhance the uptake and accumulation of toxic substances in plants grown on polluted soil (De La Torre-Roche et al., 2012).

In nanomedical literature, it is well described that the surface of fullerenes can be chemically modified, mainly by using the Bingel-Hirsch and Prato reactions, altering fullerene physical and chemical properties (reviewed in detail in: Goodarzi et al., 2017). Such a change affects the fullerenes biological activities and modulates their application as photosensitizers in photodynamic therapy and as in vivo transfection agents (Maeda-Mamiya et al., 2010; Sharma et al., 2011). The effects of water-soluble derivatives of fullerenes containing multiple hydroxyl (fullerol or fullerenol), amine and carboxyl groups were extensively studied in plants and animals (Ma & Liang et al., 2010). Fullerenol may penetrate through the cell membrane (Foley et al., 2002). Carbon nanomaterials smaller than 500 nm in length can easily get through the plant cell wall. Fullerenes may passively pass across cell membranes (due to their high affinity to the hydrophobic phase) (Bedrov et al., 2008), but diffusion of fullerol is a few orders of magnitude lower (Qiao et al., 2007). However, fullerene derivatives can be absorbed by endocytosis in animal (Zhang et al., 2009) and possibly plant cells, as endocytosis of carbon nanotubes was evidenced in the plant cells (Liu et al., 2009). Both, the fullerenes and fullerol were shown to accumulate in the cytoplasm of living tobacco (Kole et al., 2013; Husen & Siddigi, 2014) and rice cells (Lin et al., 2009).

Fullerenol and its derivatives can act both, as prooxidative (Sayes et al., 2004; Grebowski et al., 2013; Huang et al., 2014; Yin et al., 2015) and antioxidative agents (Prylutksa et al., 2008; Injac et al., 2013; Sachkova et al., 2017; Roy et al., 2018; Tyurin et al., 2018). It was found that...
fullerenol had generally no effect or tended to stimulate growth of photosynthetic organisms. In Arabidopsis thaliana, the hypocotyl length was increased and no other effects were observed (Gao et al., 2011). Fullerol treatment of Momordica charantia resulted in biomass increase by 54%, an increase in yield by 128% and a significantly enhanced accumulation of phytomedicines (Kole et al., 2013). Fullerol binds water molecules, thus greatly improving resistance of the sugar beet (Beta vulgaris) to drought stress (Borisev et al., 2016). In addition, fullerol application had decreased oxidative stress elicited by water-deficient conditions (Borisev et al., 2016). Little is known about its impact on algae. Fullerol treatment had increased cell density of Pseudokirchneriella subcapitata algae cultures (Gao et al., 2011). However, the negative effects of surface-modified fullerenes were also evidenced. The C_{al}(C(COOH))_{2}-4 derivative had caused auxin transport abnormalities and deformation of the root tip, as well as had decreased the shoot growth in Arabidopsis thaliana (Liu et al., 2010). Fullerol had also severely damaged the root cells of Allium cepa (Chen et al., 2010). However, the use of nanoparticles, including carbon allotropes, raises a question about their safety in the food chain and their environmental safety (Rico et al., 2011; Wang et al., 2018). Presence of C_{al} in the sewage had a negative effect on activated sludge and methanogenesis during anaerobic digestion (Zhao et al., 2018), but removal of fullerenols was efficient (>90%) and had no significant effect on the microorganism’s activity (Wang et al., 2011). Thus, the presence of fullerenol poses little risk of pollution in terms of wastewater production. Taking the above into consideration, it can be assumed that at low cost and low risk of serious environmental pollution, fullerenes may be potentially used as stimulants of growth of microorganisms cultured in bioreactors.

Chlamydomonas reinhardtii is a green microalga serving as a model in scientific research and a promising industrial biotechnology platform for production of biofuel, hydrogen and recombinant proteins (Scranton et al., 2015; Rasala et al., 2015; Scoma et al., 2015). Here, a novel type of water-soluble [60]fullerene derivative containing 12 glycine residues (GF) (Fig. 1) has been synthesized and tested for acute toxicity, and as a potential regulator for improvement of algal growth. Particularly, the effect of GF on pigment composition and growth rate of Chlamydomonas reinhardtii were systemically investigated. Our results suggest that GF was not toxic and could moderately stimulate pigment accumulation in a Chlamydomonas culture.

**MATERIALS AND METHODS**

**Materials for synthesis of fullerene derivatives.** All compounds were of reagent grade or better, solvents were used as received unless otherwise specified. The following reagents were used as received: C_{al} (99.5+%, MER Corps), glycine (Acros Organics), DBU (1,8-diaza-bicyclo (5. 4. 0) undec-7-ene, Sigma Aldrich), malonic acid (Sigma Aldrich), CBr_{3} (Sigma Aldrich), and sodium hydride (Acros Organics). Nuclear magnetic resonance spectra were measured on a Bruker Avance III 500 MHz NMR Spectrometer with tetramethylsilane as an internal standard. MS spectra for water-insoluble compounds were collected using an Autoflex II MALDI-TOF (Matrix Assisted Laser Desorption and Ionisations Time Of Flight) mass spectrometer, and for water-soluble [60]fullerene derivatives by an MS electrospray ionization time-of-flight (ESI-microTOF) mass spectrometer, both instruments from Bruker DaltonicsInc. High resolution spectra were performed using Shimadzu IT (Ion Trap) and TOFLC-MS System, and flash chromatography was performed using Isolera Flash Purification System. The purity of all compounds was assessed using an Agilent1260 equipped with a DAD detector at 260 nm, RP-column: Eclipse plus C18 (3.5 μm); flow rate 0.5 ml/min.

Highly water-soluble glycine derivative of [60]fullerene was synthesized using previously developed methodology (Serda et al., 2018a; Serda et al., 2018b).

**Biological material and growth conditions.** Axenic cultures of Chlamydomonas reinhardtii (WT 2137) P. A. Dang. (Volvoxales, Chlorophyceae) were obtained from Dr. Itzhak Ohad, Hebrew University, Department of Biological Chemistry, Givet Ram, Jerusalem, Israel, in the 1990s and cultured in our laboratory (Prasad et al., 1998). Cultures were grown under continuous white light (80 μmol × m^−2 × s^−1) OSRAM L36W/77, Germany) with shaking (125 rpm) in a Sager-Granick medium (Sager-Granick et al., 1953), supplemented with 100 mM mannitol as an osmoprotectant, and sodium acetate (75 mM), and citrate (1.7 mM) as sources of organic carbon, with addition of soluble, surface-modified (containing amino acid residues) fullerenes to final concentration of 0; 20; 40 and 50 μg/ml. Samples were collected after 3, 6 and 9 days of cultivation.

**Cell counting.** The cells were counted using LUNA-FI Dual fluorescence cell counter (Logos Biosystems, South Korea).

**Fluorescence of chlorophyll a in Photosystem II.** Photosynthetic parameters were measured using HANDY PEA fluorimeter equipped with Liquid-Phase Chlorophyll Fluorescence Adapter for Handy PEA (Hansatech Instruments, United Kingdom). All fluorescence measurements were conducted after 15 minutes of adaptation of algal samples to darkness. Then, the maximal quantum efficiency of Photosystem II (F_{v}/F_{m}), oxygen-evolving complex efficiency (F_{v}/F_{o}), the force generated by the chlorophyll in reaction center concentration per antenna chlorophyll (RC/ABS) and performance index (PI) were simultaneously measured. Additionally, the non-photochemical quenching (NPQ) was measured us-
Pigment composition. Chlorophyll and carotenoid content were estimated spectrophotometrically (UV-Vis spectrophotometer, JASCO, United States), according to Lichtenthaler (1987).

Statistical analysis. For group comparison, we used the Kruskal-Wallis rank sum test (Hollander & Douglas, 1973). Multiple comparisons between concentrations after Kruskal-Wallis test were done by the kruskalmc function from the pgirmess package (Siegel & Castellan, 1988), using the R (version 3.2.4) system for statistical computing (R Core Team, 2019). P-values less than 0.05 were considered to be significant. Graphs were produced using Origin 7.0 (OriginLab). Each experiment was repeated in pentaplicate.

RESULTS

The GF had no statistically significant effect on accumulation of carotenoids and chlorophyll b (Fig. 2B and D). The chlorophyll a content was significantly (P<0.05) increased in 3-day-old cultures treated with 20 µg/ml of GF (Fig. 2A) and the same was observed for total (a + b) chlorophyll concentration (Fig. 2C). Any other combination of GF concentration and time had no visible effect on the chlorophyll (a, b or total) content. No statistically significant effect on chlorophyll a/b ratio (Fig. 2E), nor chlorophyll to carotenoid ratio (Fig. 2F) was observed. The number of cells (Fig. 3A) in the control and GF-treated samples was similar, and the cell size decreased during the growth period in both, the control and tested cultures (Fig. 3B). Measurement of the chlorophyll a fluorescence in Photosystem II is a widely used technique of estimation of the physiological state of plants, for details see the following reviews (Maxwell & Johnson, 2000; Misra et al., 2012; Kalaji et al., 2014). We have measured selected parameters related to chlorophyll a fluorescence in Photosystem II. That allowed estimation of Photosystem II (Fv/Fm) efficiency, the number of chlorophyll molecules per reaction center (RC/ABS) that in turn allows the estimation of the number of antennas in one reaction center, and Fv/Fo related to the efficiency of oxygen-evolving complex and the Performance Index (PI), which allow estimation of the overall photosynthesis efficiency. GF had no significant effect on any of these parameters (Fig. 3C–F). We have also measured fluorometrically the non-photochemical quenching (NPQ), the efficiency of thermal dissipation of energy form excited chlorophyll molecules during stressful, high-light conditions) of 9-day-old cultures and no differences between the GF-treated and the control samples were observed (Fig. 4).

DISCUSSION

Our results indicate that at a concentration up to 50 µg ml\(^{-1}\), GF had no toxic effect on *C. reinhardtii*. GF had no negative effect on the pigment accumulation and did not interfere with photosynthesis and cell growth. No stress symptoms were observed. Pigment content and the chlorophyll to carotenoid ratio change dramatically under suboptimal conditions (e.g. salt stress) (Sairam & Tyagi, 2004; Hussein et al., 2014) and may be accompa-
nied by a significant increase of NPQ (Pak et al., 2009; Dongsansuk, 2013), suggesting weaker protection from higher intensities of light. No such change was observed during our experiment. On the contrary, we observed transient stimulation of chlorophyll a accumulation that might be beneficial. No other stimulating effect was observed at the tested concentrations, thus at the current stage of research there is no evidence that GF may be considered as a biostimulant of algal growth. However, its low toxicity and little interference with physiology of *C. reinhardtii* suggest that it can be developed as an excellent delivery system for entrapped ions and growth regulators (as fullerenes may act as cages with time-delayed release of molecules) or as a tool for DNA delivery. Since the GF surface is negatively charged, it may bind cationic molecules. It was shown that such properties may be used for reducing toxicity of heavy metals (Anderson & Barron, 2005), which may be complexed using hydroxyfullerenes. Furthermore, GF-based technology will be further developed and focused on the role of GF under stress conditions (as surface modified fullerenes enhance particular stress tolerance in plants), lipid accumulation and production of important biomolecules.

### Conflicts of interest

The authors declare that there is no conflict of interest regarding publication of this article.

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