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Regular paper

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

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***Chlamydomonas reinhardtii* (WT 2137) P. A. Dang. (*Volvocales, Chlorophyceae*) is a green microalgae serving as a suitable model in scientific research and a promising industrial biotechnology platform for production of biofuel, hydrogen and recombinant proteins. Fullerenes (C_{60}) are allotropic carbon nanoparticles discovered in 1985 and used in biomedical studies since the early 1990s, when water solubilization methodologies were developed. Recently, surface-modified hydroxylated derivatives of fullerenes were proven to enhance algal growth and drought tolerance in plants. Here, a novel type of 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, F_v/F_m, F_v/F_0, 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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Abbreviations: GF, water-soluble [60]fullerene derivative with 12 glycine residues; F_v/F_m, maximal quantum efficiency of Photosystem II; F_v/F_0, oxygen-evolving complex efficiency; PI, performance index; RC/ABS, the force generated by the RC concentration per antenna chlorophyll

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 C_{60} 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 (Lucafo *et al*., 2013; Franskevych *et al*., 2017), and as carriers for targeted drug-delivery in cancer therapies (Prylutska *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 therapies 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 fullerenol 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 fullerenol were shown to accumulate in the cytoplasm of living tobacco (Kole *et al*., 2013; Husen & Siddigi, 2014) and rice cells (Lin *et al*., 2009).

Fullerene 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 (Prylutska *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). Fullerenol binds water molecules, thus greatly improving resistance of the sugar beet (*Beta vulgaris*) to drought stress (Borišev *et al*., 2016). In addition, fullerenol application had decreased oxidative stress elicited by water-deficient conditions (Borišev *et al*., 2016). Little is known about its impact on algae. Fullerenol 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_{70} $(C(COOH)_2)_{4-8}$ 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). Fullerenol 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_{60} 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 microalgae 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 *C. reinhardtii* were systematically investigated. Our results suggest that GF was not toxic and could moderately stimulate pigment accumulation in a *C. reinhardtii* culture.

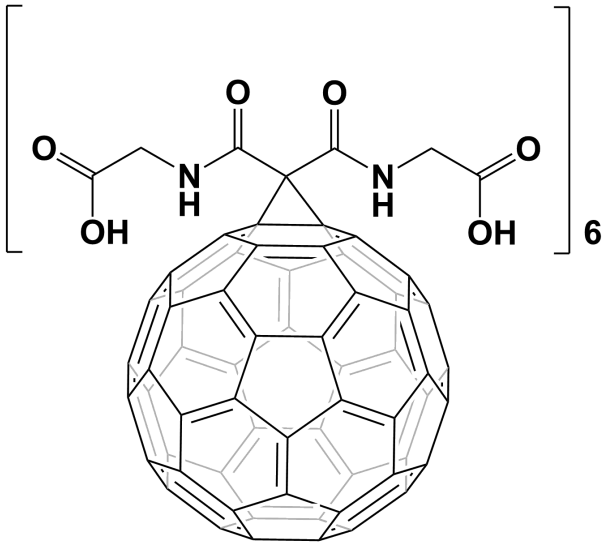

Figure 1. Chemical structure of GF, a [60]fullerene derivative containing 12 glycine residues

MATERIALS AND METHODS

Materials for synthesis of fullerene derivatives. All compounds used were of reagent grade or better, solvents were used as received unless otherwise specified. The following reagents were used as received: C_{60} (99.5+%, MER Corp.), glycine (Acros Organics), DBU (1,8-diaza-bicyclo (5. 4. 0) undec-7-ene, Sigma Aldrich), malonic acid (Sigma Aldrich), CBr_4 (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 Ionisation- 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. (*Volvocales*, *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 $\mu mol \times m^{-2} \times s^{-1}$ 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-Fl 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_0), 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-

ing Open FluorCam FC 800-O (Photon Systems Instruments, spol. s r. o., Czech Republic).

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 (F_v/F_m) 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 F_v/F_0 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-

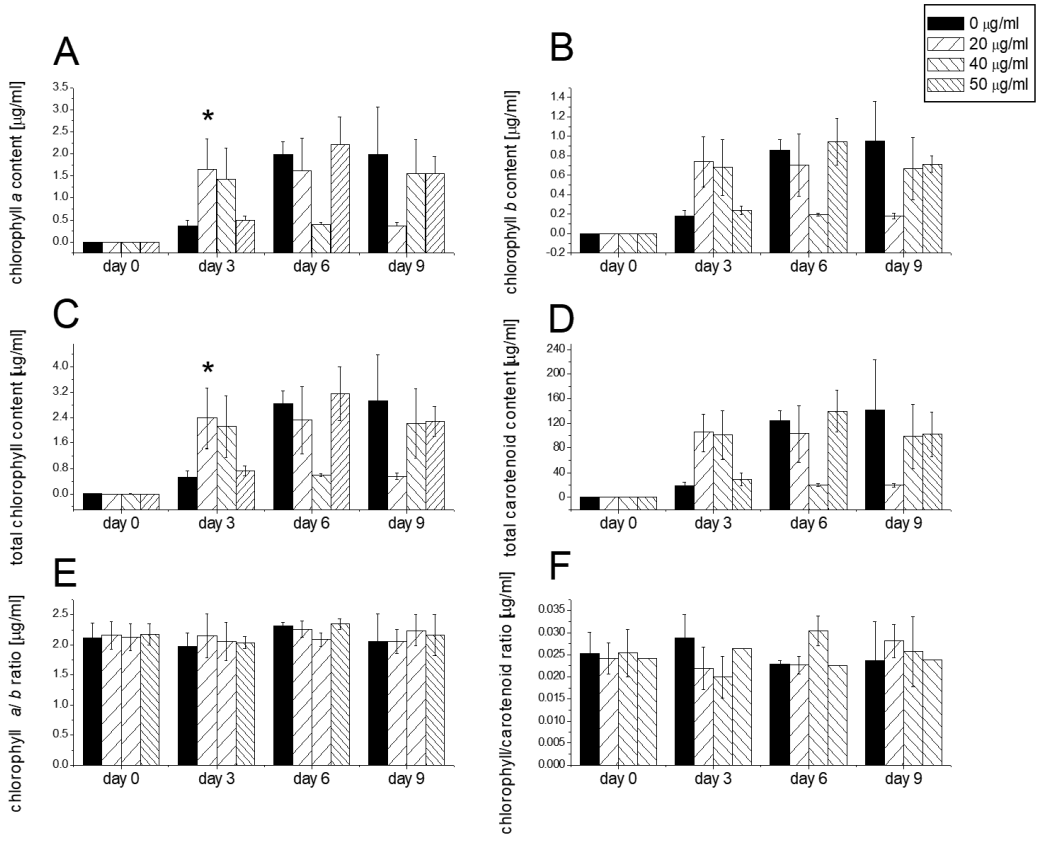

Figure 2. The effect of GF on pigment composition of *Chlamydomonas reinhardtii*.
Changes in the content of chlorophyll *a* (**A**) and *b* (**B**), as well as total chlorophyll (**C**), and total carotenoid (**D**); (**E**) the chlorophyll *a*/*b* ratio; (**F**) the chlorophyll to carotenoid ratio. Statistically significant differences ($P<0.05$) are indicated with *. Error bars – S.D.

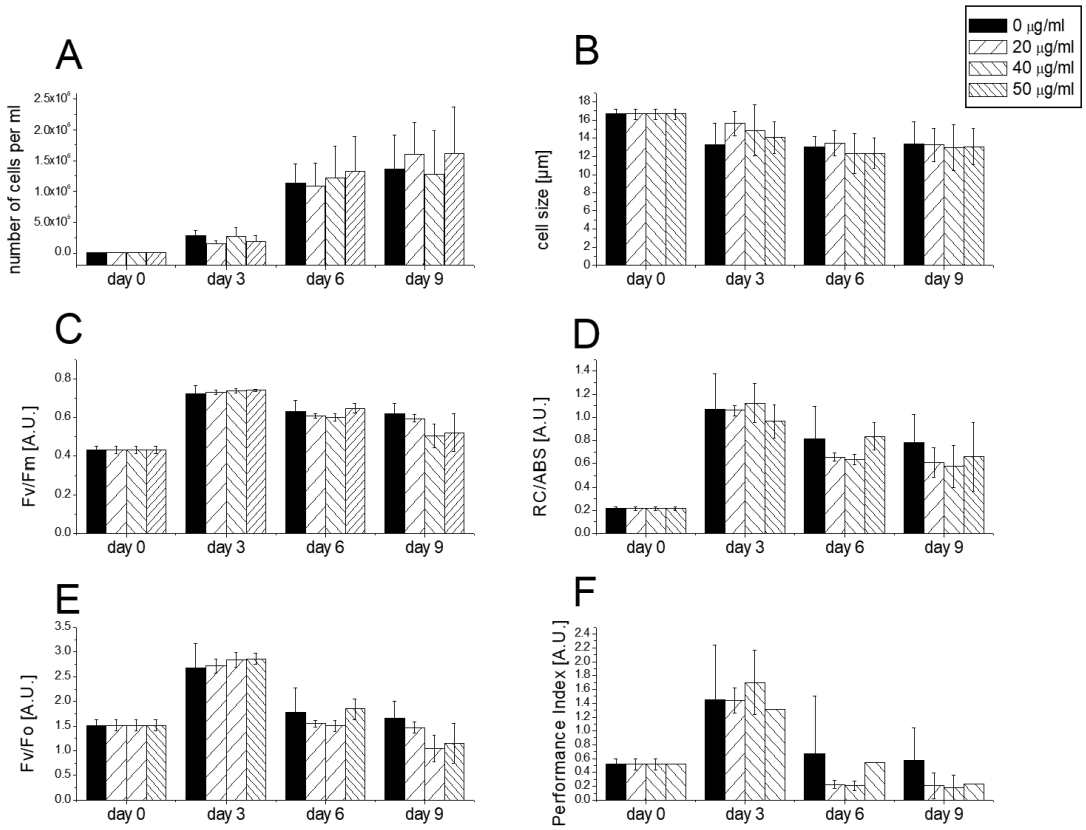

Figure 3. The effect of GF on cell size, number of cells per ml and selected photosynthetic parameters.
The number of counted cells (**A**) and cell size (**B**) measured by a cell counter. The results of fluorimetric measurements of the efficiency of photosystem II – F_v/F_m (**C**), the number of chlorophyll molecules per reaction center (**D**), the efficiency of water-evolving complex (**E**) and Performance Index (**F**) indicating overall photosynthesis efficiency. All data were checked for statistically significant differences ($P<0.05$). Error bars – S.D.

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.

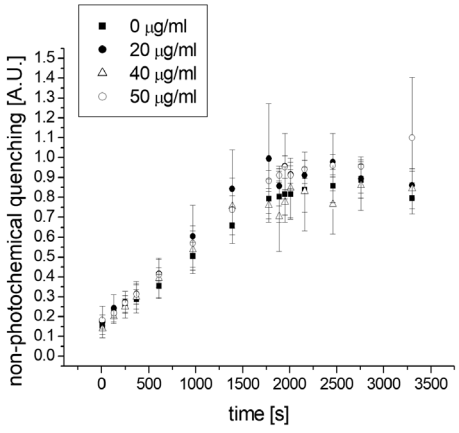

Figure 4. The effect of GF on non-photochemical quenching in *C. reinhardtii*
The fluorimetric measurements of NPQ in 9-day old cultures of *C. reinhardtii*. Dark-adapted cultures were irradiated with actinic (saturating photosynthesis) light per 1700 s, then the fluorescence was recorded under pseudo-dark conditions. Statistically significant differences ($P<0.05$) are indicated with *. Error bars – S.D.

Conflicts of interest

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

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