Journal of Bioscience and Bioengineering
VOL. 128 No. 6, 751–754, 2019

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# Polyethyleneimine-induced astaxanthin accumulation in the green alga *Haematococcus pluvialis* by increased oxidative stress

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Received 6 April 2019; accepted 4 June 2019
Available online 25 June 2019

**The unicellular green microalga *Haematococcus pluvialis* accumulates large amounts of the red ketocarotenoid astaxanthin under stress conditions such as nitrogen deficiency. In this study, we discovered an astaxanthin accumulation in *H. pluvialis* cells by the addition of a synthetic cationic polymer, polyethyleneimine (PEI), into the cell culture. With an increase in PEI amount, amount of astaxanthin accumulation was increased. To investigate the mechanism for the accumulation of astaxanthin by the addition of PEI in *H. pluvialis* cells, we measured a localization of PEI in the cells and a production of reactive oxygen species. PEI was internalized in the cells through the negatively-charged cell walls, leading to excessive production of reactive oxygen species in the cells. Thus, the increased oxidative stress by cellular uptake of PEI resulted in the acceleration of astaxanthin accumulation in *H. pluvialis*.**


[**Key words:** Polyethyleneimine; *Haematococcus pluvialis*; Astaxanthin; Cellular uptake; Reactive oxygen species; Flocculant; Oxidative stress; Microalgae]

Phototrophic microalgae are ubiquitous organisms in both fresh and sea waters. They convert photosynthetically atmospheric carbon dioxide to storage lipid, starch, and high value-added products such as astaxanthin. Recently, due to these features, these microorganisms have attracted much attention as feedstock for biofuels, food, feed, and cosmetics in a wide range of field (1). The photosynthetic microalga *Haematococcus pluvialis* is a potential source of astaxanthin, which is a fat-soluble carotenoid pigment with extraordinary antioxidant capability (2). Astaxanthin has protective and therapeutic effects on oxidative stress-related diseases by scavenging reactive oxygen species (ROS) (3). Moreover, astaxanthin is used world-wide as feed supplement in fish farming in order to grow healthy and well colored fish. Furthermore, it is used as a natural dyestuff in the food and cosmetic industries (4). Accumulation of astaxanthin in *H. pluvialis* occurs *via* morphological changes in cells, from green vegetative cells moving with flagella to red resting cyst cells. These changes occur under environmental stress conditions such as nitrogen deficiency (5) and exposure to strong light (6). The astaxanthin accumulation occurs due to the response of the cells to protect themselves from the oxidative stress caused by ROS (7).

Cationic polymers have been used as a flocculant of microalgae harvesting as they neutralize the negative charge of an algal cell wall (8). The first aim of this experimental investigation was to evaluate the utility of polyethyleneimine (PEI) as a microalgal flocculant in the culture of *H. pluvialis* cells. At the amount of 20,000 fmol/cell, PEI showed effective flocculation without the astaxanthin accumulation. Interestingly, the astaxanthin accumulation in *H. pluvialis* cells was found at a lower amount of 1000 fmol/cell, without cell flocculation. So far, there has been no report about astaxanthin accumulation by the addition of polycation. In this study, we investigated the mechanism for the accumulation of astaxanthin by the addition of PEI in *H. pluvialis* cells.

## MATERIALS AND METHODS

**Algal strain, medium composition, and culture condition** *H. pluvialis* NIES-144 was obtained from the National Institute for Environmental Studies (Tsukuba, Japan). The strain was cultured in NIES-C medium (pH 7.5). The constituents of NIES-C medium have been previously described (9).

**Addition of PEI to culture of *H. pluvialis*** Cells were harvested during the exponential phase of growth. The cell number was counted microscopically using a hematocytometer. Branched PEI with a number-average of molecular weight of 10 kDa (cat No. 9002-98-6, Sigma–Aldrich, St. Louis, MO, USA) was added to 30 mL of cell culture ($5.0 \times 10^3$ cells/mL) at amounts of 0, 10, 100, 1000 and 20,000 fmol/cell, in which final concentrations of PEI were 0, 0.5, 5, 50, and 1000 μg/mL, respectively. Cells were grown for three days in NIES-C medium at 25°C under continuous illumination that was provided by fluorescent lamps at a photon flux density of 90 μmol $m^{-2}$ $s^{-1}$. Cells were observed with a Zeiss Axio Observer Z1 microscope equipped with a CCD Axiocam 105 color camera (Zeiss, Oberkochen, Germany).

**Extraction of astaxanthin and thin-layer chromatography analysis** Freeze-dried cells were suspended in 1 mL of dichloromethane-methanol (25:75, v/v%) solution and homogenized using a tip ultrasonicator (Qsonica Sonicators model Q55, Qsonica, Newtown, CT, USA) for 1 min to extract the astaxanthin. After extraction, cells were collected by centrifuging the homogenized suspension at 10,000 ×*g* for 10 min at 10°C. The extraction

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1389-1723/$ – see front matter 
https://doi.org/10.1016/j.jbiosc.2019.06.002

procedure was repeated three times until cell debris was almost colorless (10). The extracted solution was evaporated and then the extract was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Osaka, Japan). Carotenoids extracted from the cells were separated on silica gel 60 TLC plates (cat No. 105721, Merck Millipore, Burlington, MA, USA) using acetone:*n*-hexane (30:70 v/v%) solvent. Astaxanthin amounts on the thin-layer chromatography (TLC) were analyzed by imageJ software. The relative amount of astaxanthin is expressed as the value relative to that without the addition of PEI.

**Preparation of fluorescent labeling of PEI and its cellular uptake** Fluorescein-labeled PEI (F-PEI) was prepared *via* an amide bond between PEI and 5-carboxyfluorescein in *N*,*N*-dimethylformamide (Wako). Briefly, 0.1 mg of PEI, 1.0 mg of 5-carboxyfluorescein succinimidyl ester (Molecular Probes, Eugene, OR, USA), 6.8 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Dojindo, Kumamoto, Japan), and 6.8 mg of *N*-hydroxysuccinimide (NHS; Sigma–Aldrich) were dissolved in 20 mL of *N*,*N*-dimethylformamide and stirred for 2 h at room temperature. The F-PEI solution was transferred into a pre-swollen membrane tube (Spectra/Por; molecular-weight cutoff size: 3500) and dialyzed for 24 h against 2 L water.

F-PEI was added to the culture medium at the amount of 1000 fmol/cell. Images by wide-field fluorescence microscopy of F-PEI (excitation: 400–490 nm, emission: 500–550 nm) and chlorophyll autofluorescence (excitation: 540–580 nm, emission 593–668 nm) were captured using a Zeiss Axio Observer Z1 microscope equipped with an AxioCam MRm digital camera. Confocal microscope images of F-PEI (excitation: 488 nm, emission: 500–550 nm) and chlorophyll autofluorescence (excitation: 561 nm, emission 575–615 nm) were captured using a Nikon $C2^{+}$ confocal imaging system (Nikon Instech, Tokyo, Japan) mounted on a Nikon Eclipse Ti-E inverted microscope.

**Analysis of ROS generation in cells** General levels of ROS were measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Cayman Chemical Company, Ann Arbor, MI, USA), which has been used for the measurement of the ROS levels in *H. pluvialis* (11–13). The DCFH-DA was dissolved in DMSO and used as a stock solution at a concentration of 0.5 mg/mL in order to analyze cellular oxidative stress status. Briefly, after branched PEI with a number-average of molecular weight of 10 kDa was added to 100 μL of cell culture ($5.0 \times 10^3$ cells/mL) at amounts of 1000 fmol/cell, 1 μL of DCFH-DA solution was added to the cell suspensions and mixed, followed by incubation for 1 h in the dark. Fluorescence from the DCF was detected using a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA), quantifying 488 nm-excited fluorescence signals at 525/50 nm. Microscope images of DCF fluorescence (excitation: 400–490 nm, emission: 500–550 nm) and chlorophyll autofluorescence (excitation: 540–580 nm, emission 593–668 nm) were captured using Zeiss Axio Observer Z1 microscope equipped with AxioCam MRm digital camera.

## RESULTS AND DISCUSSION

Since microalgal cells possess cell walls with the negative charge, polycations neutralize the charge of the cell walls, thus promoting the flocculation of algal cells that settle *via* gravitational forces. As shown in Fig. S1, the addition of PEI at a high amount of 20,000 fmol/cell induced the flocculation of *H. pluvialis* without astaxanthin accumulation; in the present condition, the pH value of culture medium was increased from 7.5 to 8.9. An exposure to alkali results in a cell death and a significant loss of astaxanthin because astaxanthin which possess the 3-hydroxy, 4-keto end-group is unstable in the presence of alkali (14). Therefore, we investigated the effect of PEI on astaxanthin accumulation at the PEI amounts ranging from 10 to 1000 fmol/cell. The pH value of the condition at the PEI amounts of 1000 fmol/cells was 7.6; this slight increase in pH can be ignored for astaxanthin accumulation (data not shown). Interestingly, we noted that the color of the cells changed from green to red with PEI amounts ranging from 10 to 1000 fmol/cell, without cell flocculation (Fig. 1A). To confirm the accumulation of astaxanthin, TLC was performed. Astaxanthin diester and monoester were separated on the TLC plate (Fig. S2), which corresponded with the results in previous report (15). The accumulation of both astaxanthin diester and monoester increased with increasing PEI amount. Total amount of astaxanthin diester and monoester at the PEI amount of 1000 fmol/cell was 5.5-fold higher than that obtained without the addition of PEI (Fig. 1B).

To investigate the cellular uptake of PEI, F-PEI was synthesized. After F-PEI was added into the culture at the amount of 1000 fmol/cell, fluorescent images of the cells were observed. One hour after incubation, fluorescent signal was detected in the cell walls (Fig. 2).

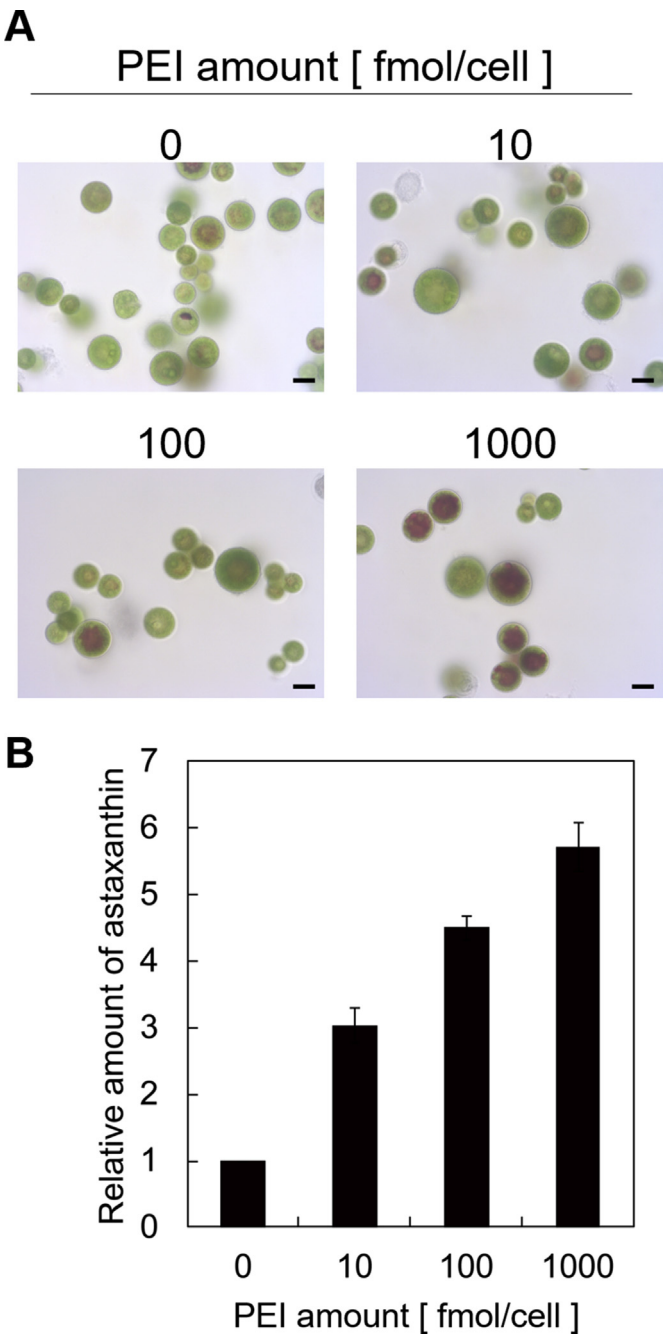


FIG. 1. Astaxanthin accumulation in *Haematococcus pluvialis* by the addition of PEI. (A) Images of *H. pluvialis* incubated with PEI at different amounts for three days. Scale bar: 20 μm. (B) TLC analysis of astaxanthin accumulation by PEI addition. Relative amounts of astaxanthin after extraction of astaxanthin from *H. pluvialis* cells incubated with PEI for three days, as determined by TLC results. Normalized astaxanthin accumulation was calculated by the analysis of TLC using *imageJ* software.

Fluorescent signal in the cells was gradually increased after incubation. Fig. S3 shows the time course of fluorescent intensity in the cells after addition of F-PEI. Even 1 h after addition of PEI, fluorescent intensity in the cells was increased, indicating cellular uptake of PEI. On the other hand, the autofluorescence of chlorophyll gradually decreased. As shown in fluorescent images at 2 h (Fig. 2), the cells with green fluorescent signal derived from cellular uptake of F-PEI had the decreased autofluorescence of chlorophyll, while the cells with the less green fluorescent still showed the strong autofluorescence of chlorophyll. Note that the light irradiation by microscopic observation did not decrease the autofluorescence of

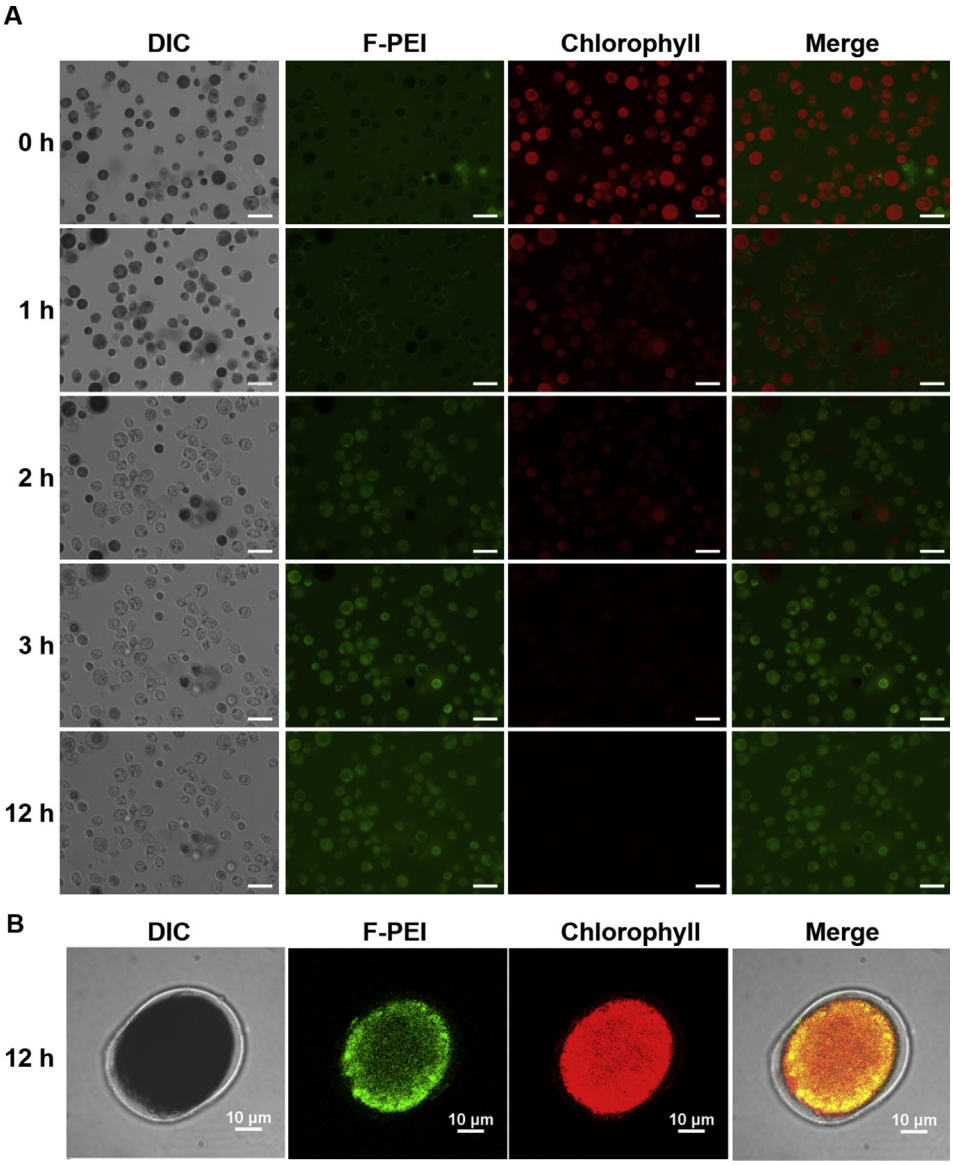


FIG. 2. Cellular uptake of F-PEI. (A) Time course of differential interference contrast (DIC) and fluorescent images by wide-field fluorescence microscopy of *H. pluvialis* after adding F-PEI at the amount of 1000 fmol/cell. In the fluorescence images, the F-PEI and the autofluorescent chlorophyll are shown as green and red, respectively. Scale bars: 50 μm. (B) DIC and fluorescent images by confocal fluorescence microscopy of *H. pluvialis* at 12 h after adding F-PEI at the amount of 1000 fmol/cell. In the fluorescence images, the F-PEI and the autofluorescent chlorophyll are shown as green and red, respectively. Scale bars: 10 μm.

chlorophyll (Fig. S4). This provides an evidence that the chlorophyll degradation was attributed by cellular uptake of PEI. At 12 h after adding F-PEI into the cell culture, the cellular uptake of F-PEI and the autofluorescence of chlorophyll were observed using confocal microscope. This result showed that the autofluorescence of chlorophyll remained at 12 h after the addition of PEI. In addition, the fluorescent signal of F-PEI was detected in the same cross section as the autofluorescence of chlorophyll, providing the evidence of the cellular uptake of F-PEI.

Here, we investigated the effects of PEI on the oxidative stress in cells using DCFH-DA in order to understand the mechanism of astaxanthin accumulation. In the cell, non-fluorescent DCFH-DA is taken up and converted into dichlorodihydrofluorescein (DCFH) by the action of cellular esterases, which is oxidized to a fluorescence dye 2′,7′-dichlorofluorescein (DCF) by ROS. Fig. 3A shows that a strong fluorescent signal was detected in the cells after the addition of PEI at the amount of 1000 fmol/cell. Note that the parts of cells were stained by DCF; the extent of DCF staining between the cells was different.

*Haematococcus* cells always alter their morphology and metabolism during a cell cycle (16). This heterogeneity by cell cycle may be the cause of difference of ROS production and astaxanthin accumulation rate in the cells. Also, flow cytometer analysis showed that the fluorescent intensity increased in the cells with the addition of PEI at the amount of 1000 fmol/cell. These results indicate that the production of ROS increased within the cells by the addition of PEI (Fig. 3B). Recently, it has been reported that the cytotoxicity is related to an increased oxidative stress in animal cells, in which PEI inhibits the electron transport system of mitochondria, resulting in cell death (17). Since not only mitochondria but also chloroplasts exist in the microalgal cells, perhaps, PEI may lead to ROS generation by the inhibition of the electron transport system of both mitochondria and chloroplasts in *H. pluvialis*.

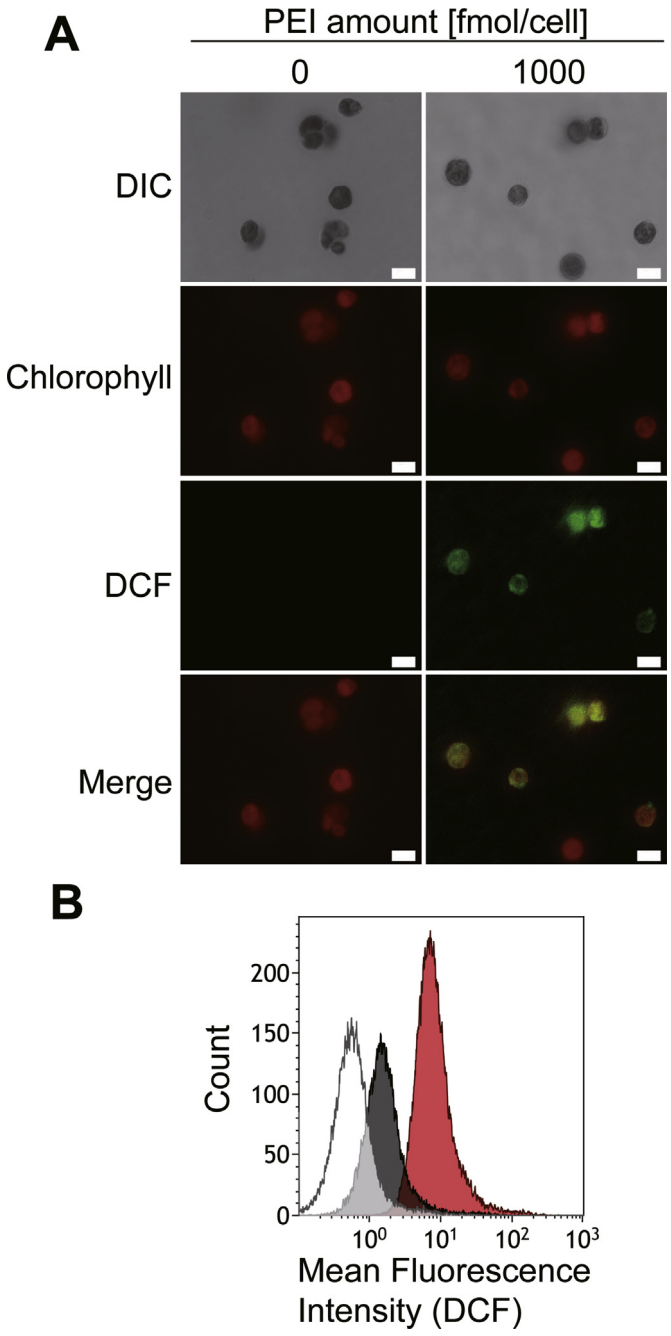


FIG. 3. Analysis of ROS generation in *Haematococcus pluvialis* cells using DCFH-DA. (A) Differential interference contrast (DIC) and fluorescent images of *H. pluvialis* 1 h after adding PEI at the amount of 1000 fmol/cell. In the fluorescence images, the fluorescent DCF and the autofluorescent chlorophyll are shown as green and red, respectively. Scale bars: 20 μm. (B) Flow cytometric histogram of *H. pluvialis* cells by DCFH-DA analysis. White: unstained cells; black: stained cells; red: stained cells 1 h after adding PEI at the amount of 1000 fmol/cell.

In summary, we demonstrated that PEI induces astaxanthin production in *H. pluvialis* by cellular uptake of PEI. To the best of our knowledge, this is the first report showing that PEI promotes astaxanthin accumulation in *H. pluvialis* cells by increasing oxidative stress *via* cellular uptake, which is new stimuli for the astaxanthin accumulation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.06.002.

## ACKNOWLEDGMENTS

We would like to thank Ms. Haruka Karita (Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo) for her technical assistance. Part of this work was supported by the Yashima Environmental Technology Foundation (to T.Y.), the Foundation for Interaction in Science & Technology, Japan (to T.Y.), and the Japan Society for the Promotion of Science KAKENHI Grants 18K04845 (to T.Y.). The authors declare no conflict of interest.

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