Supporting information

Novel Insights into the Joint Phytotoxicity of Environmental Relevant Concentration Nanoplastics and Silver Ions: A Dual Aggregation-Induced Emission Bioimaging Approach

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Synthesis of AIEgens incorporated polystyrene nanoplastics. We synthesized AIEgens incorporated polystyrene nanoplastics (AIENPs) using the following procedures that were modified from our previously reported protocols.[1] Initially, we added dodecyl benzene sulfonate (SDS), hydroxide, and deionized water into a three-necked flask. The mixture was then heated to 70°C. Next, we sequentially added styrene (St), acrylic acid (AA), methyl methacrylate (MMA), and TPA-M (AIEgen Biotech Co., Ltd., in THF, 5 wt % polymer, 2 mL) while continuously stirring for 0.5 hours. The temperature was increased to 80°C, and then the initiator potassium persulfate (KPS) was added. The detailed recipe of these different chemicals for synthesizing 40 nm AIENPs (40AIENPs) and 80 nm AIENPs (80AIENPs) can be found in Table S1. After a reaction time of 10 hours, the NPs were obtained. To purify the synthesized NPs and remove unreacted reactants, we employed a stirring ultrafilter cup with a molecular weight cutoff of 1 kDa (Merck Millipore). The AIENPs were washed three times with deionized water and then dispersed in deionized water. Finally, the purified NPs were stored at 4°C in a dark environment for subsequent use.

Characterization of AIEgens incorporated polystyrene nanoplastics. The size and morphology of synthesized different aged AIENPs were characterized using scanning electron microscopy (SEM) (JSM-6700F). The surface roughness of NPs was assessed using an atomic force microscope (AFM, Dimension Icon, Bruker, Santa Barbara, CA). Additionally, the hydrodynamic diameter and zeta potential of AIENPs, at a concentration of 1 mg/L, were determined through dynamic light scattering (DLS) using a Malvern Instrument Zetasizer Nano Series (Malvern Instruments, Westborough, MA) equipped with a He–Ne laser (λ = 633 nm, max 5 mW) and operated at a scattering angle of 173°. To investigate the stability of AIENPs, NPs with different sizes (1 mg/L) were respectively added to a modified simplified M7 medium (SM7 medium). The SM7 medium contained the following concentrations: 0.04 mM $NaHCO_3$, 0.35 mM $CaSO_4$, 0.50 mM $MgSO_4$, 0.05 mM KNO_3, and maintained a pH range of 7.0 to 8.0. The size distribution of AIENPs was then monitored at different time points (0 to 600 minutes) using DLS. The specific surface areas of the synthesized AIENPs were analyzed using a Brunauer–Emmett–Teller (BET) surface area analyzer (Model SA 3100, Beckman Coulter). Fourier transform infrared (FTIR) spectra ranging from 400 to 4000 cm^{-1} were employed to identify possible variations in functional

groups (Bruker FTIR, Vertex 70 Hyperion 1000, Germany). The hydrophilicity of the AIENPs was assessed through contact angle measurements (Krüss, Hamburg, Germany). The purity of the synthesized AIENPs was characterized using a UV-vis spectrometer (Shimadzu, Japan). The fluorescence characteristics of the AIENPs were monitored using a PerkinElmer Model LS 55 spectrofluorometer.

Algal Culture. *C. reinhardtii*, a freshwater green algae, was cultured in the WC medium under a controlled light-dark cycle of 16 hours of light and 8 hours of darkness.[2] Prior to use, the algal cells were harvested by centrifugation at 3500 rpm for 10 minutes. The supernatant was discarded, and the algal pellets were resuspended in SM7 medium. After the third wash with SM7 medium, the cell pellets were retained for dilution into the SM7 medium. The cell density of the concentrated algae was determined using a hemocytometer, allowing for accurate cell count. To measure the dry weight, the algae were collected by centrifugation at 12000 rpm for 5 minutes. The collected cells were washed twice with distilled water to remove any residual medium. Subsequently, they were dried at 105°C for 24 hours and weighed to obtain the total dry weight of the algae. This process ensure the preparation of concentrated and standardized cultures of *C. reinhardtii* for subsequent experiments.

Joint toxicity analysis. To assess the combined toxic effects of Ag^+ and NPs, the toxic unit (TU) approach was employed. This method evaluates the combined toxicity by aggregating the individual toxic effects of each compound. The TU_i can be described using the following equation:

$$M = \sum TU_i = \sum_{i=1}^{n} \frac{C_{mix,i}}{EC50_i}$$

where TU_i is the toxic unit of component i in the mixture, n is the number of components in the mixture, $C_{mix,i}$ is the concentration of component i in the mixture, and $EC50_i$ is the EC50 of component i when it acts independently on the organisms. M is the sum of the TU_i values for each component. When the M value was observed to be <1, the combined effect was interpreted as a synergistic effect. In contrast, when the M value was observed to be >1, the combined effect was interpreted as an antagonistic effect.

In mathematical terms, the degree of joint toxicity for the mixtures was evaluated using the

Additional Index (AI) method, allowing for the calculation of toxicity enhancement factors. AI was defined as follows:

$$AI=M-1.0, \quad \text{when } M=1$$

$$AI=1/M-1.0, \quad \text{when } M=1$$

$$AI=M\times(-1)+1.0, \text{ when } M=1$$

Internalization pathways of AIENPs. To investigate the internalization pathways of AIENPs, algal cells at a density of 1×10^5 cells/mL were incubated for 30 minutes with specific endocytosis inhibitors. The inhibitors used were sodium azide (NaN_3, 0.25 mM), monodansylcadaverine (MDC, 0.2 mM), 5-(N-ethyl-N-isopropyl) amiloride (EIPA, 10 μM), and filipin complex (FC, 5 μM). These inhibitors were utilized to block different endocytic routes and evaluate their impact on AIENPs internalization. The total amount of AIENPs internalized without adding inhibitors was compared with the amount internalized when inhibitors were present. A 24-hour time period was chosen to assess the endocytosis of the algae, and flow cytometry was employed to determine the intracellular presence of AIENPs after treatment with different inhibitors.

Statistical Analysis. Use GraphPad Prism 9.0 software and Origin 2022 for statistical analysis. Perform statistical analysis on confocal images using Fiji Image J. One-way ANOVA was used to determine the difference between the control group and the experimental group, with $p<0.05$.

Table S1. Detailed recipe of these different chemicals for synthesizing 40AIENPs and 80AIENPs

Particle type	Ingredients	Concentration
40AIENPs	Styrene	8.73 mM
	Ammonium bicarbonate	1.5 mg/mL
	Methyl methacrylate	0.93 mM
	Acrylic acid	1.46 mM
	Sodium dodecyl benzenesulfonate	5 mg/mL
	AIEgens (TPA-M)	1 mg/mL
	Potassium peroxodisulfate	76 mg/mL
	Water	30 mL
80AIENPs	Styrene	8.73 mM
	Ammonium bicarbonate	1.5 mg/mL
	Methyl methacrylate	0.93 mM
	Acrylic acid	1.46 mM
	Sodium dodecyl benzenesulfonate	0.72 mg/mL
	AIEgens (TPA-M)	1 mg/mL
	Potassium peroxodisulfate	50 mg/mL
	Water	30 mL

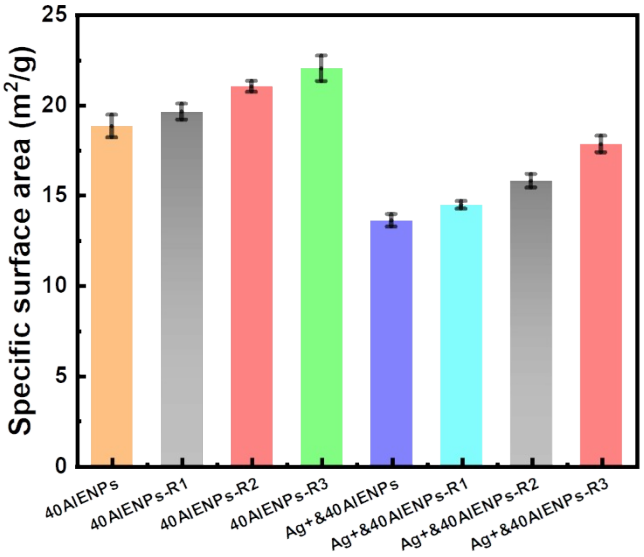

Figure S1. The specific surface area of different-sized and aged AIENPs at 1 mg/L. There were ten replicates in each measurement.

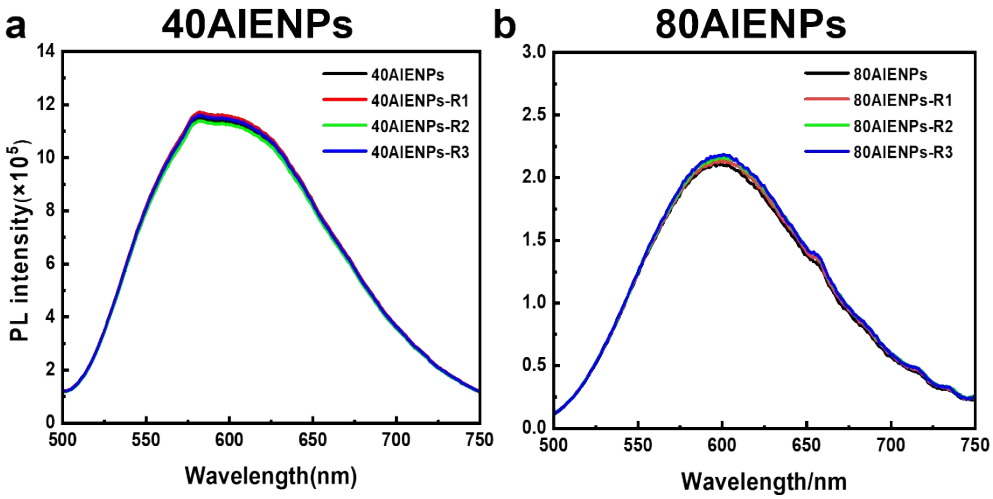

Figure S2. PL spectra of different-sized and aged AIENPs (1 mg/L) after 24 hours exposure in SM7 medium. Fluorescence signals of 40AIENPs and 80AIENPs with four different aging degrees. The excitation wavelength was 488 nm.

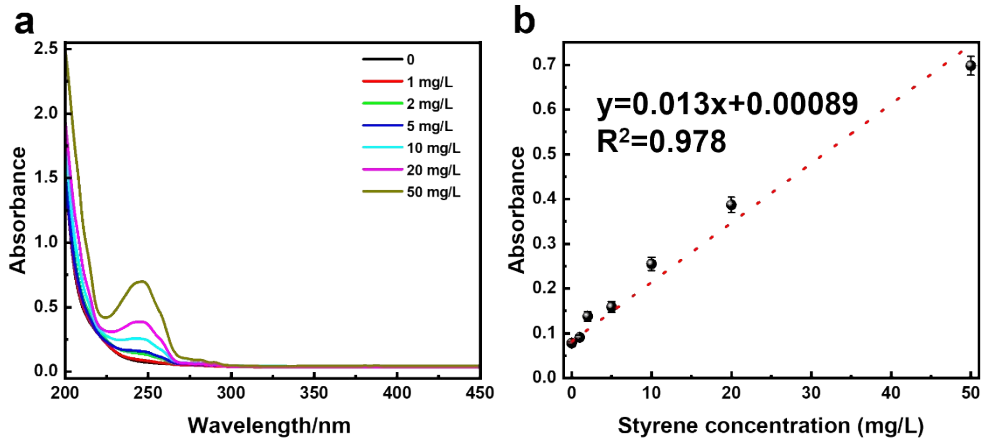

Figure S3. Quantification of the leaked residual monomer styrene from the synthesized AIENPs. (a) UV-Vis spectra of 40AIENPs at a concentration range from 0 to 50 mg/L. (b) The correlation between styrene concentration and UV-Vis absorbance.

Table S2. Toxic unit model and additional index of Ag^+ and NPs mixtures on algae.

Tested pollutants	∑TU	AI
40AIENPs&Ag^+	0.74	0.35
40AIENPs-R1&Ag^+	0.55	0.82
40AIENPs-R2&Ag^+	0.51	0.96
40AIENPs-R3&Ag^+	0.48	1.08
80AIENPs&Ag^+	1.15	-0.15
80AIENPs-R1&Ag^+	1.27	-0.27
80AIENPs-R2&Ag^+	1.32	-0.32
80AIENPs-R3&Ag^+	Not applicable	Not applicable

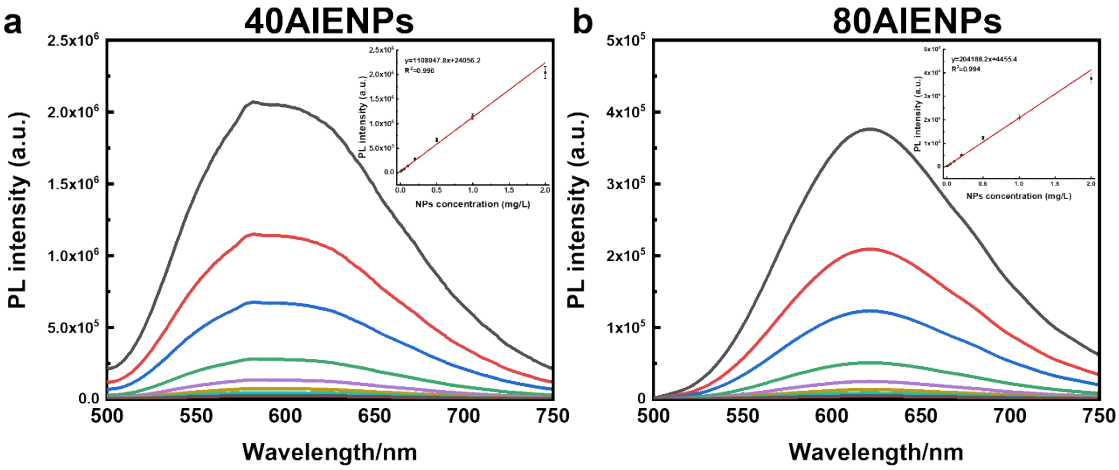

Figure S4. Correlation between the concentration of NPs (0 to 2 mg/L) and their PL intensity. Correlation between the concentration of 40AIENPs (a) and 80AIENPs (b) and their PL intensity. There were three replicates for each treatment.

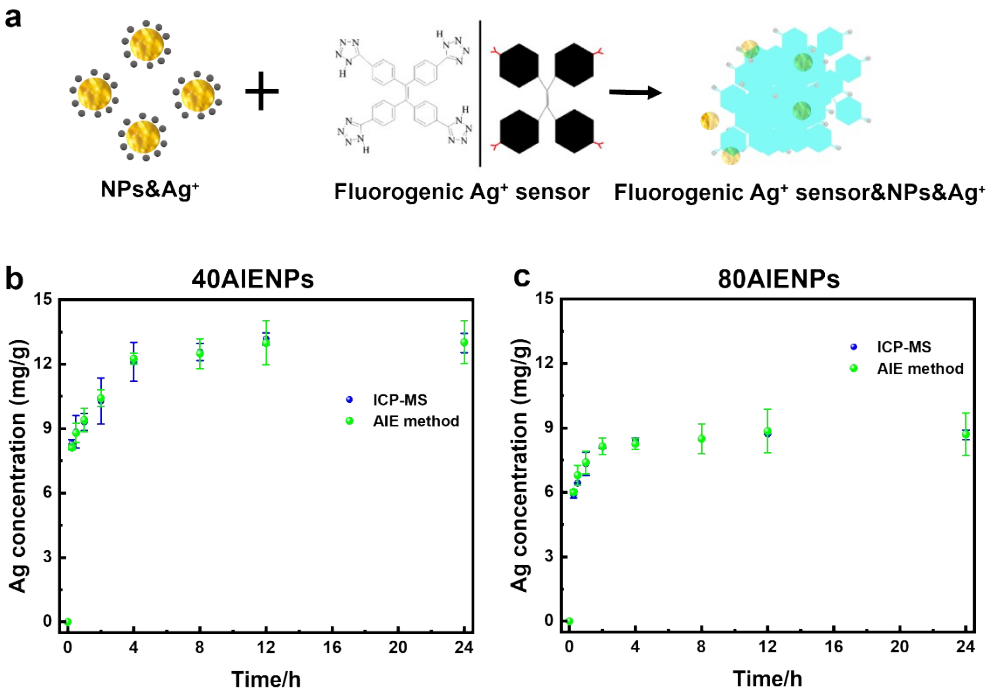

Figure S5. Detection of Ag^+ when NPs and Ag^+ were coexposed in the SM7 medium. (a) Scheme for the illustration of fluorogenic Ag^+ sensor and the Ag^+ detection via Ag^+-tetrazolate aggregation-triggered AIE process when Ag^+ were coexposed in the SM7 medium. Comparison of the absorbed Ag^+ concentration when coexposed with 40AIENPs (b) and 80AIENPs (c) using the ICP-MS detection and AIE method.

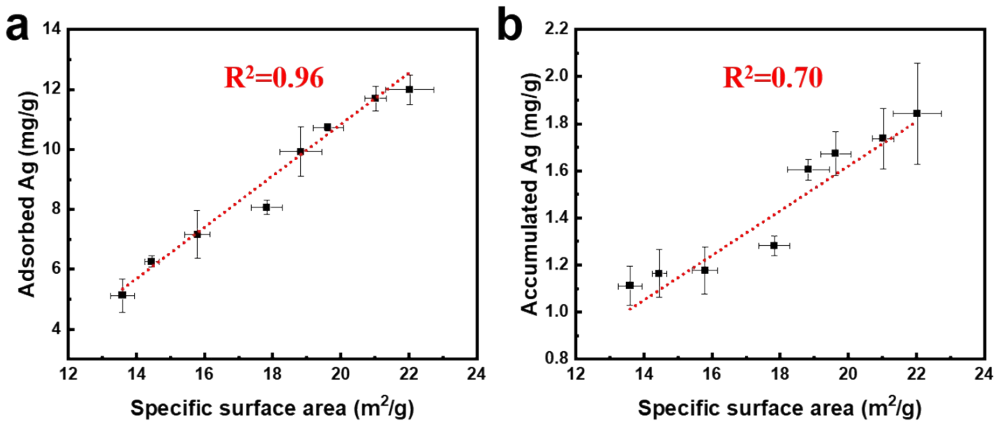

Figure S6. The correlation between the specific surface area of NPs and their absorbed Ag concentratio. The relationship between the specific surface area of 40AIENPs (a) and 80AIENPs (b) and their respective absorbed Ag concentrations, which can provide valuable insights into the interaction between nanoplastics and Ag^{+}.

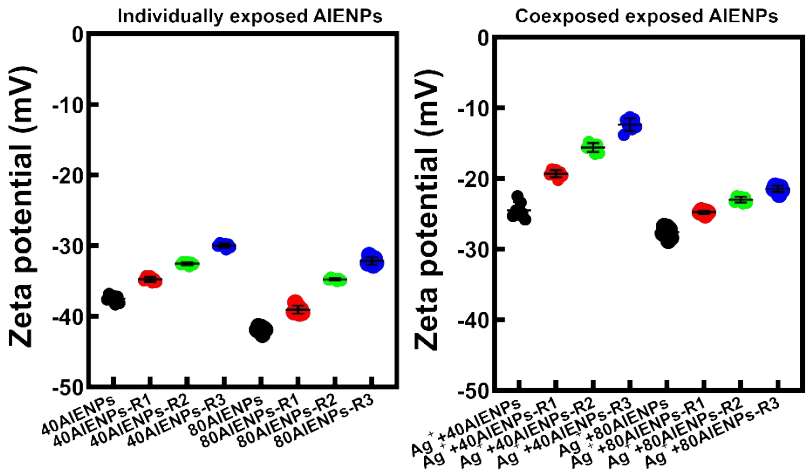

Figure S7. Zeta potential of different-aged 1 mg/L of 40AIENPs and 80AIENPs when being exposed individually and coexposed with 50 μg/L of Ag^{+} within 24 hours of exposure in SM7 medium. There were five replicates for each treatment.

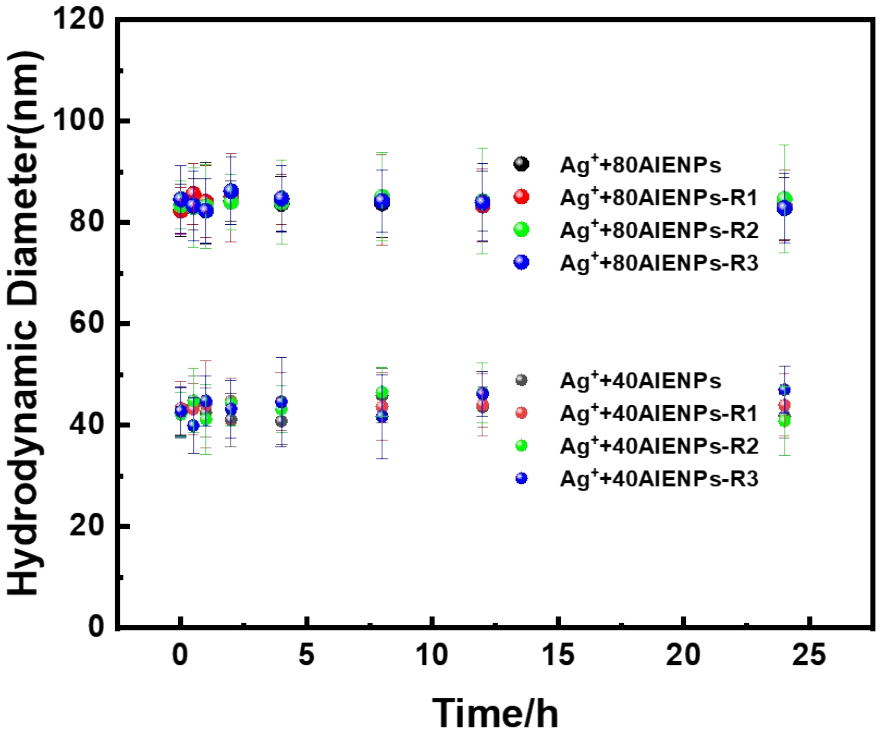

Figure S8. Stability of coexposed AIENPs in the SM7 medium within 24 hours of exposure. There were three replicates for each treatment.

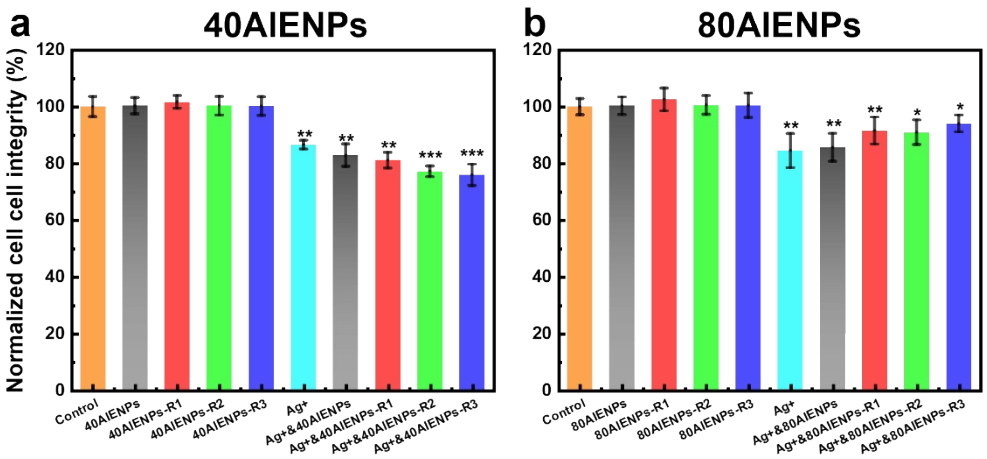

Figure S9. Variation of cell integrity when the algal cells were exposed to 40AIENPs (a) and 80AIENPs (b) individually or coexposed with 50 μg/L of Ag^{+}.

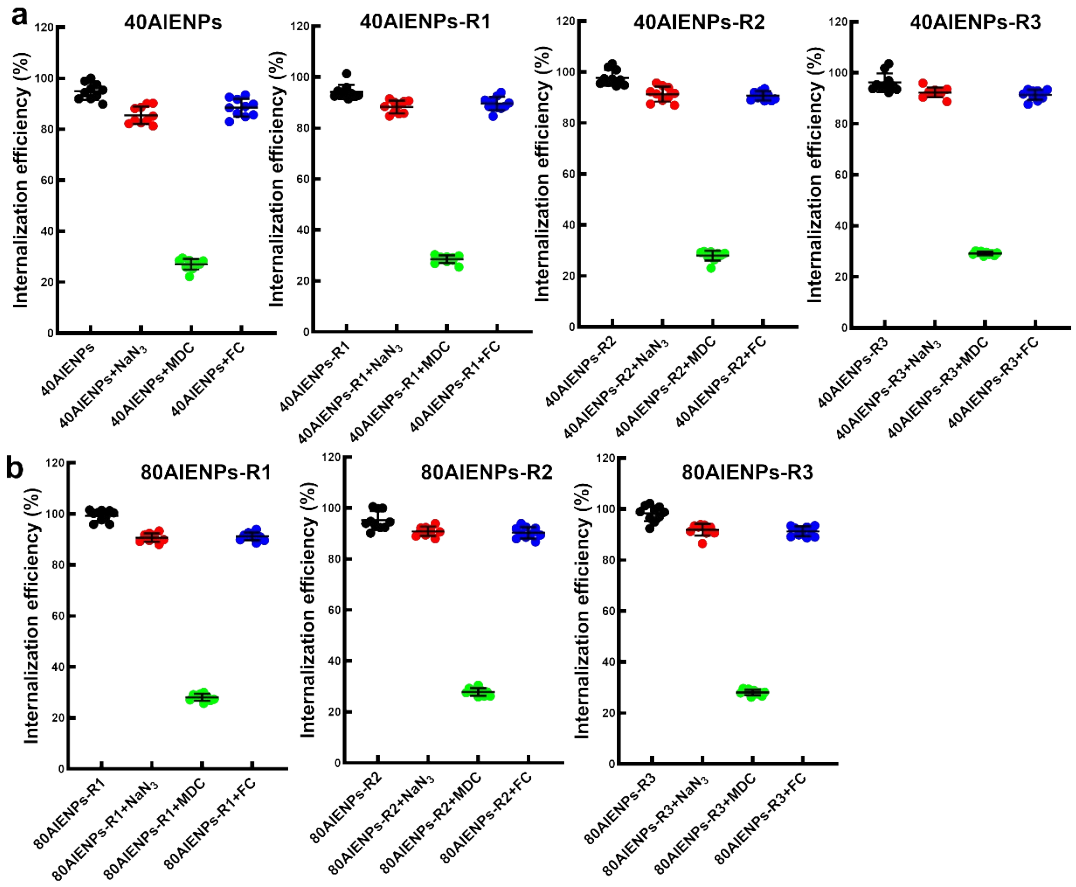

Figure S10. Impact of different endocytosis inhibitors (NaN_3, MDC and FC) on the cellular accumulation of 1 mg/L of different-sized and different-aged AIENPs after 24 hours of exposure. (a) Cellular accumulation of 40AIENPs (40AIENPs, 40AIENPs-R1, 40AIENPs-R2 and 40AIENPs-R3) and (b) Cellular accumulation of 80AIENPs (80AIENPs-R1, 80AIENPs-R2 and 80AIENPs-R3) after 24 hours of exposure in the algae that were first incubated with sodium azide (NaN3, 0.25mM), monodansylcadaverine (MDC, 0.2 mM) or filipin complex (FC, 0.07 mM).

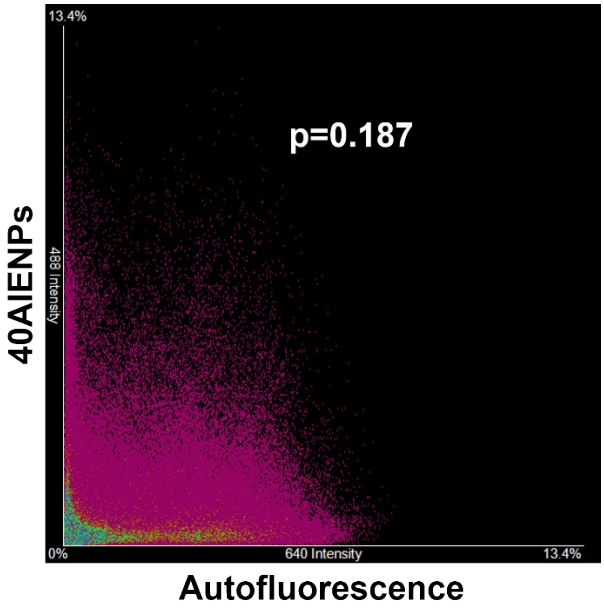

Figure S11. Colocalization between the 40AIENPs and the algal autofluorescence (chlorophyll) was analyzed using the confocal microscope software.

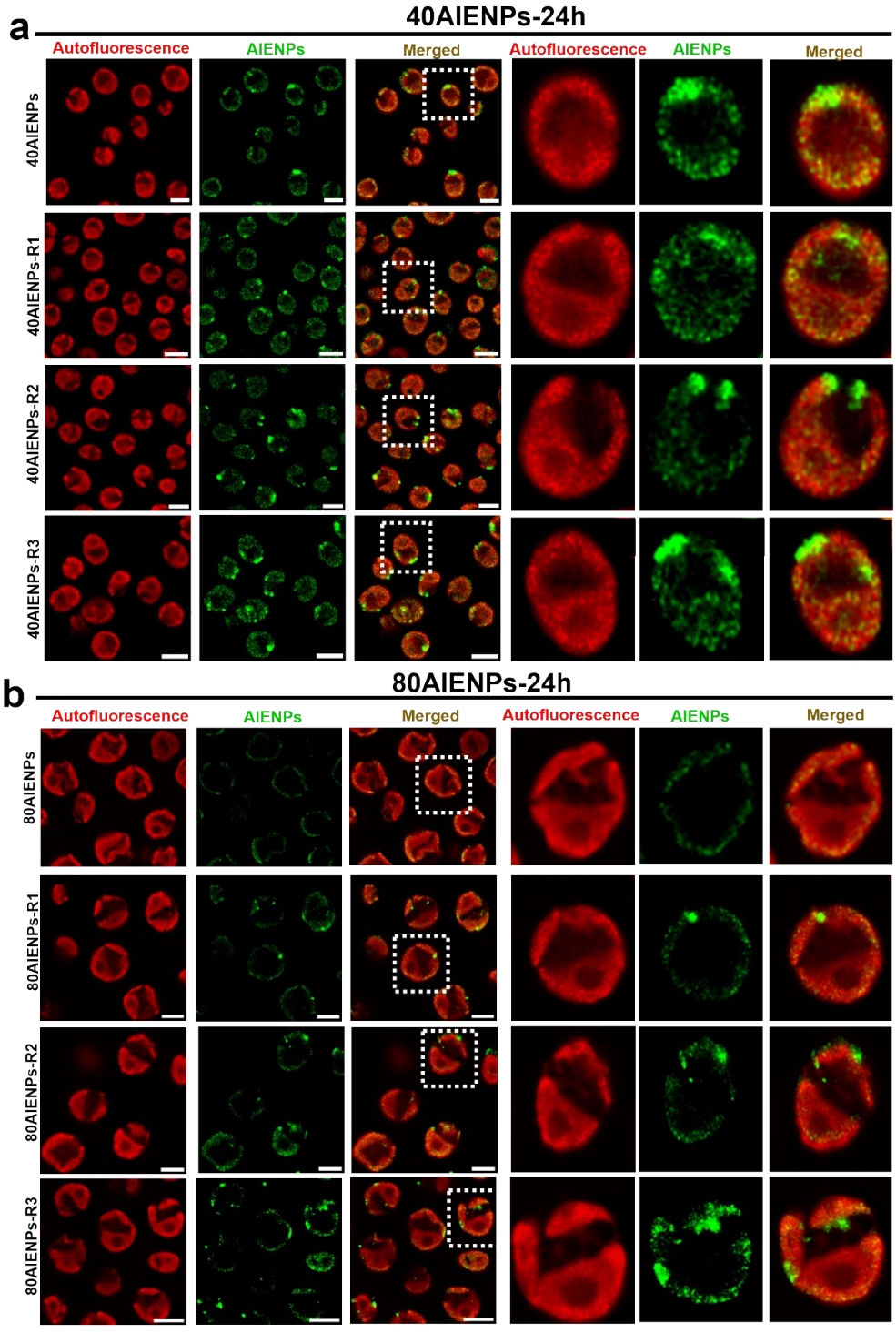

Figure S12. Bioimaging the interactions between the synthesized AIENPs and algae. Confocal images of the intracellular distribution of different aged 40AIENPs (a) and 80AIENPs (b). The scale bar was 5 μm.

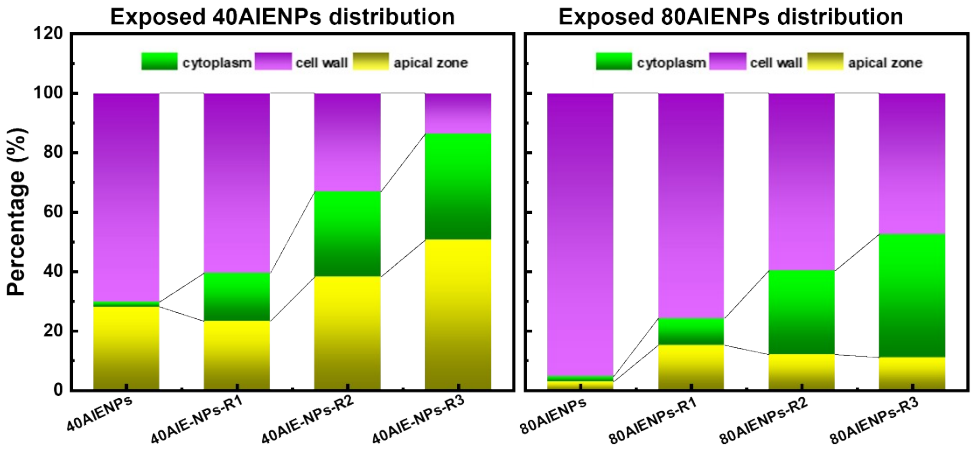

Figure S13. Biodistribution of individually exposed 40AIENPs and 80AIENPs in the algal cells.

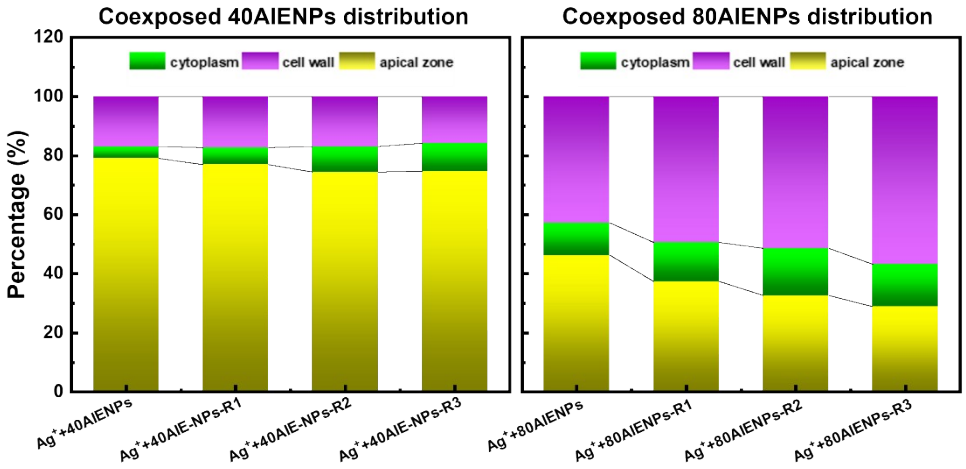

Figure S14. Biodistribution of coexposed 40AIENPs and 80AIENPs in the algal cells.

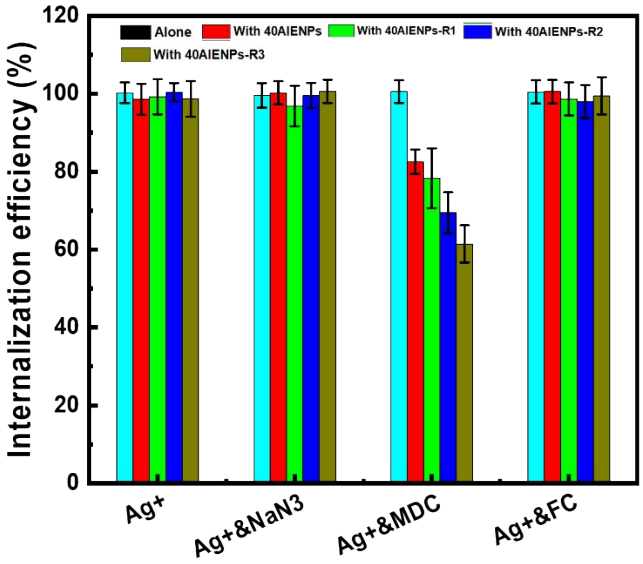

Figure S15. Impact of different endocytosis inhibitors (NaN_3, MDC and FC) on the cellular accumulation of Ag^+ when being coexposed with differently aged 40AIENPs. Algal cells were first incubated with sodium azide (NaN_3, 0.25mM), monodansylcadaverine (MDC, 0.2 mM) or filipin complex (FC, 0.07 mM) before being exposed to the mixture of Ag^+ and differently aged 40AIENPs.

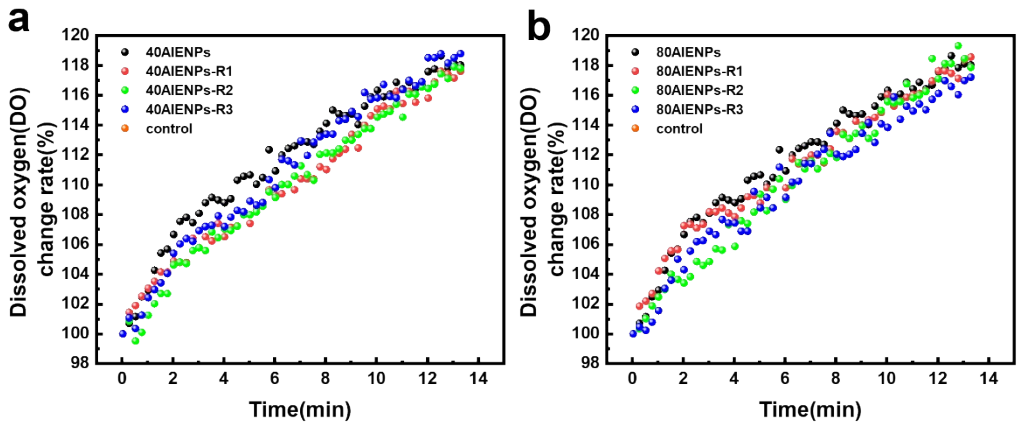

Figure S16. Oxygen production of algal cells after exposure to different-aged 40AIENPs (a) and 80AIENPs (b) for 24 hours.

Reference

1. Yan, N.; Tang, B. Z.; Wang, W.-X., Cell cycle control of nanoplastics internalization in phytoplankton. *ACS Nano* **2021,** *15* (7), 12237-12248.

2. Yue, Q.; He, X.; Yan, N.; Tian, S.; Liu, C.; Wang, W.-X.; Luo, L.; Tang, B. Z., Photodynamic control of harmful algal blooms by an ultra-efficient and degradable AIEgen-based photosensitizer. *Chem. Eng. J.* **2021,** *417*, 127890.