Cd$^{2+}$ Toxicity to a Green Alga *Chlamydomonas reinhardtii* as Influenced by Its Adsorption on TiO$_2$ Engineered Nanoparticles

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**Abstract**

In the present study, Cd$^{2+}$ adsorption on polyacrylate-coated TiO$_2$ engineered nanoparticles (TiO$_2$-ENs) and its effect on the bioavailability as well as toxicity of Cd$^{2+}$ to a green alga *Chlamydomonas reinhardtii* were investigated. TiO$_2$-ENs could be well dispersed in the experimental medium and their pH$_{pzc}$ is approximately 2. There was a quick adsorption of Cd$^{2+}$ on TiO$_2$-ENs and a steady state was reached within 30 min. A pseudo-first order kinetics was found for the time-related changes in the amount of Cd$^{2+}$ complexed with TiO$_2$-ENs. At equilibrium, Cd$^{2+}$ adsorption followed the Langmuir isotherm with the maximum binding capacity 31.9, 177.1, and 242.2 mg/g when the TiO$_2$-EN concentration was 1, 10, and 100 mg/l, respectively. On the other hand, Cd$^{2+}$ toxicity was alleviated in the presence of TiO$_2$-ENs. Algal growth was less suppressed in treatments with comparable total Cd$^{2+}$ concentration but more TiO$_2$-ENs. However, such toxicity difference disappeared and all the data points could be fitted to a single Logistic dose-response curve when cell growth inhibition was plotted against the free Cd$^{2+}$ concentration. No detectable amount of TiO$_2$-ENs was found to be associated with the algal cells. Therefore, TiO$_2$-ENs could reduce the free Cd$^{2+}$ concentration in the toxicity media, which further lowered its bioavailability and toxicity to *C. reinhardtii*.

**Introduction**

Engineered nanoparticles (ENs), defined as man-made materials smaller than 100 nm in at least two dimensions, are widely recognized as having versatile applications in a variety of areas [1]. However, the novel properties ENs possess may not necessarily be benign. Their potentially adverse effects have been intensively investigated in recent years [2–4]. The toxicity of ENs was found to be determined by several physicochemical parameters like particle size, shape, aggregation status, surface coating, chemical composition and so on [3]. Although examining the toxicity of ENs alone could give us invaluable information about the environmental and health risks of nanomaterials, they are actually present in the real world together with other pollutants, which necessitates our understanding about the combined effects of ENs and other toxicants. Colloids are substances with the size range (1–1000 nm) much wider than that of ENs (1–100 nm). They have been reported to be able to facilitate the contaminant transport in the environment (so-called ‘Colloidal Pump’) [5,6] and further influence their bioavailability in a colloid, pollutant, and organism species specific manner [7,8]. However, it remains largely unknown how ENs may interact with other pollutants already existing in the environment and how these interactions may influence the behavior, fate, and toxicity of each other.

Up till now there is still limited research about the effects of ENs on the bioavailability of other pollutants with contradictory results reported. Park et al. [9] found no accumulation of 17$\alpha$-ethynylestradiol associated with nC$_{60}$ aggregates in the zebrafish *Danio rerio* through dietary exposure. In contrast, TiO$_2$-ENs could enhance the toxicity of tributyltin to abalone embryos possibly as a result of tributyltin adsorption onto TiO$_2$-ENs followed by internalization into the embryos [10]. Similarly, the toxicity of various metals like Cd$^{2+}$, Cu$^{2+}$, As (V) was found to increase in the presence of either TiO$_2$-ENs or carbon nanotubes [11–14]. However, the synergistic toxicity of TiO$_2$-ENs and As (V) on *Ceriodaphnia dubia* was either aggravated or eliminated as determined by EN to metal ratio [15]. Pollutant-specific effects were also observed for the influences of C$_{60}$ aggregates on the toxicity of atrazine, methylparathion, pentachlorophenol, and phenantrene [16].

To further explore how ENs may influence the bioavailability of other pollutants, we investigated Cd$^{2+}$ adsorption kinetics and equilibrium isotherm on polyacrylate-coated TiO$_2$-ENs. Its bioaccumulation and toxicity in the freshwater green alga *Chlamydomonas reinhardtii* with and without TiO$_2$-ENs were compared. Potential accumulation (including surface adsorption and internalization) of TiO$_2$-ENs in the algal cells was also examined. TiO$_2$-ENs were chosen because of their wide applications in various products like sunscreens, cosmetics, paints, and surface coatings [10]. There were 50,400 tons of TiO$_2$-ENs produced in 2010, representing 0.7% of the overall TiO$_2$ market. Their production is projected to further increase to 201,500 tons.
There were five TiO$_2$-EN concentration treatments (1.0, 3.0, 0.75, 1, 2 and 6 h). At each time point, 0.2 ml aliquot from each replicate was filtered through a 10 kilo Dalton (kD) ultracentrifuge with pore size approximately 1 nm (PALL Nanosep series). Both the ultrafiltrate and what was retained on the membrane were then digested in 1 ml ultrapure concentrated HNO$_3$ under 60°C for at least 4 d. They were further diluted with Milli-Q water (10:2 M$^+$) to 7% w/v before the Cd$^{2+}$ concentrations were determined by a Thermo M6 atomic absorption spectrophotometer equipped with a GF95Z graphite furnace system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The total Cd$^{2+}$ concentration in the adsorption media without ultrafiltration was also measured at the beginning and end of the experiment for mass balance calculation.

As for the equilibrium isotherm experiment, the variation of Cd$^{2+}$ adsorption with its ambient concentration (nominal total Cd$^{2+}$ concentration - 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 mg/l) was examined in the presence of 1, 10, and 100 mg/l TiO$_2$-ENs, respectively. Since Cd$^{2+}$ adsorption got saturated when its concentration approached 0.5 mg/l with 1 mg/l TiO$_2$-ENs, the two highest Cd$^{2+}$ concentrations (3.0 and 10.0 mg/l) were not used for this EN concentration treatment. The whole experiment was similar to that of the adsorption kinetics experiment above. Based on the results of the kinetics study, Cd$^{2+}$ adsorption got equilibrated within 30 min. The duration of the equilibrium isotherm experiment was thus shortened to 4 h and Cd$^{2+}$ distribution in different fractions was measured only at the end of this experiment. A control experiment with the same concentrations of Cd$^{2+}$ but no TiO$_2$-ENs was also conducted to examine the possibility of Cd$^{2+}$ precipitation at different concentrations.

Effects of TiO$_2$-ENs on Cd$^{2+}$ toxicity

Three toxicity tests in total were performed to investigate how TiO$_2$-ENs may affect the bioavailability and toxicity of Cd$^{2+}$ to C. reinhardtii. WC$_{0.003}$ also served as the base of the toxicity media. There were seven Cd$^{2+}$ concentration treatments (nominal total Cd$^{2+}$ concentration - 0, 0.1, 0.3, 0.5, 0.8, 1.0, and 3.0 mg/l) in duplicate with and without 100 mg/l TiO$_2$-ENs, respectively, for two of the three toxicity tests. However, the nominal total Cd$^{2+}$ concentration was fixed at 1 mg/l and various concentrations (0, 1, 3, 10, and 100 mg/l) of TiO$_2$-ENs were applied in the third one. Major differences between the three toxicity tests were shown in Table S2. The polycarbonate bottles and other containers to be used in the toxicity tests were pre-equilibrated with the corresponding toxicity media similar to what was performed in the adsorption experiment above. All the toxicity media were made one day in advance and left overnight under the same conditions as the following experiment for equilibration. Their pH was kept at 7.5±0.1.

The algal cells were first acclimated in WC$_{0.003}$ without Cd$^{2+}$ or TiO$_2$-ENs until arriving at the mid-exponential growth phase. They were then collected by centrifugation at 1700 RCF, rinsed twice with 15 ml fresh WC$_{0.003}$ and resuspended into the toxicity media. Right before the addition of algal cells, 0.2 ml aliquot from each medium replicate was filtered through a 10 kD membrane. The total Cd$^{2+}$ concentration in the ultrafiltrate (non-adsorbed Cd$^{2+}$) was measured, based on which the free Cd$^{2+}$ concentration ([CdF$^{2+}$]) of each toxicity medium was calculated using the MINEQL+ software package (Version 4.3 from Environmental Research Software, Hallowell, ME, USA) with updated thermodynamic constants and the influence of ionic strength calibrated. The whole experiment lasted for 2 d with three time points (0, 1$^{st}$, and 2$^{nd}$ d). At each time point, the cell density was measured by a Z2 Coulter Counter (Beckman Coulter Inc., CA, USA). The cell specific growth rate $\mu$ was calculated as described by Miao et al.
To further examine the potential bioaccumulation of TiO₂-ENs in the algal cells, another 10 ml aliquot was filtered through a 1.2 μm polycarbonate membrane, rinsed twice with 15 ml fresh WCₘ, and then combusted in muffle furnace at 460°C for 2 h. The residue was digested in a mixture of 0.4 g (NH₄)₂SO₄ and 1.0 ml H₂SO₄ at 250°C for half an hour. After being diluted to 2.5% w/v by Milli-Q water, the Ti concentration was determined by GFAAS. Controls containing the same concentrations of TiO₂-ENs and Cd²⁺ but no C. reinhardtii were applied to eliminate any interference from the TiO₂-EN aggregates retained by the 1.2 μm polycarbonate membrane. The background Ti concentration in the cells not exposed to TiO₂-ENs was also measured.

TEM images of algal cells exposed to 100 mg/l TiO₂-ENs but without any addition of Cd²⁺ were then taken to visually examine the interactions between TiO₂-ENs and C. reinhardtii. The sample preparation was similar to our previous study [20]. Briefly, 100 ml algal culture was centrifuged and fixed with 4% glutaraldehyde at 4°C for 4 h. After the cells were cleaned with phosphate buffer (0.3 M, pH = 7.3), they were stained in 1% (mg/ml, weight to volume ratio) osmium tetroxide for 2 h, and then dehydrated with 30%, 50%, 70%, 80%, 90% and 100% acetone solution sequentially. Afterwards, they were embedded into epoxy resin (Epon 812, DDSA, MNA and DMP-30), sectioned at 100 nm thickness, further stained with uranyl acetate (5 g in 50 ml ethanol) and lead citrate (1.33 g in 30 ml H₂O₂). The elemental composition of the interesting spots on the TEM images was investigated with an energy dispersive X-ray (EDX) spectrometer.

Statistical analysis
Any ‘significant’ difference (accepted at p<0.05) was based on results of one-way or two-way analysis of variance with post-hoc multiple comparisons (Turkey or Tamhane) (SPSS 11.0 by SPSS, Chicago, USA). The normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity of variance (Levene’s test) of the data were both examined when performing the analysis of variance.

Effects of Nanoparticles on Cd²⁺ Toxicity

Adsorption of Cd²⁺ by TiO₂-ENs

The TiO₂-ENs used in the present study were coated with sodium polyacrylate and could thus be well dispersed in WCₘ as supported by their TEM images shown in Fig. 1a. Their diameter was 46.6 nm on average by measuring 1000 particles randomly chosen from the copper grids, which was consistent with what was obtained by DLS (19.0–46.8 nm). The relatively good dispersibility of TiO₂-ENs coated by the polyelectrolyte could be explained by their much lower pHₚzc (Fig. 1b), at which a particle surface has zero net electrical charge, than that of their naked counterpart [pHₚzc = 2 vs. 6] [22,23]. Such decrease in pHₚzc was mainly caused by polyacrylate’s ability to push the slip plane of the crystal lattice away from the ENs, change their charge distribution in the diffusion layer and block the active sites on the TiO₂-EN surface as well [23]. Furthermore, the extent of the shift in pHₚzc was determined by both the concentration and molecular weight of polyacrylates. Despite their good dispersibility in WCₘ, the actual diameter of TiO₂-ENs was much bigger than what was reported by the manufacturer (1–10 nm) suggesting the electric double layer of the primary nanoparticles was compressed in the adsorption medium with the ionic strength 2.65×10⁻⁵ M and aggregates were thus formed. The presence of divalent cations like Ca²⁺ (0.25 mM) and Mg²⁺ (0.15 mM) in WCₘ could further destabilize the TiO₂-EN suspension [1].

In both adsorption kinetics and equilibrium isotherm experiments, the concentrations of Cd²⁺ retained on the 10 kD membrane (TiO₂-EN surface-adsorbed) and in the filtrate (non-adsorbed) were compared with what was measured without filtration. A good mass balance (100±10%) was achieved for most treatments. Cd²⁺ was found to quickly adsorb onto TiO₂-ENs and a steady state was reached within 30 min (Fig. 2a). Rapid association of Cd²⁺, Cu²⁺, Ni²⁺, Pb²⁺ and Zn²⁺ with TiO₂-ENs was also observed by Engates and Shipley [24] and most adsorption was completed in 5 min. Meanwhile, higher proportions of Cd²⁺ were adsorbed in higher TiO₂-EN concentration treatments unless no Cd²⁺ in the medium was available any more when the concentration of TiO₂-ENs exceeded 30 mg/l. Accordingly, 0.16, 0.33, 0.70, 0.90, and 0.93 mg/l Cd²⁺ was adsorbed by 1, 3, 10, 30, 100 mg/l TiO₂-ENs, respectively, after 30 min. Such trend looked reversed in Fig. 2a as the adsorption was normalized to the TiO₂-EN concentration (mg/g). Additionally, the possibility whether Cd²⁺ with concentration up to 1 mg/l was over-saturated in WCₘ precipitated out and thus made the Cd²⁺ adsorption results overestimated was also investigated. A negligible amount (less than 1%) was retained on the 10 kD membrane without any

Figure 1. The transmission electron microscope image of TiO₂-ENs dispersed in the modified WC medium (WCₘ) (a) and their zeta potentials (mV) at different pH (b).

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addition of TiO$_2$-ENs at the end of the 6-h experiment suggesting that all the Cd$^{2+}$ was in soluble form for the kinetics experiment. Although EDTA was not used in WC$_{cm}$, no significant precipitates were formed when preparing this medium.

The Cd$^{2+}$ adsorption kinetics results were then fitted with the pseudo-first order equation as follows,

$$q_t = q_e \left(1 - \exp(-kt)\right)$$  (1)

Where $q_t$ and $q_e$ are the Cd$^{2+}$ adsorption (mg/g) at time $t$ (min) and at equilibrium, respectively. $k$ (min$^{-1}$) represents the equilibrium rate constant of the pseudo-first order adsorption. Values of the different parameters thus obtained were listed in Table 1. The adsorption of Cd$^{2+}$ onto TiO$_2$-ENs and Cu$^{2+}$ onto Fe$_3$O$_4$ magnetic ENs were also found to comply with the pseudo-first order model in previous studies [22,25]. Values of $k$ were 0.15–0.25 min$^{-1}$ for Cd$^{2+}$ and 0.64–1.05 min$^{-1}$ for Cu$^{2+}$, which were of the same order of magnitude as what was found (0.23–2.35 min$^{-1}$) in the present study even though the TiO$_2$-ENs used here were coated with sodium polyacrylate. Fitting the adsorption kinetics data points by the pseudo-second order model was also tried with unsatisfactory results (data not shown), possibly due to the high Cd$^{2+}$ concentrations we used considering that the choice of models is dependent on the solute concentration [26].

In the equilibrium isotherm experiment, a biphasic correlation between the TiO$_2$-EN normalized Cd$^{2+}$ adsorption ($q_n$, mg/g) and the non-adsorbed Cd$^{2+}$ concentration in the medium ($C_e$, mg/l) was found for each of the three TiO$_2$-EN concentration treatments (1, 10, and 100 mg/l) (Fig. 2b–d). Namely, $q_n$ went up proportionally with $C_e$ first, slowed down thereafter and even plateaued at high Cd$^{2+}$ levels especially when 1 or 10 mg/l TiO$_2$-ENs were used. In the meantime, the potential difference in the proportions of Cd$^{2+}$ adsorbed by different concentrations of TiO$_2$-ENs was small or negligible when the initial concentration of Cd$^{2+}$ was too low to saturate the ENs. However, the difference got more and more significant as Cd$^{2+}$ adsorption approached the saturation point. When 3 mg/l Cd$^{2+}$ was applied, most of it (91.7–97.3%) was adsorbed in all the three TiO$_2$-EN concentration treatments. As the initial Cd$^{2+}$ concentration increased further to 0.01 and 1 mg/l, the proportion of Cd$^{2+}$ adsorbed on 1 mg/l TiO$_2$-ENs decreased to 71.8% and 9.02%. However, nearly all Cd$^{2+}$ (96.4–100%) could still be adsorbed on 10 and 100 mg/l TiO$_2$-ENs. It was not until the initial Cd$^{2+}$ concentration exceeded 1 mg/l that significant difference ($p<0.05$) between the 10 and 100 mg/l TiO$_2$-EN concentration treatments appeared with 17.9% and 66.6% adsorption when the initial Cd$^{2+}$ concentration was 10 mg/l. The concentration of Cd$^{2+}$ retained on the 10 kD membrane in the control treatments having the same concentrations of Cd$^{2+}$ but no TiO$_2$-ENs was also negligible as compared with those adsorbed by the ENs. It further implies that Cd$^{2+}$ precipitation was insignificant for all the concentration treatments of the present study as was in contrast to what was estimated by MINEQL+ based on an equilibrium assumption.

The biphasic correlation between $q_n$ and $C_e$ was then fitted to the Langmuir isotherm for each of the three TiO$_2$-EN concentration treatments as follows,

$$q_e = \frac{K_a q_m C_e}{1 + K_a C_e}$$  (2)

Where $K_a$ is the Langmuir constant (l/mg) related to adsorption energy and $q_m$ represents the maximal monolayer adsorption

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Figure 2. Adsorption of Cd$^{2+}$ ($q_t$, mg/g) on TiO$_2$-ENs in the kinetics (a) and 4-h equilibrium isotherm (b–d) experiments, respectively. There were five treatments with different concentrations of TiO$_2$-ENs (1.0, 3.0, 10.0, 100.0, and 1000.0 mg/l) but the same concentration of total Cd$^{2+}$ (1 mg/l) in the kinetics experiment. Various concentrations of Cd$^{2+}$ (0.003, 0.01, 0.03, 0.1, 1.0, 3.0, and 10.0 mg/l initially) were used in each equilibrium isotherm experiment with different TiO$_2$-EN concentrations (b–d: 1, 10, 100 mg/l, respectively). Since Cd$^{2+}$ adsorption got saturated when its concentration approached 0.3 mg/l with 1 mg/l TiO$_2$-ENs, the two highest Cd$^{2+}$ concentrations (3.0 and 10.0 mg/l) were not used for this EN concentration treatment. Dashed lines represent the simulated curves of Cd$^{2+}$ adsorption kinetics and equilibrium isotherm by the pseudo-first order (a) and Langmuir (b–d) models. Data are mean ± standard deviation (n = 2). doi:10.1371/journal.pone.0032300.g002

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capacity (mg/g). A good correlation was found with values for the various parameters shown in Table 1 suggesting a monolayer adsorption of Cd²⁺ on TiO₂-ENs.

Cd²⁺ adsorption on TiO₂-ENs with different crystal size (7.72–145 nm) was previously investigated [27]. Values of qm thus derived from the Langmuir isotherm were in the range of 3.93–56.0 mg/g with lower adsorption by bigger particles. Much smaller difference was observed when qm was normalized to the surface area of TiO₂-ENs (0.29–0.39 mg/m²). As the TiO₂-ENs we used were made up of a TiO₂ core (4.23 g/cm³) coated with sodium polyacrylate (1.22 g/cm³), its density and specific surface area estimated to be approximately 1.50 g/cm³ and 85.8 m²/g. However, the surface area normalized qm (0.37–2.82 mg/m²) we obtained was still higher than what was reported by Gao et al. [27], especially for the two higher TiO₂-EN concentration treatments. It suggests that the polyacrylate surface coating could improve the metal ion adsorption ability of the ENs. Cd²⁺ was able to form bidentate and monodentate ligand complex with polyacrylic acid [28], which may also be the same case for its adsorption on the polyacrylate-coated TiO₂-ENs. Given that sodium polyacrylate accounts for 74% of the TiO₂-ENs we used, the Cd²⁺ to –COOH ratio at saturation would be 0.036, 0.20, and 0.27, respectively, when the concentration of TiO₂-ENs was 1, 10, and 100 mg/l. It implies that part of the carboxylate group from polyacrylate was bound with TiO₂ or other cations (e.g., Ca²⁺ and Mg²⁺ etc.) in the adsorption medium and was thus not available to Cd²⁺. The possibility that the EN surface was heterogeneous and some of the Cd²⁺ may be complexed with the TiO₂ core itself further complicated the metal-EN interactions [27].

de spoilage of Cd²⁺ toxicity as affected by TiO₂-ENs

The growth of C. reinhardtii in WC₉₀ (no addition of Cd³⁺) with or without 100 mg/l TiO₂-ENs was compared in our preliminary experiment. No obvious growth inhibition was found, which simplified our exploration how TiO₂-ENs may affect the bioavailability and toxicity of metal ions. The toxicity of bare TiO₂-ENs to various phytoplankton was investigated in the literature [29]. The median effect concentration EC₅₀ thus obtained ranged over a few orders of magnitude (e.g., 5.83–241 mg/l) as the toxicity of ENs is dependent on parameters like particle size, shape, chemical composition and so on. Surface coatings may further eliminate the toxicity of ENs [30]. The initial and final Cd²⁺ concentrations in <10 kD fraction of each replicate for the three toxicity tests were both measured. The decrease in [Cd²⁺]ₚ with exposure time was within 30% for most treatments. The relative changes of µ were thus plotted against either total dissolved Cd²⁺ concentration ([Cd²⁺]ₚ, including what was adsorbed on TiO₂-ENs) or [Cd²⁺]ₚ (Fig. 3a, b) at the beginning of each toxicity test to determine which metal ion concentration could predict its toxicity better in the presence of TiO₂-ENs. As expected, µ was substantially reduced at high Cd²⁺ levels when no TiO₂-ENs was applied. A typical dose-response correlation between the relative changes of µ and either type of Cd²⁺ concentration was observed. Namely, the cell growth was kept constant in the first three lowest Cd²⁺ concentration treatments. It was then strikingly inhibited with more than 90% reduction when [Cd²⁺]ₚ increased to 0.8 (0.50 mg/l) and completely ceased thereafter.

However, when 100 mg/l TiO₂-ENs were applied to the different treatments with [Cd²⁺]ₚ comparable to those of the first toxicity test above, the adverse effects of Cd²⁺ were substantially alleviated. There was a growth inhibition of only 13% in the highest concentration treatment with [Cd²⁺]ₚ 5 mg/l, which in contrast was lethal to the cells if no TiO₂-ENs were added. Similarly, µ went down from 0.46 d⁻¹, as was comparable to that in the control without any addition of TiO₂-ENs and Cd²⁺, to zero when [Cd²⁺]ₚ was fixed at 1 mg/l but the TiO₂-EN concentration decreased from 100 to 0 mg/l in the third experiment. However, growth inhibition to different extent at similar [Cd²⁺]ₚ but different concentrations of TiO₂-ENs, as shown in Fig. 3a, disappeared when the relative changes of µ were plotted against [Cd²⁺]ₚ. All the data points from the different toxicity tests could be well fitted to a single Logistic dose-response curve (y = min + (max–min)/(1+(x/EC₅₀)Hillslope)) (Fig. 3b). The [Cd²⁺]ₚ-based EC₅₀ thus obtained was 0.33±0.03 mg/l which was similar to 0.46 mg/l observed in our previous study [31] and was within the range of values (0.03–2.41 mg/l) reported in the literature [32,33]. As the cell growth was differently inhibited at similar [Cd²⁺]ₚ but various concentrations of TiO₂-ENs, only the dose-related responses of the first toxicity test without any addition of TiO₂-ENs were simulated with the Logistic model in Fig. 3a.

Of the limited research on the interactions between ENs and trace metals, TiO₂-ENs were frequently chosen which made the comparison of our study with the literature possible. The bioaccumulation of Cd²⁺ and As (V) in carp was found to increase remarkably in the presence of TiO₂-ENs, as was explained by ENs’ ability to facilitate the metal transport through the gills (dissolved uptake) and to induce the metal assimilation in the intestines (dietary assimilation) when EN-contaminated foods were fed to the fish [34,35]. Additionally, the enhanced oxidation of metal ions in reduced form like As (III) by TiO₂-ENs as a photocatalyst especially under the light condition could further accelerate the metal uptake [34]. As a result, the trace metal toxicity was aggravated even though part of them were still associated with TiO₂-ENs in the organisms [12]. On the other hand, metal toxicity to unicellular organisms may decline abruptly.

| Table 1. Values of the different parameters obtained when simulating the kinetics and equilibrium isotherm of Cd²⁺ adsorption by TiO₂-ENs with the pseudo-first order and Langmuir models, respectively. |
|---|---|---|---|---|---|---|---|
| --- | --- | --- | --- | --- | --- | --- |
| Pseudo-first order | k | r² | p | Langmuir | qₑ₀ | Kₑ | r² | p |
| 160.9±12.1 | 2.35±0.35 | 0.98 | <0.0001 | 31.9±17.2 | 83.5±49.6 | 0.88 | 0.0053 |
| 104.1±2.90 | 0.39±0.05 | 0.98 | <0.0001 | 177.1±77.4 | 2.14±1.12 | 0.94 | <0.0001 |
| 66.1±1.32 | 3.21±0.48 | 0.96 | <0.0001 | 242.2±23.9 | 0.113±0.04 | 0.99 | <0.0001 |
| 29.2±0.63 | 0.25±0.01 | 0.99 | <0.0001 | 8.51±0.30 | 0.23±0.03 | 0.97 | <0.0001 |

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in the presence of TiO₂-ENs if the metal-EN complexes thus formed cannot penetrate the cell membrane. The bare TiO₂-ENs used by Hartmann et al. [14] were able to reduce the Cd²⁺ toxicity to a green alga _Pseudokirchneriella subcapitata_ but the toxicity inhibition was greater than what could be explained by the concentration of Cd²⁺ not associated with TiO₂-ENs, suggesting a possible carrier effect, or mixture toxic effects of TiO₂-ENs and Cd²⁺. The latter possibility was more likely as considering the toxicity of TiO₂-ENs on the alga itself (EC₅₀ = 71.1–241 mg/l) and any direct evidence of EN internalization was lacking.

To further examine the underlying mechanisms how Cd²⁺ toxicity was lessened by the polyacrylate-coated TiO₂-ENs, the bioaccumulation of Cd²⁺ ([Cd²⁺]_cell-ads and [Cd²⁺]_intra) was quantified at the end of each toxicity test. Their changes with [Cd²⁺]_T as well as [Cd²⁺]_F were shown in Fig. 3c, d and S1. Overall, there was a positive correlation between Cd²⁺ accumulation and [Cd²⁺]_F. When [Cd²⁺]_F went up from 3.05 × 10⁻⁵ to 2.10 mg/l, [Cd²⁺]_intra was enhanced by three orders of magnitude (Fig. 3d). The cellular Cd²⁺ concentration in the same strain of alga was found to increase approximately from 0.025 pg/cell when [Cd²⁺]_F was 5.39 × 10⁻³ mg/l to 0.25 pg/cell with [Cd²⁺]_F = 3.37 mg/l [33], as was comparable to what was observed in the present study. On the other hand, [Cd²⁺]_intra was strikingly different in treatments containing the same [Cd²⁺]_T but various concentrations of TiO₂-ENs. It was decreased by 40–88% in all treatments when 100 mg/l TiO₂-ENs were applied in the second toxicity test as compared with those in the first one (Fig. 3c). Similarly, a negative correlation between [Cd²⁺]_intra and TiO₂-EN concentration was observed with fixed [Cd²⁺]_T in the third toxicity experiment.

More importantly, the difference between [Cd²⁺]_intra at similar [Cd²⁺]_T but distinct concentrations of TiO₂-ENs was substantially diminished when [Cd²⁺]_intra was plotted against [Cd²⁺]_F instead of [Cd²⁺]_T (Fig. 3d). Such trend was more obvious when all the data points from the three toxicity tests were fitted to a single Freundlich isotherm for each diagram (Fig. 3c, d and S1).

Log \left( \frac{[\text{Cd}^{2+}]_{\text{intra}} \text{ or } [\text{Cd}^{2+}]_{\text{cell-ads}}}{[\text{Cd}^{2+}]_{\text{F}} \text{ or } [\text{Cd}^{2+}]_{\text{cell-ads}}} \right) = \frac{1}{n} \log \left( \frac{[\text{Cd}^{2+}]_{\text{F}}}{[\text{Cd}^{2+}]_{\text{F}}} \right) + \log (K_F) \tag{3}

Where _K_F_ represents the Freundlich constant and _n_ is a dimensionless parameter related to the metal binding affinity. A better correlation between [Cd²⁺]_intra and [Cd²⁺]_F than that between [Cd²⁺]_intra and [Cd²⁺]_T \((r^2 = 0.93 \text{ vs. } 0.86)\) was observed. It suggests that Cd²⁺ toxicity alleviation by TiO₂-ENs was mainly caused by the decrease in [Cd²⁺]_F as a result of surface adsorption.

**Figure 3.** Relative changes of the cell specific growth rate (µ) (a–b) and intracellular Cd²⁺ concentration ([Cd²⁺]_intra, pg/cell) (c–d) with either the total dissolved ([Cd²⁺]_T, mg/l) (a, c) or free Cd²⁺ ([Cd²⁺]_F, mg/l) concentrations (b, d) at the beginning of the three toxicity experiments where 0, 100, and 1–100 mg/l TiO₂-ENs were applied, respectively. Dashed lines represent the simulated curves for the relative changes of µ (a–b) and [Cd²⁺]_intra (c–d) at different [Cd²⁺]_T (a, c) and [Cd²⁺]_F (b, d) by the Logistic dose-response and Freundlich models, respectively. Data are mean ± standard deviation (n = 2).

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**Figure 4.** Accumulation of TiO₂-ENs ([TiO₂-ENs]_cell, pg/cell) by _Chlamydomonas reinhardtii_ in the different treatments of the second (Exp_2) and third (Exp_3) toxicity experiments. In Exp_2, treatment A–G indicates different initial concentrations of Cd²⁺ (0, 0.1, 0.3, 0.5, 0.8, 1.0, and 3.0 mg/l) with the TiO₂-EN concentration fixed at 100 mg/l. In Exp_3, the initial Cd²⁺ concentration was fixed at 1 mg/l and various concentrations (0, 1, 3, 10, 30, and 100 mg/l) TiO₂-ENs were used for treatments A–F. Data are mean ± standard deviation (n = 2).

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Effects of Nanoparticles on Cd\(^{2+}\) Toxicity

In contrast, there was an unsatisfied correlation (\(r^2 = 0.68\) vs. 0.62) between [Cd\(^{2+}\)]\(_{\text{cell-ads}}\) and [Cd\(^{2+}\)]\(_{T}\) or [Cd\(^{2+}\)]\(_{F}\) (Fig. S1). At similar [Cd\(^{2+}\)]\(_{F}\), higher Cd\(^{2+}\) adsorption was usually found when TiO\(_2\)-ENs were applied. Therefore, a certain amount of TiO\(_2\)-ENs might be associated with the algal cells or just highly aggregated in the medium and were retained by the 1.2 \(\mu\)m polycarbonate membrane. Cd\(^{2+}\) adsorbed on these ENs could also be removed by EDTA, and thus made [Cd\(^{2+}\)]\(_{\text{cell-ads}}\) overestimated.

Potential TiO\(_2\)-EN accumulation (including both cell surface adsorbed and intracellular accumulated ones) by C. reinhardtii was then quantified with GFAAS. As shown in Fig. 4, the bioaccumulated concentration of TiO\(_2\)-ENs ([TiO\(_2\)-ENs]\(_{\text{bio}}\)) was in the range of 3.85–7.06 pg/cell for the second toxicity experiment in the presence of 100 mg/l TiO\(_2\)-ENs. Although [TiO\(_2\)-ENs]\(_{\text{bio}}\) decreased slightly with the enhancement of Cd\(^{2+}\), it was statistically insignificant (\(p > 0.05\)). Additionally, a substantial accumulation of TiO\(_2\)-ENs was only detected in the two highest TiO\(_2\)-EN concentration treatments (30 and 100 mg/l) for the third toxicity test. Given that TiO\(_2\)-ENs had a diameter of 46.6 nm on average, there should be 4.8\( \times \)10\(^6\)–8.9\( \times \)10\(^7\) particles associated with a single algal cell as equivalent to approximately 1000 particles within each cell slice (100 nm thick) mounted on the TEM copper grid when 100 mg/l TiO\(_2\)-ENs were applied. However, no TiO\(_2\)-ENs were found either inside the cells or adsorbed on the cell surface after several cell slices were investigated with suspicious spots scanned by the EDX spectrometer (Fig. 5). It implies that most of the Ti signal determined by GFAAS might come from the additional TiO\(_2\)-EN aggregates intercepted by the 1.2 \(\mu\)m filter in the presence of C. reinhardtii, which cannot be subtracted with the control treatments containing the same concentrations of Cd\(^{2+}\) and TiO\(_2\)-ENs but no algal cells. TiO\(_2\)-ENs can attach to various algal species. The green alga P. subcapitata could even carry TiO\(_2\)-ENs with weight 2.3 times higher than their own on the cell surface [14,36,37]. The adsorption of TiO\(_2\)-ENs was found to be dependent on the pH of the medium and maximum adsorption was observed at pH = 5.5 (comparable to the pH\(_{\text{pzc}}\) of bare TiO\(_2\)-ENs) [38]. It implies that electrostatic attraction played a critical role in the interactions between TiO\(_2\)-ENs and algal cells. As the pH\(_{\text{pzc}}\) of TiO\(_2\)-ENs we used is around 2, their surface was negatively charged in WC\(_m\) (pH = 7.5) the same as that of the algal cells themselves. Therefore, a negligible amount of TiO\(_2\)-ENs would be expected to be associated with C. reinhardtii unless other forces such as hydrogen bonding overrides the electrostatic and steric repulsion between the cells and ENs as observed by Schwab et al. [39]. The lack of direct contact between polyacrylate-coated TiO\(_2\)-ENs might be another reason why they were less toxic than bare TiO\(_2\)-ENs.

Overall, the TiO\(_2\)-ENs used in the present study could adsorb Cd\(^{2+}\) rather quickly with the maximum adsorption capacity ranging from 31.9 to 242.2 mg/g. The electrostatic and potentially steric repulsions between TiO\(_2\)-ENs and algal cells could hinder their direct contact with each other, thus prevent the internalization of TiO\(_2\)-ENs into the cells. The toxicity of Cd\(^{2+}\) was alleviated considerably when TiO\(_2\)-ENs were applied, as Cd\(^{2+}\) adsorption on the ENs decreased its free ion concentration in the toxicity medium and further its bioaccumulation in the algal cells. However, the Cd\(^{2+}\) toxicity in the presence of TiO\(_2\)-ENs could still be well predicted with the classical FIAM model.

Supporting Information

Figure S1 Relative changes of the cell surface adsorbed Cd\(^{2+}\) concentration ([Cd\(^{2+}\)]\(_{\text{cell-ads}}\) pg/cell) with either the total dissolved ([Cd\(^{2+}\)]\(_{T}\), mg/l) (a) or free Cd\(^{2+}\) ([Cd\(^{2+}\)]\(_{F}\), mg/l) concentrations (b) at the beginning of the three toxicity experiments where 0, 100, and 1–100 mg/l TiO\(_2\)-ENs were applied, respectively. Dashed lines represent the simulated curves of [Cd\(^{2+}\)]\(_{\text{cell-ads}}\) at different [Cd\(^{2+}\)]\(_{T}\) (a) and [Cd\(^{2+}\)]\(_{F}\) (b) by the Freundlich isotherm model. Data are mean ± standard deviation (n = 2).

Table S1 Compounds and their concentrations in the modified WC medium used in the present study.

Table S2 Composition of the toxicity media for the three experiments.

Author Contributions

Conceived and designed the experiments: AJM LYY. Performed the experiments: WWY AJM. Analyzed the data: WWY AJM. Contributed reagents/materials/analysis tools: WWY AJM LYY. Wrote the paper: AJM.
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