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Method Article

A density gradient centrifugation method for rapid separation of nanoTiO$_2$ and TiO$_2$ aggregates from microalgal cells in complex mixtures with mercury

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A B S T R A C T

In natural environment, the microorganisms are exposed to complex mixtures of contaminants, including manufactured nanoparticles and their aggregates. Evaluation of the toxicant accumulation in biota exposed to such cocktails is a challenging task because the microorganisms need to be separated from nanomaterial aggregates often of a comparable size. We propose a method for separation of TiO$_2$ aggregates from green microalga *Chlamydomonas reinhardtii* and subsequent determination of cellular Hg concentration in algae exposed to mixture of Hg with nanoTiO$_2$, known also to adsorb Hg. The method is based on differences in specific weight of algae and TiO$_2$ aggregates, using medium speed centrifugation on a step gradient of sucrose. The efficiency of the separation method was tested with nanoTiO$_2$ of three different primary sizes at four concentrations: 2, 20, 100 and 200 mg L^{-1}. The method gives a possibility to separate nanoTiO$_2$ and their aggregates from the algae with a mean recovery of 83.3% of algal cells, thus allowing a reliable determination of Hg accumulation by microalgae when co-exposed to Hg and nanoTiO$_2$.

- A rapid and reliable method to separate algal cells and nanoparticle aggregates of comparable size.
- A method to measure the cellular amount of Hg in green alga co-exposed to Hg and nanoTiO$_2$.

A R T I C L E I N F O

Method name: A density gradient centrifugation method for rapid separation of nanoTiO$_2$ and TiO$_2$ aggregates from microalgal cells in complex mixtures with mercury
Keywords: Green microalgae, Nanoparticles, Density gradient centrifugation, Mixtures
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Specifications table

Subject Area	Environmental Science
More specific subject area	*Environmental (nano)toxicology*
Method name	*A density gradient centrifugation method for rapid separation of nanoTiO_2 and TiO_2 aggregates from microalgal cells in complex mixtures with mercury*
Name and reference of original method	–
Resource availability	–

1 Method details

1.1 Background

In the aquatic environments, contaminants are found as complex mixtures [7,10]. Due to the extensive use of engineered nanomaterials (ENMs) in industry and consumer products, a variety of ENMs is inevitably disposed or released into the environment [5,6], including titanium dioxide nanoparticles (nanoTiO_2) [11,12]. Thus, it is pressing to determine the interactions and effects of nanoTiO_2 in mixtures because of their high reactivity. NanoTiO_2 is known to adsorb the dissolved contaminants [3,4,13]. Our previous work found that inorganic Hg (IHg) could significantly adsorbed to the nanoTiO_2 materials within 2 h [8]. Determining the bioaccumulation potential of the contaminants coexisting with ENMs is an important component of the hazard assessment of chemical mixtures. This requires quantification of cellular contaminants, after effective removal of the unbound or loosely bound nanomaterials from the organisms which may cause overestimation of the content of cellular contaminants. However, in the conventional differential centrifugation, where particles are separated based on their size and density, the aggregates of nanoTiO_2 are expected to sediment along with algal cells. Filtration is not an option due to the presence of TiO_2 aggregates of size similar to those of the algal cells in suspension. Here we present a novel method for separation of the nanoTiO_2 and their aggregates from green microalga *Chlamydomonas reinhardtii* and subsequent determination of the bioaccumulated Hg during exposure to mixtures of Hg and nanoTiO_2. This method is adaptation of a previous methodology successfully used to separate bacterium *Pseudomonas aeruginosa* from unbound multiwall carbon nanotubes (MWCNTs) and MWCNT aggregates [9].

1.2 Materials and equipment

- Wild-type *C. reinhardtii* (CPCC11, Canadian Phycological Culture Centre, Waterloo, Canada)
- Powdered nanoscale TiO_2 particles with different structure and size (anatase, 5 nm (A5), anatase, 15 nm (A15) and anatase/rutile, 20 nm (AR20), Nanostructured & Amorphous Materials, Inc., USA)
- Sucrose (analytical grade)
- $HgCl_2$ standard solution (1.0 g L^{-1}, Sigma-Aldrich, Buchs, Switzerland)
- Algal exposure medium (8.2×10^{-4} M $CaCl_2 \cdot 2H_2O$, 3.6×10^{-4} M $MgSO_4 \cdot 7H_2O$, 2.8×10^{-4} M $NaHCO_3$, 1.0×10^{-4} M KH_2PO_4 and 5.0×10^{-6} M NH_4NO_3, pH 7.0 ± 0.1)
- Acid-washed and autoclaved 15 and 50 mL centrifuge tubes
- Cooling centrifuge for cell harvesting
- Flow cytometer (FCM, BD Accuri 6, BD Biosciences, San Jose, CA)
- Advanced Hg Analyzer AMA 254 (Altec s.r.l., Czech Republic)

1.3 Procedure

The experimental procedure consists of three steps: (i) Exposure of algal cells to mixtures containing mercury and nanoTiO_2; (ii) Separation of algal cells from TiO_2 aggregates based on two-step centrifugation and (iii) Determination of the cellular Hg in alga. Here we present in details the steps (ii) and (iii).

Separation of algal cells from TiO_2 aggregates is based on two-step centrifugation: (i) differential centrifugation; and (ii) density gradient centrifugation in sucrose solutions. The variables to optimize

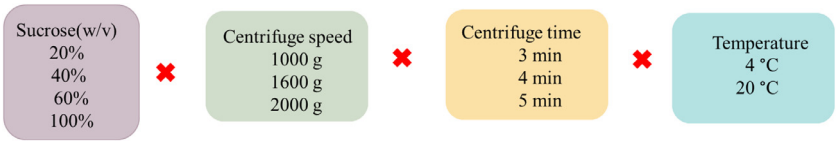

Fig. 1. Experimental conditions for density gradient centrifugation tested to select the best combinations of sucrose density, centrifuge speed, time, and temperature for nanoTiO_2 separation from algae.

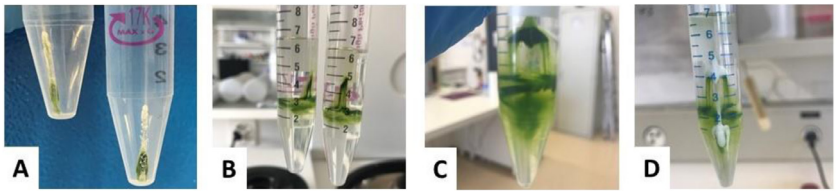

Fig. 2. Examples of unsuccessful tests. (A) TiO_2 aggregates sediment along with algal cells during conventional differential centrifugation step; (B) centrifugation time is too short to get all the alga settle down during density gradient centrifugation step; (C) density of sucrose was too low; (D) density of sucrose was too high, which prevented the TiO_2 aggregates to sediment.

include sucrose density gradient, centrifuge speed and time, and temperature. The selection of optimal conditions depends on the size and density of the materials being analyzed. According to the published literature, marine plankton is localize in an approximately 25–60% sucrose layer, meaning that their buoyant density is equal to the density of sucrose in this concentration range [2]. Theoretically, alga will settle down faster than individual nanoparticles but slower than nanoparticle aggregates. Thus, the localization of *C. reinhardtii* in sucrose was verified in 20–60% sucrose in nanoTiO_2-free exposure medium. The localization of nanoTiO_2 in sucrose was tested experimentally in 20–100% sucrose (Fig. 1), employing nanoTiO_2 suspensions in alga-free exposure medium. These two series of tests allowed to determine the optimal sucrose gradient concentrations. Because the ultimate goal was to assess bioaccumulation of Hg to *C. reinhardtii,* the duration of the separation procedure should be kept as short as possible (3–5 min). Two centrifugation temperatures were tested: 4 and 20 °C. To confirm that the cellular content of Hg was not affected by the separation procedure, the amounts of Hg accumulated in *C. reinhardtii* in the absence of nanoTiO_2 were quantified by atomic absorption spectrometry using the Advanced Hg Analyzer AMA 254 (Altec s.r.l., Czech Republic) before and after the separation by differential centrifugation followed by density gradient centrifugation. Some examples of different tentative for separation (Fig. 2) clearly demonstrated the need of the performing differential centrifugation (Fig. 2A) and careful optimization of the experimental condition of density gradient centrifugation.

Based on a number of different tests with 10% step increase of the sucrose percentage, 40% (w/v) sucrose was chosen for separation of individually dispersed nanoTiO_2 and algal cells. Under this condition almost all algae were localized in sucrose layer. 100% (w/v) sucrose was chosen for separation of algal cells and compact TiO_2 aggregates, as TiO_2 aggregates concentrated exclusively in this sucrose layer. After centrifugation for 4 min at 1600 *g* at 4 °C, the upper layer of 100% sucrose containing algae could be clearly distinguished by its green color. During the tests, algal cell numbers and nanoTiO_2 particle numbers were determined by flow cytometry. The method summarized in Fig. 3 and described herein in detail is used to assess the bioaccumulation of Hg by *C. reinhardtii* exposed to the mixtures of Hg and nanoTiO_2.

1.3.1 Preparation

1. Stock solution of 2.0 g L^{-1} of nanoTiO_2 were prepared by dispersing nanoparticles in ultrapure water and applying sonication for 10 min (50 W L^{-1} at 40 kHz), and a further 10 min sonication was conducted immediately before dosing.

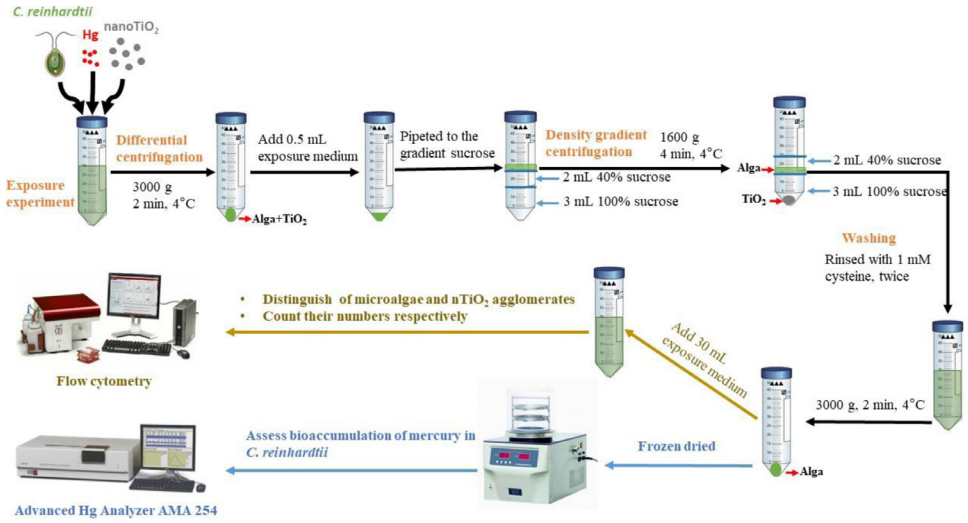

Fig. 3. Summary of developed procedure for separation of algal cells from nanoTiO$_2$ and TiO$_2$ aggregates using density gradient centrifugation.

2. All the glassware was soaked for at least 24 h in 5% v/v HNO_3, rinsed three times with ultrapure water and autoclaved prior use.

1.3.2 Exposure of C. reinhardtii *to Hg, nanoTiO$_2$, and their mixtures*

3. For each trial, the algal cells at mid-exponential growth phase were re-suspended (~10^6 cells mL^{-1}) in 30 mL exposure medium which contained Hg (10^{-9} M) or mixtures of nanoTiO$_2$ (2, 20, 100, and 200 mg L^{-1}) and Hg (10^{-9} M) for 24 h. Cells exposed in the absence of Hg and nanoTiO$_2$ were used as control. Exposures and analyses were performed on three biological replicates.

1.3.3 Differential centrifugation

4. 30 mL suspensions were centrifuged at 3000 g for 2 min at 4 °C to collect the pellet containing algae and nanoTiO$_2$, to get enough algal cells for quantitative analysis of their intracellular mercury.
5. Wash the cells twice with 30 mL 10^{-3} M ethylene diamine tetraacetic acid (EDTA; Sigma-Aldrich, Buchs, Switzerland) and 10^{-3} M cysteine (Sigma-Aldrich, Buchs, Switzerland) [1], respectively, by 1 min vortexing followed by centrifugation at 3000 *g* for 2 min at 4 °C to remove the extracellular loosely bound nanoTiO$_2$ and Hg.
6. Pellet was resuspended in 0.5 mL of exposure medium, vortexed for 1 min to obtain a homogeneous suspension.

1.3.4 Density gradient centrifugation

7. 2 mL of 40% sucrose were pipetted carefully over 3 mL of 100% sucrose into sterile conical polypropylene centrifuge tubes of 15 mL.
8. Algal suspensions obtained in step 5 were carefully pipetted over the sucrose gradient of 40% and 100% (w/v) and transferred to sterile conical polypropylene centrifuge tubes of 15 mL.
9. Tubes were centrifuged for 4 min at 1600 *g* at 4 °C.
10. Pellet was resuspended in 0.5 mL of exposure medium, vortexed for 1 min to obtain a homogeneous suspension.

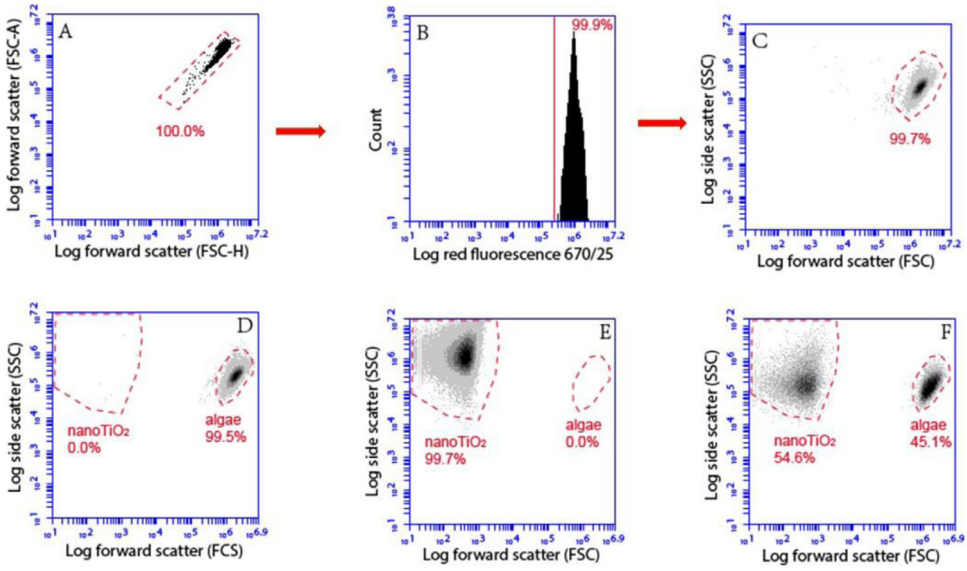

Fig. 4. Schematic representation of the FCM data analysis procedure used to define the gate corresponding to algae and nanoTiO$_2$ aggregates. As a first step FSC-H/FSC-A dot-plot (A) was used to remove eventual instrument background. Then signals present in the gated region were plotted in a count versus red fluorescence plot (B) and SSC/FSC dot-plot (C) to verify cellular characteristics of size, granularity and chlorophyll autofluorescence. (D) cytogram of algal suspension (1.0×10^6 cells/mL) in the absence of nanoTiO$_2$, (E) cytogram of 200 mg L^{-1} nanoTiO$_2$ suspension only (F) cytogram of mixture containing 1.0×10^6 cells/mL algaeand 200 mg L^{-1} nanoTiO$_2$.

1.4 Separation method validation

To evaluate the efficiency of the separation of the microalgae from the aggregates of nanoTiO$_2$, the cell numbers and the numbers of TiO$_2$ aggregates were determined by using flow cytometry before and after density gradient centrifugation. 488 nm argon excitation laser and fluorescence detection channel with band pass emission filters at a long pass emission filter for > 670 nm (FL3) were used. Data acquisition and analysis were performed with the BD Accuri C6 Software 264.15. The primary threshold was set to 20,000 events on FSC-H. Algal cells were discriminated from nanoTiO$_2$ aggregates applying the gating strategy shown on Fig. 4. The log FSC-H versus log FSC-A dot-plot was used first to remove cell doublets or artefacts. Then two different plots (log SSC-A versus log FSC-A and count versus log FL3) were used to distinguish algal cells and TiO$_2$ aggregates based on the difference in their size and chlorophyll autofluorescence specific for alga.

After density gradient centrifugation, the fractions collected from the region containing the green colored bands were resuspended in the same volume of initial algal suspensions and the cell densities were measured by FCM. The numbers of algae and TiO$_2$ particles were counted before and after density gradient centrifugation step (Fig. 5). Before the density gradient centrifugation, the ratios between alga and 2–200 mg L^{-1} TiO$_2$ were 16.54, 0.82, 0.14, and 0.07, respectively (Fig. 4 A–D). Our data show that above 98% of the three types of nanoTiO$_2$ with a large range of concentrations (2-200 mg L^{-1}) were removed from the algal pellet (Fig. 4E–H). A mean recovery of 83.3% algal cells was found by flow cytometry measuring cell density in suspensions after density gradient centrifugation and prior centrifugation. Initial density was (1.0 ± 0.056) x10^6 cells mL^{-1}.

1.4.1 Quantification of Hg accumulation by C. reinhardtii *in complex mixtures with nanoTiO$_2$*

After 24 h exposure, 30 mL of algal suspensions were centrifuged using the density gradient centrifugation method developed. Algal pellets were stored frozen at −20 °C until analysis in clean centrifugation flasks. The samples were dried in a freeze-dryer for at least 24 h. Freeze-dried

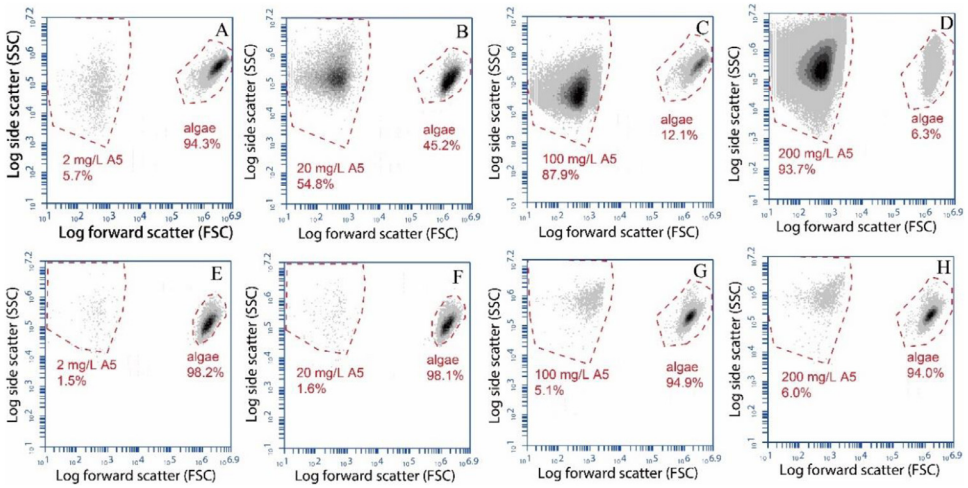

Fig. 5. FCM results of *C. reinhardtii* mix with 2 mg L^{-1} (A), 20 mg L^{-1} (B), 100 mg L^{-1} (C), and 200 mg L^{-1} (D) A5, taken before (A, B, C, D) and after (E, F, G, H) density gradient centrifugations.

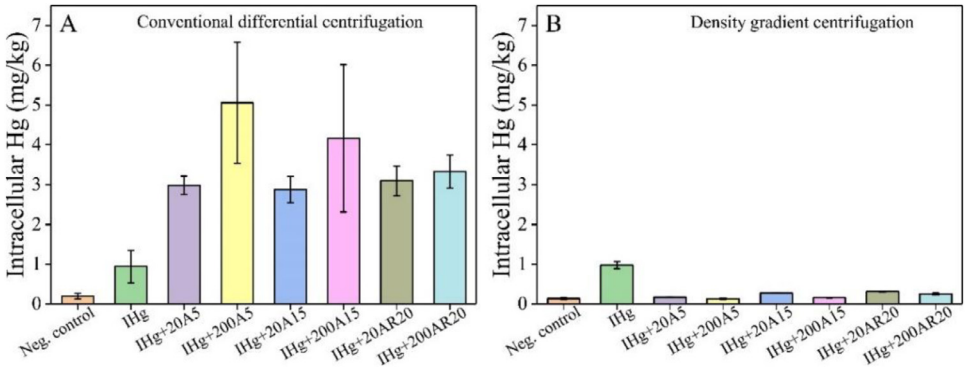

Fig. 6. Amount of intracellular (non-extractable by mixture of EDTA and cysteine) Hg in *C. reinhardtii* after the conventional differential centrifugation step (A) and the density gradient centrifugation step (B). Exposure conditions: 10^{-9} M inorganic mercury (IHg) in the absence and presence of 20 or 200 mg L^{-1} of different nanoTiO_2 materials A5, A15 and AR20, exposure duration 24 h.

samples were weighed (±0.00001 *g*) and the amount of accumulated Hg were determined by atomic absorption spectrometry using the Advanced Hg Analyzer AMA 254 (Altec s.r.l., Czech Republic). The accuracy of the measurements was checked by analyzing the certified reference material (CRM) MESS-3 (100 ± 0.1% recovery). Initial THg concentrations in the exposure medium were determined using MERX® Automated Total Mercury Analytical System (Brooks Rand Instruments, Seattle, WA, USA). Detection limit was 0.03 ng THg L^{-1}. The accuracy of THg measurements was verified by analyzing the CRM ORMS-5 (116.0 ± 3.5% recovery).

In the conventional differential centrifugation method, the presence of nanoTiO_2 significantly increased the bioaccumulated amount of Hg compared with alga exposed to Hg only. This increase was more pronounced at high nanoTiO_2 concentrations (Fig. 6A). To be specific, in the treatment of 10^{-9} M IHg, cellular Hg amount in the presence of 20 mg L^{-1} A5/A15/AR20 nanoTiO_2 was about 3 times higher than in the absence of nanoTiO_2. In the presence of 200 mg L^{-1} A5/A15/AR20, 5.4, 4.4 and 3.5-time increase of the intracellular Hg was found in comparison with the intracellular Hg

amount in the absence of nanoTiO$_2$. When density gradient centrifugation method was used (Fig. 6B), the intracellular Hg concentrations in the Hg + nanoTiO$_2$ mixture exposure decreased. Intracellular Hg amount in the presence of 20 mg L^{-1} A5/A15/AR20 was around 0.3 times less than the intracellular Hg amount in the absence of nanoTiO$_2$. In the presence of 200 mg L^{-1} A5/A15/AR20, intracellular Hg amount was around 0.2 times of the intracellular Hg amount in the absence of nanoTiO$_2$. This may be due to the co-sedimentation of the aggregated nanoTiO$_2$ with alga. Therefore, neglecting of these effects can lead to significant overestimation of the accumulation of Hg by alga in the presence of TiO$_2$ aggregates.

In the present study we used washing of the cells with 10^{-3} M EDTA and 10^{-3} M cysteine (step 5 of the procedure), which was previously shown to allow an extraction of the loosely-bound metals and mercury and allowed to operationally determine the intracellular or non-extractable metal [1]. Therefore, we assume that the possible contribution of Hg bound to the nanoTiO$_2$ that could be adsorbed on the algal cells and can not be separated by the proposed methodology will be negligible, since removed by cysteine wash given the strong affinity of Hg to SH- groups. In addition, no significant changes in the SSC and FSC signals of cells were found by FCM, suggesting no measurable adsorption of nanoTiO$_2$ to alga after washing with 10^{-3} M EDTA and 10^{-3} M cysteine took place.

2 Conclusions

We propose a novel density gradient centrifugation-based method for rapid and efficient separation of green microalga *C. reinhardtii* from nanoTiO$_2$ and TiO$_2$ aggregates followed by quantitative determination of the Hg accumulated in microalgae co-exposed to nanoTiO$_2$ and IHg. A key step of the method is a sucrose gradient centrifugation step allowing to separate efficiently phytoplankton from nanoTiO$_2$ and their aggregates and thus to determine the amount of the contaminant that is taken up by the microorganism, avoiding artefacts. The method was developed and validated in a specific case of green microalgae, nanoTiO$_2$ and Hg, however, the approach is highly transferable to other ENPs, which do not dissolve significantly, and trace metals in complex environmental mixture.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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