THE EFFECT OF TITANIUM DIOXIDE NANOPARTICLES SYNTHESISED USING GREEN TEA EXTRACT AND PURE EPIGALLOCATECHIN-3-GALLATE ON GLOBAL 5-METHYLCYTOSINE LEVEL IN PHYSARUM POLYCEPHALUM

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Titanium dioxide (TiO_2) is a crystalline material widely used in industrial applications due to its photocatalytic and superhydrophobic properties. Nano-sized TiO_2 is used in some consumer products, including paints, cosmetics, aerosols, or functionalized surfaces [1]. The release of TiO_2 NPs into soil, water or air during manufacturing or utilisation of the above mentioned products could be significant [2], leading to the possible bioaccumulation of this inorganic and stable nanomaterial into the environment. The toxic effects of TiO_2 NPs that were already demonstrated are multiple [3], including oxidative stress, DNA damage and inflammation (Figure 1). Green approaches are emerging as possible solutions for diminishing the toxic effects of conventional NPs as they use biomolecules in order to obtain more biocompatible NPs. Therefore, the aim of the present study was to obtain TiO_2 NPs by green routes, involving green tea (*Camellia sinensis*) extract and one of its major components in pure form, epigallocatechin-3-gallate (EGCG), and to study their effect on the growth rate and global 5-methylcytosine (5-mC) level by using microplasmodia of *Physarum polycephalum* as a model organism. *P. polycephalum*, a decomposer, eukaryotic organism naturally living under the forest leaf litter, tolerates the oxidative stress produced by relatively high doses of TiO_2 NPs as shown by recent studies [4,5]. However, epigenetic marks might respond differently to the presence of TiO_2 NPs, as the epigenome is generally more sensitive to the external factors. In this context, three types of TiO_2 NPs were tested (Figure 2): i. chemically obtained TiO_2 NPs derived from hydrolysis of titanium (IV) isopropoxide (TTIP) and condensation of the reaction products (chem-TiO_2 NPs); ii. green route obtained TiO_2 NPs from green tea extract assisted hydrolysis of TTIP (GT-TiO_2 NPs) and iii. green route obtained TiO_2 NPs from EGCG assisted hydrolysis of TTIP (EGCG-TiO_2 NPs). The method of synthesis of chem-TiO_2 NPs was adjusted from the previous work of Mahshid et al. [6]. The adjustments were described in detail in a previous publication of our research group [7]. Briefly, the synthesis of chem-TiO_2 NPs was performed by adding dropwise a solution of 8 mL TTIP:isopropanol (1:3) into 100 mL of distilled water. The mixture was stirred overnight on a heating plate at temperatures between 60-70°C until all the water was released by evaporation. A white precipitate was retrieved and dried for 2 h at 100°C. Green NPs were produced in a similar manner, excepting the water involved in hydrolysation of TTIP that was replaced by: i. a solution of 1 mmol EGCG in case of the EGCG-TiO_2 sample and ii. green tea extract, obtained from chopped aerial plant parts boiled in distilled water, for the GT-TiO_2 NPs sample, respectively. The physicochemical characterisation of NPs was performed using UV-VIS spectroscopy, scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and dynamic light scattering (DLS). The identification and quantification of phenolic compounds in the green tea extract was analysed by UHPLC-MS/MS. Microplasmodial cultures of *P. polycephalum* maintained in a liquid semi-defined medium were exposed to 0.01, 0.1 and 1 mg/mL for 24 and 72 h for each of the three NPs samples. Culture solutions and procedures were detailed in a previous paper [8]. DNA from each treated culture was purified by performing the organic phenol-chloroform extraction and quantified using the commercially available Qubit™ dsDNA Quantification Assay Kit (Invitrogen, Life Technologies Corporation, Oregon, USA). Global 5-mC level in DNA samples was measured by Dot Blot technique performed on a nitrocellulose blotting membrane (pore size: 0.45 µm; Amersham™ Protran™, Germany) incubated with an anti-5mC antibody. UHPLC-MS/MS analysis of the green tea extract revealed 25 phenolic compounds, EGCG being the most abundant (0.527 mmol) followed by epicatechin (0.2 mmol). Formation of TiO_2 NPs was preliminarily confirmed by UV-VIS spectroscopy. Bulk TiO_2 absorbs radiation in the UV region of the spectrum, between 275 and 405 nm. The UV-VIS spectra of the three types of NPs synthesised by us had a similar pattern. Chem-TiO_2 sample had a maximum absorption (λ_{max}) at 226 nm, while for EGCG-TiO_2 NPs and GT-TiO_2 NPs it occurred at 232 nm and 212 nm, respectively. An additional peak at 273-278 nm was observed in the three UV-VIS spectra. FTIR spectra depicted a large absorption band (1000-400 cm^{-1}) with peaks around

550 cm^{-1} corresponding to Ti-O bonds stretching. Both NPs synthesised by green route presented a distinct region of absorption bands between 1040 and 1520 cm^{-1}. As these peaks had no correspondence in the spectrum of chem-TiO_2 NPs, they suggest the presence of moieties from EGCG solution and green tea. SEM images depicted spherical, uniformly dispersed and clustered particles with variable sizes within the nanometer range (<50 nm). XRD analysis proved that biomolecules might affect the crystalline structure of NPs. XRD spectra of both samples synthesised by the green route suggested their amorphous nature. In contrast, chem-TiO_2 NPs acquired a distinct crystalline structure comprising 73.3% anatase and 26.7% brookite. Zeta potential of the samples was variable, suggesting that EGCG-TiO_2 NPs were the most stable (-56.5 ± 6.43 mV). Electronegative NPs are considered stable under a zeta potential value of -25 mV. Chem-TiO_2 NPs and GT-TiO_2 NPs had a zeta potential of -20.3 ± 1.27 mV and -24.5 ± 0.50, respectively. The hydrodynamic diameter of TiO_2 almost reached 1500 nm. Both green synthesised TiO_2 NPs had a significantly lower average size when dispersed in water, 470 nm (GT-TiO_2 NPs) and 438.5 nm (EGCG-TiO_2 NPs). The amount of DNA in each tested culture was an indicative of the growth rate of microplasmodia. It should be noted that the *P. polycephalum* cultures exhibited similar growth in presence of the different NPs and in their absence. Dot blot revealed chem-TiO_2 NPs had a significant hypermethylation effect on the 5-position of cytosines in the DNA of *P. polycephalum* at 72 h, the effect being dose-dependent. Differences at 24 h were not statistically significant. EGCG-TiO_2 NPs acted reversely, causing a significant hypomethylation effect at 0.1 and 1 mg/mL after 72 h of exposure. The response of cytosine methylation state in *P. polycephalum* to exposure at GT-TiO_2 NPs was two-phased: i. hypomethylation was observed at 0.01 mg/mL in a time dependent manner and ii. at 0.1 and 1 mg/mL after 24 h of exposure the signal suggested global DNA methylation increased, while at 72 h decreased under the 5-mC level in the control culture. We concluded that a better control and standardisation of the NPs synthesis process were acquired by using pure EGCG instead of the entire green tea extract. The main advantage of using EGCG in the synthesis of TiO_2 NPs was the increased particle stability. Results suggested that EGCG inverted the effect of TiO_2 NPs on global 5-mC level in *P. polycephalum*. While EGCG and green tea acted barely similar on the particle properties, the epigenetic effect of GT-TiO_2 NPs did not follow an evident tendency. Response of *P. polycephalum* to GT-TiO_2 NPs could be described by a hormesis effect that might be caused by the variable biomolecules content of green tea.

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Figures

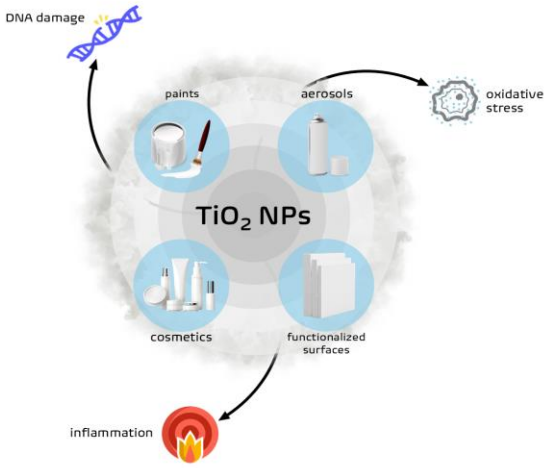

Figure 1. Main toxic effects of TiO_2 NPs and some of the consumer products prone to release NPs

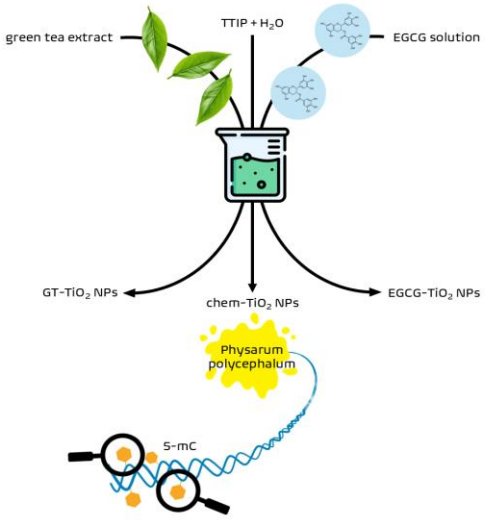

Figure 2. General flowchart of the experiment