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# Influence of carbon-based nanomaterials on *lux*-bioreporter *Escherichia coli*

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ABSTRACT

The cytotoxic effects of carbon-based nanomaterials are evaluated via the induction of luminescent genetically engineered *Escherichia coli* bacterial cells. Specifically, two engineered *E. coli* bacteria strains of DPD2794 and TV1061 were incubated with aqueous dispersion of three carbon allotropes (multi-wall carbon nanotubes (MWCNTs), graphene nanosheets and carbon black nanopowders) with different concentrations and the resulting bioluminescence was recorded at 30 °C and 25 °C, respectively. The corresponding optical density changes of bacterial cells in the presence of various carbon nanomaterials were recorded as well. Based on these results, *E. coli* DPD2794 bacterial induction responds to a greater degree than *E. coli* TV1061 bacteria when exposed to various carbon-based nanomaterials. Finally, the surface morphology of *E. coli* DPD2794 bacteria cells before and after carbon-based nanomaterials treatment was observed using a field emission scanning electron microscope (FESEM), from which morphological changes from the presence of carbon-based nanomaterials were observed and discussed.


## 1. Introduction

Owing to their excellent mechanical and tunable electronic properties, carbon-based nanomaterials (carbon nanotubes, graphene, fullerene, carbon black, etc.) have found wide applications in a range of fields such as biochemical sensors [1–3], alternative energy [4,5], nanomedicine [6,7] and high performance composite materials [8,9]. However, the environmental impact of carbon-based nanomaterials is crucial to understand before they are released in large scale into the environment. Issues such as dispersion, ecotoxicology and human health effects need to be systematically evaluated prior to their large-scale commercial use. Previously, it has been reported that cytotoxicity of carbon-based nanomaterials is dependent on their shape, size, surface modification and electronic structures [10]. For instance, single wall carbon nanotube (SWCNT) dispersions are highly cytotoxic to bacterial cells when dispersed uniformly in solution [11]. By using specialized microscopic (AFM or SEM) characterization, as well as gene expression methods, carbon nanotube cytotoxicity mechanisms to bacterial cells were evaluated as possibly involving a combination of direct cellular membrane stress and oxidative stress [12–14]. In addition, emerging 2D graphene nanosheet composites were shown to exhibit high toxicity to bacterial cells possibly due to the direct contact of their extremely sharp edge [15,16], thus hinting to their putative use as a novel antibacterial agent [17,18] or material, such as antibacterial paper, or film [19,20].

Over the years, bioluminescence of genetically modified bacterial cells has been explored in whole cell biosensor devices in the detection of various toxicants such as pesticides [21–23], heavy metals [24–27] and organic pollutants [28]. In this system, the plasmids of bacterial cells are genetically modified to include toxicant-specific promoter genes that regulate the expression of bioluminescence proteins [29]. For example, *Escherichia coli* strain TV1061 harbors a fusion of *luxCDABE* reporter gene and the promoter for the heat-shock gene *grpE*, while in the *E. coli* strain DPD2794, the promoter of *recA* gene is fused with *luxCDABE*. While TV1061 bacterial strain is responsive to many toxic compounds [30], the responses of DPD2794 bacterial cells are mainly restricted to that of genotoxicity [31]. When these bacterial cells are incubated with certain toxicants, bioluminescence is generated once the lux operon has been transcribed and translated to produce luciferase (encoded by *luxAB*) and its associated substrate (reduced flavin mononucleotide ($FMNH_2$) and long-chain fatty aldehyde, encoded by *luxCDE*), an event that is triggered by the adjacent promoter that is sensitive to certain given toxicants.

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In addition, one must take into account that the luminescence is indirectly influenced by a series of biochemical reactions in the living bacterial cells, which are highly dependent on the metabolism of the said bacterial cells, itself putatively altered by the incoming toxicant [32,33]. Therefore, the toxic effects of the added compounds on the bacterial cells can be readily revealed via the kinetic evolution of bacterial bioluminescence, while keeping in mind their influence on the general cellular metabolism.

Although there are some reports studying the cytotoxicity of carbon-based nanomaterials [12,34], only one group of materials was studied and using wild-type bacterial cells [13,35]. In addition, toxicity experiments were conducted by using either tedious microbial plating methods [36] or using expensive bio-reagents and specialized bioanalytical kits [35]. Of special note is the fact that the bioluminescence assay described herein based on genetically modified bacterial cells is both sensitive and cost-effective, as bacterial cells produce all the needed ingredients involved in bioluminescence generation without the addition of any exogenous bio-reagents [37]. Even though engineered bacterial cells have been used to detect various toxic compounds, their application in an assay resulting from the exposure to nanomaterial toxicity has not been much exploited, with the exception found in cases of metal nanoparticles, like silver [38] or copper [39].

In this work, three different commercial carbon allotropes of 0D carbon black nanopowder (CB), 1D multiwall carbon nanotube (MWCNT) and 2D graphene nanosheet have been incubated at the same dispersal concentrations (or suspensions) with two of the aforementioned genetically engineered *E. coli* bacteria strains of DPD2794 and TV1061. The resulting bacterial bioluminescence (toxicity induction) and optical density changes (survival) are used to evaluate the cytotoxicity of carbon-based nanomaterials at two different temperatures of 30 °C and 25 °C. The field emission scanning electron microscope (FESEM) was used to observe any possible interaction or morphological effect on the bacterial cells, by the said carbon based nanomaterials. To our best knowledge, this is the first report using engineered *E. coli* bacterial bioluminescence in the toxicity study of several carbon-based nanomaterials.

## 2. Materials and methods

### 2.1. Materials

Kanamycin sulfate (K1377), LB-broth (L3032), glutaraldehyde solution (G7651), and ethanol (02877) were purchased from Sigma (Germany). Phosphate buffer saline (PBS buffer, pH 7.4) was prepared in our lab using sodium chloride (S7653), sodium phosphate dibasic (94046) and sodium phosphate monobasic (71505) received from Sigma (France). Deionized water was produced by a Millipore water purification system (Molsheim, France). Short multiwall carbon nanotubes (MWCNTs) modified with carboxylic group were purchased from Nanostructured & Amorphous Materials. Inc., graphene nanosheets were acquired from Graphene Supermarket. Inc. and carbon black nanopowders (CB, Monarch 1300) were obtained from Cabot Inc. *E. Coli* bacteria strains of DPD2794 and TV1061 were acquired from Prof. Shimshon Belkin of The Hebrew University of Jerusalem in Israel. Non-sterile white polystyrene 96 well microtiter plates (Costar, Corning Incorporated, USA) were used in the bioluminescent assays.

### 2.2. Medium preparation

LB (Luria-Bertani) medium was prepared by adding 2 g LB broth powder into 100 mL double distilled water in a 200 mL bottle. The bottle with the cap partially closed and covered by a piece of aluminum paper was sterilized in an autoclave-steam sterilizer (Tuttnauer, 2540 ML) for 15 min at 121 °C. After freely cooling down to room temperature, the LB medium was immediately used for the cultivation of bacteria and the preparation of various dispersed carbon-based nanomaterials dilutions.

### 2.3. Instrumentation

Growth of *E. coli* strain DPD2794 and TV1061 bacteria was performed using a water bath (Grant Instruments, Cambridge Ltd., UK). The optical density of bacterial suspensions was measured using a DR/ 2500 spectrophotometer Odyssey (HACH Company, USA), while various dilutions of bacterial cultures and carbon-based nanomaterials solutions were mixed using a G560E Genie2 vortex. For the centrifugation experiments, a model type Hitachi Universal 320R (Germany) was used. Bioluminescence measurements were conducted in a luminometer (Luminoskan Ascent, Thermo Fisher Scientific, United States). The morphology of carbon-based nanomaterials and bacterial cells was characterized by using the field emission electron scanning microscope (Raith e-line, Germany). We wished to evaluate the possibility that the carbonaceous materials may have been affected by the presence of the bacteria via any of their metabolites and therefore we checked for changes in their surface or structure and this by the use of infrared and Raman spectroscopy and compared against the original material.

### 2.4. Bacterial strains

*E. coli* DPD2794 and TV1061 strains were used as recombinant luminescent bioreporter bacteria. *E. coli* DPD2794 strain contains the promoter of DNA damage sensitive *recA* gene, while *E. coli* TV1061 strain harbors the promoter of heat-shock *grpE* gene; the operon of *lux*CDABE gene, used as the bioluminescent reporter, was fused with these promoters in the plasmid of two *E. coli* strains. The *grpE* gene is sensitive to metabolic changes that can be activated due to the presence of cytotoxic substances, while *recA* gene is mainly responsive to the genotoxical compounds. Before the initial growth step, the bacterial suspensions were stored at −20 °C in a sterile medium mixture containing equal volumes of LB medium and glycerol aqueous solution (40%, v/v).

### 2.5. Bacterial growth conditions

*E. coli* bacterial cultivation, prior to bioluminescence measurements, was performed in 10 mL LB medium supplemented with 10 μL of 50 mg $mL^{-1}$ kanamycin antibiotics, with a final concentration of antibiotic at 50 μg $mL^{-1}$. The cells were grown for 9 h at 37 °C in a thermostated water bath. Thereafter, 200 μL of the resulting bacterial suspension were inoculated into 10 mL fresh LB medium free of antibiotics at 30 °C, to get the optical density of 0.08 as determined by a spectrophotometer, corresponding to a bacteria cell concentration number of $1.1 \times 10^8$ cfu $mL^{-1}$.

### 2.6. Preparation of the 96-well microtiter test plate

Stock solutions (0.1 mg $mL^{-1}$ were prepared by dispersion of 1 mg of various commercial carbon-based nanomaterials in 10 mL $ddH_2O$, followed by ultrasonication at room temperature for 1 h. Then, 100 μL stock solution was diluted into 900 μL LB medium in a 1.5 mL Eppendorf tube to obtain a suspension of 1:10 of carbon-based nanomaterials (10 μg $mL^{-1}$, and thereafter tenfold serial aliquots of $10^{-2}$ (1 μg $mL^{-1}$), $10^{-3}$ (0.1 μg $mL^{-1}$), $10^{-4}$ (10 ng $mL^{-1}$), $10^{-5}$ (1 ng $mL^{-1}$) and $10^{-6}$ (0.1 ng $mL^{-1}$). To obtain uniform carbon nanomaterials/bacterial suspension, Eppendorf tubes containing mixtures of 360 μL bacterial suspension of a specific