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Probing the toxicity mechanism of multi-walled carbon nanotubes on bacteria

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Probing the toxicity mechanism of multi-walled carbon nanotubes on bacteria

Carbon nanotubes (CNTs) have emerged recently as superior adsorbent materials for the removal of recalcitrant pollutants. The potential of combining the sorption capability of CNTs with bacterial degradation for pollutant removal, however, necessitates further investigation of the mechanisms of CNTs' toxicity towards bacteria cells. In this study, we used a panel of stress-responsive recombinant *Escherichia coli* bioluminescence bacterial strains to explore the possible mechanisms of toxicity of the multi-walled carbon nanotubes (MWCNTs). The effects of MWCNTs on markers of oxidative stress, protein, DNA, and membrane damage enabled the exposition of some of the mechanisms of their antimicrobial properties. Using a bioluminescence panel and live/dead staining, we observed that membrane damage played a role in the toxicity of MWCNTs. A subsequent viability study using three strains of bacteria – two gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and one gram-positive (*Bacillus subtilis*) – showed significant MWCNT toxicity in hypotonic water and phosphate-buffered saline solution, compared with the MWCNT toxicity towards the same bacteria incubated in isotonic-rich media. Using a field-emission scanning electron microscope, we demonstrated that membrane damage is caused largely by MWCNTs trapping bacteria and piercing the cell walls. As a result of our observations, we propose integrating MWCNTs and bacteria degradation for pollutant removal in nutrient-rich media to minimize the toxicity effect of CNTs.

Keywords: carbon nanotubes; nanotoxicity; bacteria; membrane damage

Introduction

Carbon nanotubes (CNTs) are one of the most promising emerging sorbent materials for water treatment, as they are capable of effectively removing low concentrations of toxic recalcitrant pollutants over a wide range of pHs (Long and Yang 2001; Gupta et al. 2013); they have also been reported to be a more effective adsorbent than activated carbon (AC) (Lu et al. 2007) owing to their greater surface area, short equilibrium times, hydrophobicity, and selective adsorption for aromatic compounds through π - π electron donor-acceptor interaction (Hartono et al. 2014, 2016). The use of CNTs as a new-generation sorbent may reduce the cost of water conditioning due to the shorter contact times and lower-volume tanks required for treatment (Bohdziewicz and Kamińska 2013). Moreover, the increase in market demand for CNTs, due to their emerging applications across different fields, is expected to cause the price of CNTs to decrease in the future (De Volder et al. 2013). Since the 1970s, the biological activated carbon process (BAC), which combines the adsorption capability of AC with bacteria for the removal of organic pollutants, has been steadily developed (Sirotkin et al. 2001; Martin et al. 2004). Greater adsorption and biological oxidation capacity in the combined process have been observed in several studies using AC (Orshansky and Narkis 1997; Morinaga et al. 2003). A combination of bacterial biodegradation with CNT adsorption in a single system, however, may provide additional complexity due to the perceived antimicrobial nature of CNTs. Despite this, a recent study of the integration of pollutant-degrading microorganisms with CNTs for the removal of herbicide atrazine reported promising improvement in pollutant removal, depending on the number of CNTs added (Zhang et al. 2015).

Although significant efforts to utilize CNTs as sorbent materials, as well as in other applications, have been reported, there is insufficient research dedicated to

addressing CNTs' interaction with and possible toxicity towards living entities, including bacteria. A clear understanding of the toxicity mechanism governing CNTs' antimicrobial capability will be important in the further development and integration of CNTs for potential applications, including wastewater treatment.

The antimicrobial properties of single-walled carbon nanotubes (SWCNTs) were first reported by Kang et al. (2007). These properties appear to be affected by the concentrations (Dong et al. 2012), length (Yang et al. 2010), buffers, and surface functional groups of the SWCNTs, and by the shapes of the affected bacteria (Chen et al. 2013); they were also found to be less influenced by the charge of the functional group on the CNTs (Arias and Yang 2009). The antimicrobial properties of SWCNTs were also observed to increase with the fraction of metallic SWCNTs (Vecitis et al. 2010) as well as the presence of metal catalysts introduced during the synthesis of CNTs (Cortes et al. 2014). One of the propounded mechanisms of CNT toxicity is through direct piercing leading to cell-membrane damage, which subsequently results in the leakage of cytoplasmic materials (Nagai and Toyokuni 2012). Moreover, thinner SWCNTs have been considered more effective in piercing the membrane of the bacteria than multi-walled CNTs (MWCNTs) (Chen et al. 2013). On the other hand, MWCNTs have also been reported to have higher toxicity than SWCNTs, possibly due to their efficiency in piercing the cell (Young et al. 2012). Other studies have posited that it is unlikely that direct piercing of the bacteria membrane by individual SWCNTs occurs during incubation, and proposed that the bacterial death is caused by the accumulated effects of agglomerated nanotube networks trapping the surface of the cells, a process that enhances bacterial cellular stress and inhibits microbial growth (Liu et al. 2010; Olivi et al. 2013). From these conflicting reports, it is apparent that the interactions of CNTs with bacterial cells may be multifaceted, and require further exploration. In

1 addition, the variability of the chemical and physical characteristics of the CNTs used in
2 the previous studies has added to the difficulties in assessing the modes of toxicity.
3

4 Bioluminescent bacteria provides an attractive platform for rapid screening of
5 the in-situ bio-availability of pollutants toxic to microorganisms, due to their relatively
6 low cost, rapidity, and technical simplicity (Eltzov et al. 2008; Li et al. 2013; Jia et al.
7 2014; Hua et al. 2015). For this work, a bioluminescent bacterial panel comprising a
8 series of genetically engineered recombinant *E. coli* strains, each sensitive to different
9 toxicity stressors, was used. This was made possible by the insertion of a specific
10 regulatory promoter and *luxCDABE* gene within the plasmid. These markers, including
11 protein, DNA, membrane damage, and oxidative stress, were used to screen commercial
12 MWCNTs based on the mode of toxicity triggered by the CNTs' presence. In addition,
13 live/dead fluorescent bacterial staining (Le et al. 2015) was used as a qualitative
14 indicator of the membrane damage caused by CNTs. Previous work (Arias and Yang
15 2009) has suggested that the ionic strength of the incubation medium may influence the
16 resulting toxicity of CNTs, with a lower ionic strength medium exhibiting higher
17 toxicity. To better understand the effect of the incubation medium, the toxicity capacity
18 of MWCNTs in an isotonic rich medium, phosphate buffered saline, and hypotonic
19 ultrapure water was tested against three bacterial strains (*Escherichia coli*,
20 *Pseudomonas aeruginosa*, and *Bacillus subtilis*) using viability plate count and electron
21 microscope imaging.
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Materials and Methods

Chemicals

All chemicals used in this study were of analytical grade and used as received. Lysogeny broth (LB) agar, LB broth, and 50% Glutaraldehyde were purchased from Sigma. MWCNTs (724769, d: 6–9 nm with 5.5 nm mode, L: 5000 nm) were purchased from Aldrich. 10X phosphate buffered saline (PBS) stock solution was prepared by dissolving 1.3 M NaCl, 70 mM Na₂HPO₄·2H₂O, and 30 mM NaH₂PO₄·2H₂O and adjusting the pH to 7.0; 1x PBS solution was subsequently prepared by diluting the 10X PBS stock with Milli-Q ultrapure water. All aqueous media used were sterilized by autoclave at 121 °C for 15 min.

Bioluminescence assays

The four recombinant *E. coli* strains used in the bioluminescence assay are described in Table 1. Bioluminescent bacterial strains were cultivated in 10 ml LB broth with the addition of the antibiotic kanamycin, and grown for 17 h at 37 °C and 150 rpm. The overnight cultures were diluted 100x to approximately 10⁷ CFU/mL and re-grown in static LB broth in the absence of antibiotics for 1.5 h at 37 °C. The OD_{600nm} after static incubation was adjusted to ~0.02–0.03. For stock solutions, 500 µg·mL⁻¹ of as-received MWCNTs were diluted in LB broth, followed by sonication to allow better dispersion. The stock solution was immediately diluted into the desired concentrations in LB and vortexed prior to luminescence measurement. Bioluminescence production by the bioreporter bacteria was followed overnight using a Luminoskan Ascent luminometer (Thermo Fisher). To conduct the luminescence measurement, 90 µl of a bacterial culture was added to each well of white opaque 96-well microtiter plates, followed by the addition of 10 µl of toxicant solution in different concentrations (n=3 for each

concentration, triplicate assays). The specified temperature of the luminometer was adjusted and maintained at ambient temperature throughout the measurement. The luminescence values were characterized as relative light units (RLUs).

The bioluminescence signal produced by the bacteria as a response to different inducers was represented as an area under the curve (AUC) (Jia et al. 2014). The AUC was calculated using the integration function of the graph of emitted RLU vs incubation time in Origin Pro 8.5 software. The subsequent data was presented as ΔAUC , which was calculated as follows:

$$\Delta AUC (\%) = \frac{\left(\frac{AUC_{CNTs} - AUC_{bac}}{AUC_{bac}} \right)}{AUC_{bac}} \times 100\% \quad (1)$$

$AUC_{CNTs/bac}$ refers to the total luminescence produced by the CNT-bacteria suspension, while AUC_{bac} refers to the total luminescence produced by the control bacteria without the addition of CNTs.

Unless otherwise specified, the assay was repeated in triplicate with 3 wells for each tested concentration per assay (n=9). The value reported was the mean of the readings. The optimization of the monitoring temperature used in all bioluminescence measurements and the adjustment of the signal due to attenuation effects introduced by CNT interference at high concentration are described in **Supporting Information (S1)**.

Staining assay

The Live/Dead BacLight Bacterial Viability Kit (L7012, Molecular Probes Invitrogen) was used to assess the viability of the cells after luminescence measurements. The kit comprises two stains: SYTO9 and propidium iodide (PI). SYTO9 exhibits green fluorescence and stains viable cells, and PI exhibits red fluorescence and only stains cells with severely damaged cytoplasm membranes. To prepare the dye stock mixture

solution, 1.5µl of PI and 1.5µl of SYTO9 were added to 1 ml of sterile double-distilled water. The stock solution was kept at -20 °C in the dark. 100 µl of the CNT-luminescence bacteria suspension was collected from one well in a microplate after overnight measurement and centrifuged at 14,000 rpm for 15 min. The supernatant was removed and the pellets re-suspended into 0.85% sterile NaCl, followed by incubation for 40 min at ambient temperature. After another round of centrifuge-washing, the pellets were re-suspended in a 100-µl dye mixture solution and incubated for 15 min in the dark. Prior to imaging, 5 µl of the stained bacterial suspension was added to clean glass slides and fixed with a cover slip. The optimum excitation/emission for SYTO9 and PI dyes are approximately 480/500 nm and 490/635 nm respectively. An inverted fluorescence microscope (Leica), equipped with two filter cubes of L5 (excitation: BP 480/40, emission: BP 527/30, for SYTO9 green fluorescence) and N3 (excitation: BP 546/12, emission: BP 600/40, for PI red fluorescence), and LAS X software (Leica) were used for visualization. The frames were captured at, at least, three different sites for each sample, under 20x magnification. ImageJ software was used to estimate the number of viable (green-stained) and non-viable (red-stained) cells in each frame after converting the images to 8-bit, adjusting to a similar threshold, and analyzing the particle size that fell within 0.05–1.00 circularity. For this work, the live/dead stain was used to approximate the percentage of non-viable cells collected from a 96-well plate upon overnight exposure to CNTs, based on the following calculation:

$$\text{Membrane – damaged cells (\%)} = \frac{\#Red-stained\ cells}{\#Red-stained\ cells + \#Green\ stained\ cells} \times 100\% \quad (2)$$

Incubation of bacterial cells with CNTs

Escherichia coli (ATCC 8739, TV1061), *Pseudomonas aeruginosa* (ATCC 9027), and *Bacillus subtilis* (ATCC 6051) strains were used for the incubation experiment. Here, three incubation media (an isotonic LB medium, a PBS solution, and hypotonic ultrapure water) were utilized to assess the possible effect of the medium. Ultrapure water was added as one of the incubation mediums to enable comparison of the effects of nutrient-deficit and hypotonic conditions on the toxicity of CNTs. First, a colony of each strain pre-grown on LB agar was inoculated into a 10-ml LB broth, in a 50-ml sterile tube, and placed inside an incubator shaker at 37 °C with 150 rpm agitation for overnight growth. To reduce the influence of residue originating from the growth medium, 1000 µL of the overnight culture was collected and centrifuged at 5,000 rpm for 2 min. The supernatant was removed while the pellets were re-suspended and stored in 1000 µL of sterile ultrapure water, PBS, or LB broth. Approximately 100 µL of each cell suspension was added to 9.9 mL of ultrapure water, PBS, or LB solutions containing a pre-defined amount of CNTs in 15 ml-tubes. Prior to the addition of the cell suspensions, the solution containing the CNTs was sonicated for 30 min at 37 kHz (Elmasonic) under ambient conditions to improve the dispersion of the CNTs; the solution was then irradiated under UV light inside a biosafety hood for 15 min to render it sterile. The inoculated tubes were placed inside an incubator shaker at 26 °C with 150 rpm agitation, and 500 µL aliquots were taken after one day and seven days of incubation for further analysis. Control experiments were performed by incubation in ultrapure water, PBS, or LB broth without the addition of CNTs. The details of the strains used and the experimental conditions are summarized in **Table 2**.

Cell-Viability Assay

The viability of the cells was assessed using a plate-counting method. The serial dilutions of the aliquots were made in a sterile PBS solution and spread on LB agar plates. The number of colony-forming units (CFUs) was counted after overnight incubation at 30 °C. The relative decrease in cell viability upon exposure to MWCNTs was calculated as follows:

$$\text{Relative decrease in viability}(\%) = \frac{\#CV_{bac} - \#CV_{MWCNTs/bac}}{\#CV_{bac}} \times 100\% \quad (3)$$

In the above equation, CV_{bac} refers to the average number of counted colonies produced by the control bacteria without the addition of MWCNTs, while $CV_{MWCNTs/bac}$ refers to the average number of counted colonies in $CFU.mL^{-1}$ that originated from MWCNTs-bacteria suspensions.

Field emission scanning electron microscopy (FESEM) imaging

The preparation of samples for electron microscopy imaging was performed as follows. The bacterial suspensions were vortexed and 500 μ L of the suspensions were taken and centrifuged at 10,000 rpm for 10 min. The supernatant was then removed and the pellets were re-suspended into 500 μ L of 1XPBS. This centrifuge-washing step was repeated twice. Following the removal of the PBS supernatant, 500 μ L of a 2.5 wt% glutaraldehyde-1XPBS solution was added, and the pellet was re-suspended and incubated at 4 °C for at least 4 h, after which the suspension was centrifuged at 10,000 rpm for 5 min and washed via the addition of 500 μ L of ultrapure water. Dehydration was then performed by adding 30% (v/v) ethanol solution and carrying out immersion for 15 min at 4 °C, followed by centrifugation at 10,000 rpm for 5 min and the removal of the supernatant. This step-wise dehydration was subsequently carried out for 50%, 70%, and 100% ethanol. Prior to imaging, the dehydrated cell suspension was vortexed

and 10 μL was spread onto an ethanol-cleaned silicon substrate; this was followed by air-drying inside a biosafety hood. A platinum layer was then used to sputter-coat the dry sample under 20 mA current for 20 sec to reduce the charging effect. A Zeiss Supra55VP FESEM was used for imaging of the samples.

Results and discussion

Bioluminescence panel response

In general, positive bioluminescence responses were recorded for all *E. coli* reporter strains, which would indicate that the presence of CNTs triggered cellular damage in the bacteria. The bioluminescence panel responses to MWCNTs at different concentrations are presented in **Figure 1**. The RLU curves versus incubation time for all tested strains are provided in **Supporting Information (S2)**. A significant (ANOVA $p < 0.05$) dose-dependent effect of MWCNTs was observed in the case of *E. coli* DPD2544, a marker that is sensitive to membrane damage. The luminescence responses to MWCNTs by *E. coli* DPD2544 were recorded at the lowest concentration tested of $10^{-4} \mu\text{g.mL}^{-1}$, with luminescence loss starting at $10^{-2} \mu\text{g.mL}^{-1}$ (**Fig 1**). *E. coli* DPD2794, which as mentioned is a marker sensitive to DNA damage, showed marked reductions in luminescence response with increases in CNT concentrations (ANOVA $p < 0.05$). Indeed, at $10 \mu\text{g.mL}^{-1}$ the amount of luminescence produced for DNA damage was less than the negative control. The *E. coli* TV1061 strain, which is sensitive to protein damage, showed bioluminescence responses independent of dose (ANOVA $p > 0.05$, insignificant effect of CNT dose). The *E. coli* DPD2511 strain, which is sensitive to oxidative stress, showed similar dose-independent (ANOVA $p > 0.05$) positive luminescence response for CNTs at the tested concentrations.

Liu et al. (2009) proposed that the role of oxidative stress in the antimicrobial effect of CNTs is minor. Since un-purified MWCNTs were used in this study, a possible source of the oxidative stress response exhibited by *E. coli* DPD2511 may have been the trace metal catalysts introduced during the production of CNTs (Cortes et al. 2014). The presence of these metals may mediate the formation of reactive oxygen species (ROS) through the Fenton reaction (Stohs and Bagchi 1995), thus causing the effects exhibited by *E. coli* DPD2511. Oxidative stress has been shown to stimulate protein, membrane, and DNA damage on bacteria (Li et al. 2013), and therefore it may be assumed that the bioluminescence response induced in the other three stress-responsive strains beside *E. coli* DPD2511 was influenced by the presence of ROS, albeit as a secondary effect. Clear differences in bioluminescence responses were observed with regard to markers of membrane damage. A negative luminescence could suggest bacterial death due to too much toxicity (Eltzov et al. 2008). The loss of luminescence in *E. coli* DPD2544 exposed to MWCNTs indicated the role of membrane damage in the toxicity of MWCNTs.

Membrane-integrity assay

To further establish the prominence of membrane damage on the CNT-exposed bacteria, another indicator for membrane permeability, based on the uptake of fluorescence dye propidium iodide, was utilized (Le et al. 2015). The fluorescence live/dead staining includes SYTO 9™, which penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes; the combination of the two stains produces red fluorescing cells. A rapid estimation of bacteria with damaged membranes was performed using fluorescence live/dead staining on *E. coli* (DPD2544). Bioluminescence bioreporter bacterium *E. coli*

(DPD2544) was exposed to various concentrations of MWCNTs, and the emitted luminescence signal was monitored overnight. In order to estimate the percentage of bacteria damaged after the exposure, the culture was then collected from the wells and stained with both fluorescent dyes. As shown in **Figure 2**, the percentage of membrane-damaged cells from total counted cells appeared to follow a dose-dependent trend in the case of *E. coli* (DPD2544) exposed to MWCNTs. The fluorescence-staining results showed that the bacterial membranes were indeed compromised after exposure to MWCNTs, and therefore indirectly support the results obtained from the bioluminescence panel assay.

Effect of incubation medium

It has been reported that the toxicity effect of a pollutant depends on the physicochemical state of the pollutant as well as the physiological state of the bacteria tested, which may vary in the type of medium used (Virto et al. 2005; Li et al. 2011). For this study, the interaction between MWCNTs and three different bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*) in three different incubation media was further investigated. The first medium used was LB broth (the same as that used in our bioluminescence study), while the second and third mediums were PBS solution and nutrient-free hypotonic ultrapure water respectively. They were chosen to provide an additional measure for the assessment of the toxicity of MWCNTs. A traditional plate assay was performed after incubation to assess their viability after exposure to MWCNTs in different media. In order to mimic our bioluminescence experiments and to ensure the presence of a toxic effect, the bacteria-MWCNT suspensions were incubated at 26 °C with an elevated MWCNT concentration of 100 $\mu\text{g.mL}^{-1}$.

The effects of MWCNTs on the viability of *E. coli*, and on additional bacterial strains *P. aeruginosa* and *B. subtilis* after incubation for one and seven days at 26 °C in a different incubation medium, are shown in **Figure 3**. These results showed a relative decrease in viability for gram-negative *E. coli* and *P. aeruginosa* bacteria in all media after a one-day incubation, with higher toxicity observed in PBS and ultrapure water.

For MWCNT/bacteria suspensions in LB, proliferation still occurred after a one-day incubation for both gram-negative bacteria, while there was a relative decrease in viability estimated to be approximately 20% for *E. coli* and 12% for *P. aeruginosa*. More significant viability decreases for *E. coli* and *P. aeruginosa* were observed in PBS and ultrapure water. Continued incubation for seven days resulted in a similar level of viability decrease for these gram-negative strains in both PBS and ultrapure water, while the MWCNT/bacteria suspension in LB experienced a relatively lower decrease of viability with respect to control.

An unexpected result was observed for the viability assay of gram-positive *B. subtilis* in both LB broth and PBS solution. Interestingly, when compared with the control, 48% and 33% increases in the mean viable cell count were recorded for LB and PBS respectively after a one-day incubation. This finding is similar to that of a previous study of the other gram-positive bacterium *Arthrobacter sp*, which suggests that MWCNTs have a stimulatory effect for the up-regulation of genes involved in bacteria growth (Zhang et al. 2015). Since gram-positive bacteria possess thicker peptidoglycan layers than gram-negative bacteria, it can be argued that *B. subtilis* possesses higher resistance to piercing by MWCNTs. However, akin to what was observed for gram-negative *E. coli* and *P. aeruginosa*, the exposure of *B. subtilis* to MWCNTs/the nutrient-deficient hypotonic medium caused a significant and greater toxicity, resulting in a 90% decrease in cell viability after a one-day incubation. The estimated CFU.mL⁻¹ value of

the control in ultrapure water after a one-day incubation was almost a 2-log order of magnitude lower than the starting cell density. The hypotonic effect of water together with the MWCNTs may result in a synergistic detrimental effect. Nevertheless, extended incubation for seven days was also shown to exacerbate the toxicity effect of MWCNTs on *B. subtilis* in LB and PBS solution.

FESEM imaging of *E. coli* (Figure 4 a–d) and *P. aeruginosa* (Figure 4 e–h) following a one-day exposure to MWCNTs in LB and ultrapure water showed that the MWCNT/bacteria suspension in LB produced a profusion of bacteria on top of the MWCNTs. This result might be explained by the agglomeration of MWCNTs over time reducing the momentum and decreasing the number of CNTs exposed to the free-moving cells in the suspension. Figure 4 d,h,i shows the strains exposed to MWCNTs in ultrapure water after one day. Piercing of the cell membrane by the MWCNTs (black arrow), as well as torus-shaped objects (white arrow) resembling flattened cells among rod-shaped cells, was found in both the *E. coli* and *P. aeruginosa* samples. FESEM micrographs of *B. subtilis* (Figure 4 i–l) taken after exposure to MWCNTs in different media show the trapped cells (black arrow) in a similar state to that that occurred in gram-negative bacteria. Both the “trapping” and “piercing” of bacteria by the MWCNTs was observed in our study, which further established the membrane-damage capability of CNTs, consistent with the bioluminescence and fluorescence-staining results.

Proposed mechanism

The antimicrobial properties of single-walled carbon nanotubes (SWCNTs) have been previously reported (Kang et al. 2007; Liu et al. 2010; Dong et al. 2012; Chen et al. 2013). Based on these studies, it is apparent that the interactions of CNTs with bacterial cells may be multifaceted and require further exploration and better understanding of

1 the toxicity mechanisms for the bacterial cell. The bioluminescence toxicity-induced
2 results and fluorescence microscopy studies suggest that membrane damage plays a role
3 in governing the antibacterial nature of CNTs. Visualization through electron
4 microscopy showed the “piercing” and “trapping” of bacteria cells by the CNT network,
5 indicating that the damage was caused by physical interaction, which necessitates close
6 proximity of the CNTs to the bacteria. For this study, non-functionalized CNTs were
7 used, which resulted in relatively lower solubility and bio-availability in comparison
8 with the functionalized form. Despite this, the toxicity effect was still observed in both
9 nutrient-rich and nutrient-poor media in the case of gram-negative *E. coli* and *P.*
10 *aeruginosa*, and in a nutrient-poor medium in the case of gram-positive *B. subtilis*.

21 The toxicity of pollutants is influenced by their bioavailability as well as the
22 bacterial physiology in certain environments. It was observed that the toxicity of CNTs
23 in an LB medium was reduced compared with a PBS solution and ultrapure water. One
24 of the main reasons would be the fact that an LB medium is rich in nutrients that
25 promote the growth of bacteria.

26 On the other hand, the nutrient deficiency in a PBS solution and ultrapure water
27 renders the bacteria unable to replicate, and thus may have decreased the tolerance of
28 bacteria to CNTs. Moreover, the osmotic stress experienced by the bacteria in hypotonic
29 ultrapure water on its own may disturb their physiological condition and have an
30 adverse effect on cells (Hall and Anderson 1995). Toroid-shaped conformations were
31 also clearly observed for bacteria incubated in hypotonic water solutions in the absence
32 of MWCNTs (**Figure 4 c, g, k**) or in the presence of a low concentration of MWCNTs
33 (**Figure S3**). It may be possible that these structures were a form of membrane vesicles
34 produced by the bacteria under stress (MacDonald and Kuehna 2013). Briefly, the more
35 pronounced antibacterial capability of MWCNTs in a PBS solution and ultrapure water

1 can be explained by a combination of nutrient deficiency, coupled with protein, DNA,
2 oxidative, and membrane damage caused by the contact with CNTs for an extended
3 period which resulted in the loss of membrane integrity and eventually led to a lower
4 count of viable cells in comparison with the control that did not have CNTs added.
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10 11 12 **Conclusions**

13 A bioluminescence panel of bacterial strains was used to investigate the toxicity
14 mechanisms of MWCNTs, and the results suggest that different cellular responses were
15 activated upon exposure to MWCNTs. Different toxicity responses were observed with
16 different concentrations of CNTs. In particular, membrane damage appears to play a
17 major role in CNT toxicity. The electron microscopy studies of bacteria after exposure
18 to MWCNTs showed that the loss of membrane integrity could occur through either
19 piercing or trapping. The toxicity effect of CNTs appeared to be intensified in the
20 hypotonic and nutrient-free solution. The potential integration of bacteria with CNTs for
21 pollutant removal, therefore, would be dependent on the type of incubation medium and
22 the bacteria strain, with a nutrient-rich medium appeared to minimize the toxic effect of
23 CNTs.
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LIST OF FIGURES

Fig. 1. The bioluminescence responses produced by bioluminescence strains (Error bar represents standard error).

Fig. 2. The percentage of membrane-damaged cells based on at least triplicate screen-captures for each treatment

Fig. 3. The cell viability assay for 100 $\mu\text{g.mL}^{-1}$ MWCNTs at different incubation medium

Fig. 4. FESEM photographs of *E.coli* (ATCC 8739), *P.aeruginosa* (ATCC 9027) and *B.subtilis* (ATCC 6051) taken after overnight incubation in (a, e, i) LB broth, (b, f, j) LB broth with 100 µg.mL⁻¹ MWCNTs and (c, g, k) ultrapure water and (d, h, l) ultrapure water with 100 µg.mL⁻¹ MWCNTs. Scale bar is 1 µm, 10000×

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Table 1: Strains and plasmid used

Bacteria		Antibiotic		Temp.		Reference	(+) control	Stress regulator	Stress
Strain	Plasmid	Name	Concentration µg.mL ⁻¹	Growth °C	Refreshment °C				
<i>E.coli</i> DPD2794	pRecALux	Kanamycin	50	37	37	(Vollmer et al. 1997)	80 ppb Mitomycin C	SOS	DNA damage
<i>E. coli</i> DPD2511	pKatGLux	Kanamycin	50	37	37	(Van Dyk et al. 1994)	2 µg/mL H ₂ O ₂	Oxidative (peroxides) Fatty acid availability	Oxidative damage
<i>E.coli</i> DPD2544	pFabALux	Kanamycin	50	37	37	(Premkumar et al. 2002)	0.52 mM phenol	(general stress)	Membrane damage
<i>E.coli</i> TV1061	pGrpELux	Kanamycin	50	37	37	(Premkumar et al. 2002)	2%(v/v) ethanol	Heat shock (general stress)	Protein damage

Table 2: Strains and experimental conditions for CNTs incubation

Bacteria strain	MWCNTs concentration (µg.mL ⁻¹)	Incubation media
<i>Escherichia coli</i> (ATCC 8739)	0; 100	LB PBS Ultrapure water
<i>Pseudomonas aeruginosa</i> (ATCC 9027)	0; 100	LB PBS Ultrapure water
<i>Bacillus subtilis</i> (ATCC 6051)	0; 100	LB PBS Ultrapure water

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Supplementary Material

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