Bacterial remediation from effluent containing multi-walled carbon nanotubes

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**Abstract.** Multi-wall carbon nanotubes (MWCNT) were functionalized with functional groups containing oxygen, mainly carboxylic groups (-COOH), through reaction with a mixture of $\text{H}_2\text{SO}_4/\text{HNO}_3$ (3:1 v/v). The oxidized multi-wall carbon nanotubes (MWCNTOOH) were used to prepare an effluent, 2 mg L$^{-1}$ in a saline solution of NaCl (0.9%), to study of remediation of MWCNTOOH in aqueous suspension by utilization of *Escherichia coli*. The suspensions of *E. coli* (4.5 x 10$^5$ CFU mL$^{-1}$ and 4.5 x 10$^8$ CFU mL$^{-1}$) in test tubes with MWCNTOOH effluent caused the precipitation of a large amount of MWCNTOOH and supernatant clearing. The scanning electron microscopy (SEM) analysis of the precipitate and supernatant showed the adhesion and interlace of MWCNTOOH in bacteria surface. Although the precipitate consist of a large quantity of MWCNTOOH and bacteria, it was verified their presence in the supernatant. The spread plate technique showed that MWCNTOOH caused no cellular death of *E. coli* in the supernatant.

**1. Introduction**

The carbon nanotubes (CNT) have been one of the most interesting nanomaterials due their unique optical, electrical, mechanical and thermal properties, which lead to numerous applications [1]. However, many researchers have verified a high toxicity of CNT dispersed in solution against microorganisms. The CNT can absorb significant amounts of bacteria with serious impact on the cellular membrane, metabolic activity, and their morphology [2,3].

The results from toxicity studies with CNT and other nanomaterials are often contradictory, because of the use of CNTs of different purities and functionalizations. Therefore, detailed purification and physicochemical characterization of CNT are extremely important before testing their toxicity [3,4].

Many data in the literature have been using carbon nanotubes to remove pathogenic organisms from aqueous solutions [2,5-8]. The bacteria are adhered in the CNT surfaces and they are removed of solution by CNT precipitation.
Most of these studies using carbon nanotubes without any modification and then the precipitation of CNT occur spontaneously in aqueous solution due to their high hydrophobicity. The high van der Waals attractive force of CNT provide the production of wide aggregates that precipitate in aqueous solution after periods that depend on CNT dimensions and the dispersion method used [9].

However, the use of surfactants and the functionalization of carbon nanotubes can produce suspensions that are stables for a very long period, which may consist in environmental risks due the CNT toxicity. In these cases, efficient and low cost methods for CNT removal of aqueous suspensions are desirable.

Thus, based on results of researches which no modified CNT are used to eliminate pathogenic organisms by precipitation, would the opposite way be viable? Would it be possible to use bacteria to remove carbon nanotubes of stable aqueous suspension?

This work evaluated the utilization of *Escherichia coli* to remove of oxidized multi-wall carbon nanotubes (MWCNTOOH) in stable aqueous suspensions, as a model of bioremediation for this nanomaterial.

2. Experimental

2.1. Functionalization of Carbon Nanotubes

The carbon nanotubes used in this work were multi-wall (MWCNT) with 10-40 nm in outer diameter, 5-20 μm length and 93% purity. The MWCNT were produced by thermal chemical (CVD) process and supplied by CNT Co. Ltd. Korea. In order to produce a stable suspension the MWCNT were treated in a mixture of H₂SO₄/HNO₃ (3:1 v/v) and sonicated in an ultrasonic bath for 1 h. This suspension was filtered in a PVDF membrane and washed with distilled water until neutral pH. The chemical oxidation with H₂SO₄/HNO₃ mixture promote the functionalization of MWCNT with oxygen-containing groups, mainly carboxylic groups (-COOH). These functional groups present in the oxidized carbon nanotubes (MWCNTOOH) allow that their suspension remain stable by long periods.

2.2. Remediation tests with Escherichia coli

The MWCNTOOH were used to prepare an effluent with concentration of 2 mg L⁻¹ in a solution of NaCl (0.9%). A suspension of *E. coli*, previously grown in a culture medium (LB broth) was inoculated into 10 mL of MWCNTOOH effluent. The test tubes with MWCNTOOH effluent and *E. coli* 4.5 x 10⁵ and 4.5 x 10⁸ CFU mL⁻¹ were incubated at 37°C for 24 h at 120 rpm.

2.3. Characterization

The precipitates of test tubes were characterized by Scanning Electron Microscopy (SEM). A low mass of precipitate was deposited in a PVDF membrane (0.22 μm) and immersed for 1 hr in 1.5% glutaraldehyde solution at 5-8°C and the samples were dehydrated by different ethanol volumes starting; 30%, 50%, 70%, 80%, 90% and 100%, the period in each ethanol solution was 10 minutes. After, the samples were sputtered with Pd/Au alloy in a Balzer MED 020 machine and analyzed in a Jeol JSM-T300.

For supernatant analysis, 100 μL of supernatant were deposited in the PVDF membrane and after the same process with glutaraldehyde solution (1.5%) and ethanol gradient was realized before SEM analysis.

The supernatant was removed and centrifuged at 2000 rpm by 20 min. Thus, 100 μL of the 2nd supernatant (after centrifugation) was characterized by SEM, as well as, the 1st supernatant (before centrifugation).

Aliquots of 100 μL were used to evaluate the *E. coli* growth in the test tubes by spread plate method in LB agar. The incubation of plates occurred at 37°C for 16h.
3. Results and Discussion
The Figure 1 shows the test tubes with MWCNTOOH effluent (A) and the mixture of MWCNTOOH and bacteria (B) after the period of incubation.

Figure 1. (A) MWCNTOOH effluent; (B) MWCNTOOH effluent with (1) $4.5 \times 10^5$ $E. coli$ CFU mL$^{-1}$ and (2) $4.5 \times 10^8$ $E. coli$ CFU mL$^{-1}$, after incubation at 37ºC, 120 rpm for 24h.

The test tube with MWCNTOOH effluent (without bacteria) did not show any significant change after incubation. However, the test tubes with MWCNTOOH effluent and bacteria showed the formation of precipitate and the supernatant clearing in both concentration of bacteria. This indicates that the presence of bacteria promoted the precipitation of a high amount of MWCNTOOH, and consequently, the MWCNTOOH removal from aqueous suspension.

The adhesion of bacteria in the MWCNTOOH surface provides the formation of large agglomerates that decant in solution due to their high weight.

Figure 2. SEM images of precipitate present in the tubes with MWCNTOOH effluent and $E. coli$: $4.5 \times 10^5$ CFU mL$^{-1}$ (A e B) and $4.5 \times 10^8$ CFU mL$^{-1}$ (C e D).
The characterization of precipitate by SEM can be observed in the Figure 2. Both concentrations of *E. coli* resulted in the formation of agglomerates composed by MWCNTOOH and bacteria (Figure 2). In these agglomerates we can observe possible points of adhesion and interlace between MWCNTOOH and bacteria, as indicated by the arrows in Figure 2-B and D.

The LB agar plates incubated with 100 µL of 1<sup>st</sup> and 2<sup>nd</sup> supernatants came from test tube containing only *E. coli* $4.5 \times 10^5$ CFU mL<sup>-1</sup> (Figure 3 A and B) show a significant reduction of CFU mL<sup>-1</sup> after the centrifugation at 2000 rpm for 20 min. Therefore, the centrifugation condition was efficient to promote the removal of a high concentration of *E. coli* from aqueous solution.

In the LB agar plates of 1<sup>st</sup> and 2<sup>nd</sup> supernatants of samples with MWCNTOOH effluent and *E. coli* $4.5 \times 10^5$ CFU mL<sup>-1</sup> (Figure 3 C and D) we can verify again the reduction of bacteria in the supernatant after centrifugation and the high concentration of bacteria in the 1<sup>st</sup> supernatant. This result indicates that the MWCNTOOH did not cause the cellular death of bacteria present in the supernatants.

**Figure 3.** Photographs of LB agar plate of test tubes with *E. coli* $4.5 \times 10^5$ CFU mL<sup>-1</sup> (A) 1<sup>st</sup> supernatant and (B) 2<sup>nd</sup> supernatant (after centrifugation), and test tubes with MWCNTOOH effluent and *E. coli* $4.5 \times 10^5$ CFU mL<sup>-1</sup> (C) 1<sup>st</sup> supernatant and (D) 2<sup>nd</sup> supernatant (after centrifugation).

In the case of test tubes with higher *E. coli* CFU mL<sup>-1</sup> ($4.5 \times 10^8$ CFU mL<sup>-1</sup>), although it was not possible to clearly visualize the CFU due to high concentration of colonies in Figure 4 (A) and (C), it was possible to verify again that MWCNTOOH did not promote the bacterial death.

This agrees with the results observed by Kang *et al.* [10]. The authors observed that single- wall carbon nanotube (SWCNT) promoted the bacterial death in aqueous suspension, while the bacteria in the presence of MWCNT remained alive.

The centrifugation of supernatant provided a decrease of *E. coli* in the aqueous medium but it remained still high, comparing the 1<sup>st</sup> supernatant to 2<sup>nd</sup> supernatant of each sample with *E. coli* $4.5 \times 10^5$ CFU mL<sup>-1</sup>. Therefore, it is necessary more severe conditions of centrifugation to remove efficiently the bacteria from supernatant at that *E. coli* culture.
Figure 4. Photographs of LB agar plates of test tubes with *E. coli* $4.5 \times 10^8$ CFU mL$^{-1}$ (A) 1st supernatant and (B) 2nd supernatant (after centrifugation), and test tubes with MWCNT-OOH effluent and *E. coli* $4.5 \times 10^5$ CFU mL$^{-1}$ (C) 1st supernatant and (D) 2nd supernatant (after centrifugation).

MWCNT-OOH and bacteria were verified only in the SEM images of 1st and 2nd supernatants of sample with MWCNT-OOH effluent and *E. coli* $4.5 \times 10^8$ CFU mL$^{-1}$ (Figure 5 A and B). In the SEM images of all supernatants of other samples nothing was observed.

Figure 5. SEM images of 1st supernatant (A) and 2nd supernatant (B) of MWCNT-OOH effluent with *E. coli* $4.5 \times 10^8$ CFU mL$^{-1}$.

A low number of bacteria were evidenced on the 100 µL of 1st supernatant (Figure 5 A) and only one bacterium was found in the case of 2nd supernatant (Figure 5 B). In both SEM images we can verify the presence of MWCNT-OOH, however in Figure 5 (A) the arrows show adhesion points between MWCNT-OOH and isolated bacteria. In this case the adhesion was better visualized than in the agglomerates that compound the precipitate, as shown in Figure 2. The SEM images in the Figure 5 show that a determined amount of MWCNT-OOH and bacteria remain suspended in the supernatants, even after of centrifugation. In this case, higher centrifugation conditions can lead to better removal of MWCNT-OOH.
4. Conclusion
The addition of *E. coli* in the MWCNT-OH effluent promoted the precipitation of MWCNT-OH. The *E. coli* showed a normal growth in the MWCNT-OH effluent, indicating that the MWCNT-OH did not cause their cellular death.

It was possible to observe bacteria and MWCNT-OH in the supernatants of test tubes with MWCNT-OH and *E. coli* $4.5 \times 10^8$. Thus, although the removal of MWCNT-OH was not total in this case, the presence of bacteria *E. coli* resulted in a high decrease of MWCNT-OH concentration in aqueous suspension, indicating that the growth of *E. coli* in MWCNT-OH effluent can be a promissory method for MWCNT-OH bioremediation.

Acknowledgements
FAPESP, CNPq and Brazilian Network of Carbon Nanotubes (MCT/CNPq).

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