Research Article

The Adsorption Properties of \textit{Bacillus atrophaeus} Spore on Functionalized Carbon Nanotubes

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An equilibrium study of \textit{Bacillus atrophaeus} (\textit{B.a}) spores on functionalized Single-Wall Carbon Nanotubes (SWCNTs) has been performed in order to characterize the adsorption properties of the spores/nanotubes complex. The carbon nanotubes here investigated were subjected to a two-step purification and functionalization treatment in order to introduce chemical groups on their basal planes. The inclusion of carboxyl functional groups on the nanotubes was corroborated by Raman and infrared spectroscopy. These carboxyl groups appear to enhance the nanotube-\textit{B.a} interaction by reacting with the proteinaceous pili appendages present on the spore surface. The adsorption data demonstrate that bacillus spores diffuse faster on functionalized carbon nanotubes than on as-received and purified nanomaterials. Transmission Electron Microscopy also shows that the chemically treated nanotubes resulted in a swollen nano-network which seems to further enhance the bacillus adsorption due to a more extensive spore-nanotube contact area.

1. Introduction

Presently, carbon nanotubes (CNTs) are one of the most promising materials in the areas of electronics, sensors, bio-manipulation, and medicine due to their unique mechanical, electrical, and chemical properties [1–4]. A considerable amount of research in the area of sensors has positioned single wall carbon nanotubes as one of the most capable materials for detecting diverse biological agents [5]. Recent studies have also shown the potential of carbon nanotubes as amperometric sensors for detecting analytes in solution without the requirement of any mediator due to their high electron transfer rates [6]. It has been shown that CNTs are very sensing materials for components such as cytochrome c, horseradish peroxidase, myoglobin, and glucose oxidase [7–10]. Li et al. [11] have studied the sensing properties of polymer-coated nanotubes for detecting hydrochloric acid (HCl) vapors and demonstrated that the coated nanotubes are capable of sensing concentrations of HCl at levels as low as 2 ppm. Developments of nanotubes as biosensor platforms have shown that these nanomaterials can detect pathogens microorganisms such as \textit{E. coli} or \textit{Salmonella} in food supplies [12]. However, major challenges remain in making devices that differentiate between adsorbed species in complex mixtures and provide rapid forward and reverse response.

In a recent study regarding the adsorption properties of CNTs-spore systems, we have shown that the adsorption kinetic strongly depends on the purity of the nanomaterial involved [13]. In that study, it was shown that the adsorption of \textit{B. atrophaeus} upon purified-CNTs was faster than that observed with as-received CNTs, apparently due to the removal of amorphous carbon. A research work carried out by Deng et al. [14] on the adsorption equilibrium of \textit{B. atrophaeus} upon CNTs has also shown that the kinetics can be successfully predicted by the Freundlich adsorption equation. These results are encouraging since they suggest a potential outcome sensing signal simulated by established adsorption parameters. The study of \textit{B. atrophaeus} (a non-human pathogen) and CNTs system provides a good surrogate
for studying nanotubes interaction with pathogenic microorganisms such as *B. anthracis*. Indeed, the detection of pathogenic organisms is of great interest due to the hazard that they represent; as naturally occurring disease agents or as biological weapons [15]. Indeed, microbial pathogens can be introduced into the main water supply systems in urban places representing a public health epidemic crisis. To date, the analytical detection methods of pathogenic contaminants on drinking water systems are complex and time consuming [16]. Thus, the development of rapid detection systems is crucial for a fast response against bioterrorists acts. In order to build effective sensor devices based on nanotubes, it is essential that these nanomaterials are free of their metal catalyst as well as of impurities such as amorphous carbon material. It has been shown that the presence of amorphous carbon considerably reduces the electrical properties of the nanostructures [17]. The elimination of the amorphous carbon is a critical step for subsequent surface functionalization. Indeed, by functionalizing the surface structure of CNTs, specific chemical groups can be introduced to enhance their adsorption properties. Undoubtedly, the study of the physical attachment of biological organisms onto chemically modified nanotubes has to be initially performed in order to characterize their adsorption properties. The purpose of this research program is to document the adsorption properties of *B. atrophaeus* spores onto functionalized single-wall carbon nanotubes for the development of bioagent sensing device platforms based on SWCNTs.

2. Experimental Methodology

2.1. Absorbent Material. Single-Wall Carbon Nanotubes purchased from Carbolex, Inc. (Lexington, KY, USA) were studied in this research program. These nanostructures are synthetized through an arc-discharge method yielding nanotubes with a diameter around 1.4 nm. A thermogravimetric analysis performed in air by Carbolex, Inc. on these raw-nanotubes, indicated that this material contains between 20 to 30 wt% of amorphous carbon. Removal of amorphous carbon and impurities was performed by introducing 0.5 grams of carbon nanotubes into 100 mL of a 2.6 M HNO₃ solution. It is well documented that nitric acid (a strong oxidizing chemical) eliminates the metal catalysts used in the nanotube synthesis as well as disaggregating amorphous carbonaceous agglomerates [18, 19]. The acid solution containing the nanotubes was placed in a shaker at 150 r.p.m. for 48 hrs at room temperature. Subsequently, the CNTs-acid solution was vacuum filtered through a 5 μm pore size polycarbonate membrane and the filtrate was washed with 2 liters of deionized water, after which the nanotubes were dried for 24 hours at 70°C. The introduction of carboxyl chemical groups in the nanotubes was carried out by introducing purified nanotubes in a 10 M HCl solution. It has been widely shown that chloridric acid is an effective reagent for functionalizing carbon nanotubes [19]. The nanotubes-HCl suspension was vigorously shaken at 300 r.p.m. for 10 hours and subsequently vacuum filtered through a 5 μm pore size polycarbonate membrane. The filtrate was subsequently washed with 2 liters of deionized water until neutral pH was reached.

2.2. Absorbate Material. *Bacillus atrophaeus* was received from the U.S. Army Dugway Proving Grounds, Utah (courtesy of Patricia Cox). Here, a stock culture was prepared following the spore purification method established in previous work with *B. atrophaeus*-carbonaceous system [14]. The purified spores were stored in 40% ethanol at 4°C. These spores presented an elliptical shape with dimensions of 1 μm × 0.3 μm.

2.3. Adsorption Stage. Shaker experiments were performed to determine the kinetics and adsorption equilibrium of *B. atrophaeus* onto functionalized Single-Wall Carbon Nanotubes. Here, 0.1 grams of the functionalized CNTs were added to 250 mL flasks containing 99 mL of sterilized DI water. Then, one milliliter of a spore solution of *B. atrophaeus* at concentrations of either $9.5 \times 10^4$, $5.0 \times 10^5$, or $6.8 \times 10^6$ CFU/mL (CFU: Colonies Forming Unit) was added to each flask. Subsequently, the flasks were placed in a mechanical shaker at 200 r.p.m. at room temperature. Two milliliters of each CNTs/spore system were sampled at regular intervals and filtered via a 2 μm polycarbonate filter paper (Millipore, MA, USA). To quantify unattached spores, the filtrates were inoculated on Tryptic Soya Agar plates and incubated at 37°C for 24 hours, after which, the number of colonies was counted. The absorbed amount of spores onto the CNTs was calculated by a mass balance of the total spores in the solution before and after adsorption. The final concentration of the solution and the maximum adsorbed amount gave the respective adsorption equilibrium concentration and the nanotubes adsorption capacity. These measurements were carried out on duplicate flasks based on the three spore concentrations here considered.

2.4. Spectral and Microscopic Analyses. The presence of carboxyl groups on the nanotubes following the functionalization process was confirmed through a FTIR and Raman spectroscopy analysis. Optical analysis using a Hitachi H 7650 TEM was performed on functionalized CNTs to characterize their morphology. Microscopic observations of the CNTs/spore systems after the batch-experiment were also carried out to elucidate their interaction.

3. Results and Observations

3.1. Microscopic Analysis. Microscopically, functionalized CNTs under TEM shows a nanostructure relatively free of amorphous carbon suggesting that the acid treatments successfully removed the carbonaceous impurities from the nanotubes (see Figure 1). In our previous work, we have shown the presence of considerable amount of impurities on the SWCNTs in the “as-received” conditions [13]. Figure 1 also shows that the nanotubes display a nonaggregated state which again appears to be a result of the acid treatments (purification and functionalization) which evidently broke apart the bundles of nanotubes. It is well known that acid
spectra of the materials here studied are shown in Figure 2. was confirmed by performing a FT-IR analysis. The infrared also observed at 1400 cm$^{-1}$ is also observed and is related to C–N groups. Another peak is also observed in the aforementioned spectrum and are related to the presence of C=C stretching bonds [20]. Single peaks around 1400 and 1610 cm$^{-1}$ are also observed and is related to C–N groups. Another peak is also observed at 1400 cm$^{-1}$, and it can be related to stretching alkene groups. The spectrum also shows a distinctive peak around 1630 that suggests the presence of C=O stretching bonds [20, 21]. From this spectrum, it is apparent that the purification process performed in the research program introduced oxygen groups onto the basal planes of the nanotubes. Indeed, similar results have been reported elsewhere after treating nanotubes with nitric acid [19]. Included in Figure 2 is the IR spectrum of the functionalized nanotubes. From the figure, it is again observed the presence of C–C groups in the range of 800 to 1000 cm$^{-1}$. The spectrum also shows a double peak in the range of 1012–1028 cm$^{-1}$, which is associated to the C–O bonding of COOH groups [22]. A strong peak at 1420 cm$^{-1}$ is also visible, and this peak is associated with C–O–H bending groups [22]. The spectrum also shows a peak around 1680 cm$^{-1}$ that can be related to C=O (carbonyl) groups. In addition, a broad peak is displayed in the spectrum of the functionalized nanomaterial around 3160 cm$^{-1}$. This peak is related to the O–H bonding of the COOH groups [18]. From Figure 2, it seems that the functionalization process here performed introduced carboxylic groups onto the nanotubes. The presence of COOH groups on the nanotubes due to the HCl treatment appears to agree with the results shown by Furtado et al. [19].

The structural modifications of the nanotubes were also corroborated by carrying out Raman spectroscopy. Figure 3 shows two Raman bands in the 1200–1850 cm$^{-1}$ range. The relatively broad, disorder-induced band (D-band), appears in the region $\sim$1300 cm$^{-1}$ and the first-order-allowed band (G-band) with substructure at $\sim$1570 cm$^{-1}$. The D-band is generally associated with defects as well as presence of amorphous carbon whereas the G-band with the vibration of atoms in the basal plane [23]. Figure 3 shows that the functionalized nanotubes displays a shorter D-band than the purified and the as-received nanomaterial. It is worth noting that the G/D relationship of the functionalized sample is 17 and more 100 percent lower than that exhibited by the purified and as-received nanotubes respectively. Indeed, the ratio between the G and D bands is a good indicator of the quality of the nanotubes. Hence, if both bands have similar intensities (G/D = 1) it can be assumed that high quantity of structural defects is present on the sample. Here, a G/D relationship of 0.68, 0.39, and 0.33 was found for the as-received, purified, and functionalized nanotubes, respectively. It seems that the purification process removed structural defects from the as-received nanotubes since the G/D relationship of the purified nanomaterial is 72% lower than that shown by the as-received sample. These data show that an oxidation process with HNO$_3$ is initially required to eliminate the relatively high concentration of amorphous carbon on nanotubes before being subjected to a functionalization process.

3.3. Adsorption Kinetics and Equilibria. For the adsorption kinetic study, samples were taken at different time intervals from the spores-CNTs batch incubations; and the variation of adsorbed spores onto the CNTs was established by counting the number of unattached cells and subtracting them from the initial concentration. Figure 4 shows the number of
grown colonies of unattached spores from the sampling performed after 1 minute of starting the batch experiment on the functionalized CNTs/B. atrophaeus system. The figure shows the grown colonies at different dilutions; these dilutions were performed in order to allow a proper spore quantitation. Indeed, the number of colonies counted divided by the dilution performed and the volume inoculated gives the concentration of spores still present on the solution (spores not absorbed). Included in Figure 4 is the inoculated plate also from the functionalized CNTs/B. atrophaeus system after 14 minutes of starting the batch experiment; from the figure, it is clear that the number of colonies has dramatically decreased due to its adsorption onto the CNTs. Indeed, whereas the concentration of nonadsorbed spores at 1 minute was approximately $9 \times 10^2$ CFU/ml, at 14 minutes the concentration decreased to $3.33 \times 10^1$ CFU/ml, representing a value 27 times lower.

Figure 5 shows the grown colonies from the 3 minute time point of the functionalized CNTs/B. atrophaeus complex as well as those from the purified and as-received systems based on an initial concentration of $B.a$ in the order of $10^5$ CFU/ml. From Figure 5, it is observed that fewer colonies are present on the functionalized system, indicating that the functionalized nanotubes adsorb the $B. atrophaeus$ faster than the purified and as-received systems. This might be due to the removal of amorphous carbon from the nanotubes followed by the inclusion of chemical groups with affinity to interact with spores [24]. Indeed, it has been previously reported that the removal of impurities and the disentanglement of agglomerates increases the active surface area of the nanotubes improving their adsorption properties [13].

Figure 3: Raman spectra of the (a) as-received, (b) purified, and (c) functionalized SWCNTs.

Figure 4: Inoculated agar plates showing the growth of $B. atrophaeus$ (at different dilutions) from the functionalized CNTs-spores batch experiment. (a) Sample taken at 1 min. (b) Sample taken at 14 min.
Figure 5: Inoculated agar plates showing the growth of *B. atrophaeus* (at different dilutions) from a 3 minutes CNTs-spore batch experiment. (a) functionalized, (b) purified, and (c) as-received. Included is the final concentration of the nonabsorbed spores (colonies growth on the plate). It should be noted that the colonies shown on the plate based on the functionalized nanotubes appear to be smaller than those based on the purified and as-received systems. This size effect is only due to the incubation time for the colony growth, and does not affect the number of colonies grown.

Adsortption curves of the *B. atrophaeus*/functionalized CNTs for the three different initial spore concentrations here studied are shown in Figure 6. Here, the average fractional adsorption uptake \( \frac{M_t}{M_{\text{max}}} \) is plotted against time \( t \), where \( M_t \) and \( M_{\text{max}} \) are the adsorbed amount per unit mass of adsorbent at a specific time and at infinity time, respectively. In this study, \( M_{\text{max}} \) was calculated from the last time point in these batch experiments. Figure 6 shows that the fractional uptake of the concentrations here studied yielded similar behavior; reaching the maximum uptake value at 14 minutes. It is interesting to note that in 1 minute of the batch experiment, the functionalized nanotubes adsorbed 98% of spores; a value that is 1.4 times higher than previously reported on as-received/spores complex at a similar time [13]. Indeed, the interaction between the carboxylic groups of the nanotubes and those present in the spores seems to have an important role during the adsorption mechanism. Here, the surface of *Bacillus* spores are negatively charged, with isoelectric points reported to be about 3.0, and with a hydrophobic cortex [25, 26]. Additionally, two species of *Bacillus* have been found to have proteinaceous pili appendages (amino groups) extending from the spore surface [27]. The physicochemical attributes of *Bacillus* spore seems to certainly enhance their interaction with the carboxyl and carbonyl groups of the functionalized CNTs.

The effective diffusivity \( (D_e) \) of the spores onto the CNTs can be estimated using the following equation based on a macropore diffusion process if the fractional uptake is greater than 70% [14]:

\[
1 - \frac{M_t}{M_{\text{max}}} \approx \frac{6}{\pi^2} \exp \left( -\frac{\pi^2 D_e t}{r^2} \right),
\]

where \( r \) is the absorbent particle radius, and \( t \) the time. Indeed, it has been suggested in a previous study that the CNTs act as web during the bacteria-nanotubes adsorption process [14]. It is worth mentioning that in this research program, a number of microscopic analyses were performed on the functionalized nanotubes in order to evaluate the distance between carbon nanotubes aggregates \( (r) \). Here, \( r \)
yielded a value of around 100 μm. Hence, the diffusivity rate and the effective diffusivity \((D_e)\) can be obtained from the slope of \(\ln\left[\frac{1}{1 - \frac{M_t}{M_\infty}}\right]\) against time \((t)\). The slope is given by \((-\pi^2D_e/r^2)\) and the intercept by \(\ln(6/\pi^2)\). In this study, the effective diffusion rate \((D_e/r^2)\) of the functionalized CNTs was \(3.25 \times 10^{-4}\), \(3.59 \times 10^{-4}\) and \(3.79 \times 10^{-4}\) s\(^{-1}\) for the three different initial spore concentrations investigated \((6.80 \times 10^6, 5.00 \times 10^5, \text{ and } 9.50 \times 10^4 \text{ CFU/ml}; \text{ resp.})\). The effective diffusivity was then determined from the effective diffusivity rate. Figure 7 shows the effective diffusivity of the bacterial spores on the functionalized carbon nanotubes. From the figure, it is observed that the effective diffusivity of the functionalized carbon nanotubes is higher than that displayed by both the purified and the as-received carbon nanotubes. These results are encouraging since they suggest that functionalized nanotubes might result in faster detection platforms.

The maximum adsorbed amount of absorbate material \((M_{\text{max}})\) upon the nanotubes and its corresponding concentration in the supernant \((C_e)\) are plotted on Figure 8. Freundlich adsorption isothermal equation was used to fit the component adsorption data for the spore-CNTs complex studied. The Freundlich isothermal is given by

\[
M_{\text{max}} = k_f C_e^{1/n},
\]

where \(k_f\) is the Freundlich adsorption coefficient, and \(n\) is the adsorption intensity parameter. From the figure, the adsorption coefficient of the bacterial spore/functionalized CNTs is around 50% higher than that shown by spore-nanotubes systems based on purified CNTs and more than 5 times higher than the as-received CNTs-spores systems [13]. This again suggests that the removal of amorphous carbon during the purification process and the additional inclusion of chemical groups onto the basal planes of the CNTs increase the affinity of the nanotubes towards bacterial spores.

3.4. TEM Analysis. Optical analysis of the bacillus-nanotube system following the batch experiments were carried out on a TEM to highlight their interfacial interaction. A typical
micrograph of an isolated CNTs-spores system is shown in Figure 9, where an extensive interaction between both components can be observed. The figure shows the contact of the bacillus with the functionalized nanotubes after 1 minute in the shaker-batch experiment, and it seems that the amount of material interacting on this functionalized system is higher than that shown in a previous study within the as-received nanotubes [13]. The micrograph shown in Figure 9 supports the kinetic data here reported, where the functionalized carbon nanotubes are intimately in contact with the spores.

4. Conclusions

An adsorption equilibrium and a kinetic study of Bacillus atrophaeus on Single-Wall Carbon Nanotubes have been here performed. The carbon nanotubes used on this study were chemically modified after being subjected to different acid treatments. Raman and FTIR evidence suggest that the functionalized nanotubes show the presence of carbonyl and carboxylic groups. The results have also shown that the effective diffusion of spores onto functionalized nanotubes is considerably higher than that previously reported onto untreated and purified nanotubes. It seems that the detachment of amorphous carbon from the nanotubes, the disentanglement of agglomerates, and the inclusion of chemical groups on the CNTs structure increase the active surface area of the nanomaterial allowing a stronger spore-nanotube interaction. Optical analysis following the spores-nanotubes interaction on a shaker-batch experiment seems to support the experimental data obtained during the kinetic process. Here, the TEM micrographs showed a widespread and intimate attachment between the nanotubes and the spores. This research work has clearly shown the potential of building potential biosensors devices based on diffusion parameters of microorganisms on carbon nanotubes.

References


