Carbon Nanotube-Based Biosensor for Pathogens Concentration and Detection

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by

Dipendu Saha and Dr. Venkata K.K. Upadhyayula

Advisor: Prof. Shuguang Deng

Department of Chemical Engineering
New Mexico State University
P.O. Box 30001, MSC 3805
Las Cruces, NM 88003

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Abstract: Batch adsorption studies to determine adsorption kinetics of *Escherichia coli* (*E.coli*) K12 and *Staphylococcus aureus* (*S.aureus)* SH 1000 bacterial cells on single-walled carbon nanotube aggregates were performed at two different initial concentrations. The diffusivity of *E.coli* cells in single-walled carbon nanotube aggregates obtained was 6.54×10^{-9} and 8.98×10^{-9} cm^2/s, whereas that of *S.aureus* was between 1.00×10^{-7} and 1.66×10^{-7} cm^2/s respectively. In addition to batch adsorption studies, electron microscopy studies were also conducted. The results suggest that diffusion kinetics of bacterial cells is concentration dependent as well as bacteria dependent. Diffusivity of *S.aureus* is two orders of magnitude greater than *E.coli* cells. This proves to be beneficial from adsorption perspective where it is desired to filter microorganisms (water pretreatment and wastewater post treatment) and from nanotube biosensor perspective where it is desired to simultaneously capture and detect biothreat agents in a less span of time.

Keywords Carbon nanotubes, Microbial adsorption, Diffusivity, Ultramicrotomy, Water treatment

INTRODUCTION

Bacterial adsorption studies on single-walled carbon nanotubes provide insight on developing treatment based and sensor based applications in water and wastewater treatment industry. There are three major areas pertaining to water and wastewater plants where the use of carbon nanotube adsorption technology is foreseen. First and foremost, carbon nanotubes can be used as biosensors to concentrate and detect biothreat pathogens in drinking water treatment systems. In recent years, drinking water treatment plants have become highly susceptible to bioterrorism attacks (Nuzzo 2006). When a biothreat pathogen is introduced into a drinking water system, there is a likely chance that it is not detected with existing systems (Nuzzo 2006; Horman 2005). This is because of the fact that current detection systems are purely designed to detect sewage based contaminations and not the biothreat pathogens (Nuzzo 2006). Moreover, most of the biothreat pathogens are colorless odorless and tasteless (Horman 2005) which makes their detection almost impossible. Thus it is important to deploy a sensor comprising of a material such as single-walled carbon nanotubes which can concentrate and detect the pathogens almost

instantaneously. Another application of single-walled carbon nanotubes is to detect upsets caused by toxic contaminants upfront in a wastewater treatment plant (Timur et al. 2007; D'Souza 2001). A certain indicative strain of bacteria is made to adsorb on the surface of carbon nanotubes and the corresponding activity of the adsorbed bacteria is monitored to detect the presence of toxic materials (Timur et al. 2007). Finally, single-walled carbon nanotube filters can possibly be used as seawater pretreatment to remove bacteria from raw seawater and thereby reduce biofouling problems of reverse osmosis membranes.

Single-walled carbon nanotubes possess antimicrobial properties (Kang et al. 2004; Lee et al. 2004) that enable them to effectively concentrate and deactivate pathogens from contaminated water. When bacteria come in physical contact with nanotubes, they penetrate through the cell membrane, disrupts its activity and eventually destroys the cell viability (Kang et al. 2007; Lee et al. 2004; Narayan et al. 2005). It was proposed that the cylindrical shape of nanotube fibers coupled with a high aspect ratio are mainly responsible for death of bacterial cells. (Kang et al. 2007). Typically fibrous media provide larger accessible surface area for bacterial adsorption than powdered or granular adsorbents and higher the aspect ratio, higher is the accessible surface area. (Kang et al. 2007; Suzuki 1991). Single-walled carbon nanotubes have aspect ratios greater than 2000 (Ounaies et al. 2003) are expected to adsorb bacteria efficiently. Srinivatsava et al. (2004) provided qualitative confirmation that microorganisms get adsorbed on carbon nanotubes. Gu et. al. (2005), and Huang et al. (2004) provided basis for functionalization of carbon nanotubes using carbohydrated ligands and antibodies to detect pathogens.

In real time applications in water treatment plants, pathogenic bacteria must have faster adsorption kinetics. When a biothreat pathogen is introduced in treated water, it is required to concentrate and detect the pathogen almost instantaneously. Thus it is important to measure diffusivities of bacterial strains adsorbed on carbon nanotubes. The present study is initiated to determine adsorption kinetics of *E.coli* and *S.aureus* on single-walled carbon nanotube aggregates at two different initial concentrations. Diffusivities of these microbes in single-walled carbon nanotubes are also measured. We have also performed transmission electron microscopy (TEM) and ultramicrotomy analysis of the adsorbed bacteria to visualize the binding and association of the bacterial cells onto the carbon nanotubes. Ultramicrotomy is widely used to

image bacterial cell characteristics. Recently ultramicrotomy technique was extended to study morphology of non-biological specimens like rubber latex (Subramaniam et al. 2004). Ultramicrotomy may work well to visualize the association between large biological entities such as bacteria and mesoporous fibrous materials like carbon nanotubes. The information on association of bacteria with carbon nanotubes is important in designing a biosensor for specific applications in water and wastewater treatment plants.

MATERIALS AND METHODS

Adsorbent

The single-walled carbon nanotube samples (AP-Grade) were purchased from Carbolex, Inc. (Lexington, KY, USA), a commercial supplier of carbon nanotubes for research and development. These carbon nanotubes are synthesized with arc-discharge method and are close ended nanotubes with an average diameter of 1.4 nanometers and lengths of 2-5 μm. The reported average size of single-wall carbon nanotube aggregates using similar arc-discharge technique is about 20 μm (Yu et al., 2003; Qian et al., 2003). The as received single-wall carbon nanotube adsorbent samples have a purity of ~80% carbon nanotubes with ~10% of catalyst impurities including nickel and yttrium and ~10% amorphous carbon.

Batch Adsorption Studies

Fresh bacterial cultures of *E.coli* and *S.aureus* are prepared from their respective stock cultures. The cultures were prepared in tryptic soy broth solution in sterile glass tubes. The growth of the bacteria is confirmed with spectrophotometer by measuring the optical density of the solution. The optical densities of *E.coli* and *S.aureus* solutions were between 1.8 to 2.0. This freshly prepared bacterial solution is subsequently used for shaker experiments. Prior to the shaker experiments, the concentrated bacterial solution is aliquoted in 1.5 ml eppendorf tubes and centrifuged for two minutes. The supernatant broth solution is removed and the bacterial pellet formed at the bottom of the tubes is suspended in 0.85% saline solution and vortexed for two minutes. The same procedure of centrifuging and vortexing is repeated two more times to remove the broth content from the cells. The cells were prepared to be used for shaker studies and microscopic studies.

For shaker experiments, 100 ml of distilled water each (autoclaved) is taken in four flasks (duplicate samples two for *E. coli* and two for *S. aureus*) and bacterial solutions of *E.coli* and *S.aureus* are added to the flasks. A blank (without addition of nanotubes) is taken as a control to determine the initial concentration of bacteria in the solution. The bacteria concentration of *E.coli* in the two flasks was approximately $4.5x10^8$ CFU/ml (Colony Forming Units) and that of *S.aureus* was $4.4x10^8$ CFU/ml. To this solution, 0.1 grams of carbon nanotubes is added to each flask and the flasks are mounted on a mechanical shaker and contents are shaken at 6000 rpm. One ml of supernatant is drawn at regular intervals and filtered via 2 μm polycarbonate filter (Millipore, MA, USA). Then 100 μl of the filtrate sample was taken and diluted to several orders lesser than the initial concentration. The dilution factor was varied from 10^{-1} to one factor less than the initial concentration, i.e. if C_0 is of the order of 10^7 CFU/ml, the upper dilution factor used to for plating is 10^{-6} and if C_0 is of the order of 10^{-6} CFU/ml, the upper dilution factor is 10^{-5}. From each diluted sample, 20 μl of sample was taken and inoculated three times on selective agar plates. McKonkey agar is used to enumerate *E.coli* cells and mannitol salt agar is used for *S.aureus* cells. The plates were then incubated at 37°C for 24 hours. The number of colonies grown on the plates was enumerated. The total number of colonies was counted according to the following equation:

Number of Colonies [(CFU)/ml] = (Number of colonies for each dilution)/(dilution

172 factor)/sample volume

The same experiment was repeated one more times both for *E.coli* and *S.aureus* with different initial concentrations at

Electron Microscopy Analysis

1 ml of bacteria solution adsorbed on carbon nanotubes (not the supernatant) sample is taken from each flask after conclusion of the batch adsorption experiment and collected into four 1.5 ml eppendorf tubes. Two eppendorf tubes containing *E.coli* cells adsorbed on carbon nanotubes and two were *S.aureus* cells on nanotubes. One ml of these samples was used for TEM analysis while the rest half is reserved for TEM-Ultramicrotomy analysis.

For TEM imaging, approximately 5 µl solution was taken from the eppendorf tube containing adsorbed *E.coli* reserved for TEM analysis. The sample is directly deposited on Formvar coated nickel grids (Electron Microscopy Sciences, PA, USA) and viewed under a Hitachi H7650 TEM machine (Hitachi Technologies, CA). For *S.aureus* the same procedure was followed except that the cells were fixed using 1 % phosphate buffered formaldehyde solution for about two hours prior to the examination under TEM.

For ultramicrotomy analysis, the sample in the tubes is allowed to settle down naturally. After 15 minutes the supernatant was then carefully removed from the tubes using 1 ml disposable glass pipette. Then the contents in the tubes were fixed by adding 2.5 % cacodylate buffered (pH 7.2) glutaraldehyde and refrigerated overnight at 4°C. The fixed samples were then washed in cacodylate buffer and post-fixed in 1% osmium tetroxide (OSO_4) for an hour at 4°C. The tissue samples were dehydrated in a graded series of ethanol (50%, 70%, 80%, 90% and 100%) for 10 minutes each and treated with acetone twice for 5 minutes each. Following the acetone treatment, the samples were infiltrated with 1:1 (vol/vol) mixture of acetone and Spurr's low viscosity epoxy resin embedding media (Spurr 1969) and incubated overnight on a rotator at room temperature. In the following day, the samples were infiltrated further with a mixture of acetone and Spurr's media (1:3 volumetric ratio) for six hours on a rotator at room temperature followed by an overnight incubation in 100% Spurr's at room temperature. Finally, the samples were embedded in 100% Spurr's and cured in a 60°C oven for 24-48h.

A Leica UC-6 ultramicrotome and a diamond knife were used to make ultrathin sections (70-80nm) of the specimen blocks. The sections were collected on a 200 mesh Formvar coated nickel grids, stained with uranyl acetate (Epstein and Holt 1963), followed by lead citrate (Reynolds 1963), and examined under a Hitachi H7650 TEM(Hitachi High Technologies, CA). Images were captured using a side mount AMT XR-60 digital camera.

RESULTS AND CONCLUSION

Adsorption Kinetics

The amount of bacteria adsorbed on a given adsorbent is calculated by the following equation:

$$M_t = [(C_0 - C_t)\ V]/m \quad (1)$$

where

M_t is the amount of bacteria adsorbed on a given adsorbent (CFU/g)

C_0 is the initial concentration of the bacteria in the feed solution (CFU/ml)

C_t is the concentration of bacteria in solution at a given time t (CFU/ml)

V is the total volume of the feed solution (ml)

M is the mass of the adsorbent (g).

At adsorption equilibrium bacteria concentration in the feed C_t becomes the equilibrium concentration C_e and adsorbed amount M_t becomes M_{max}.

A plot between M_t/M_{max} and time (t) is called the fractional uptake curve (Do 1998) is drawn for two different concentrations of *E.coli* and *S.aureus* and are shown in Figures 1.1.

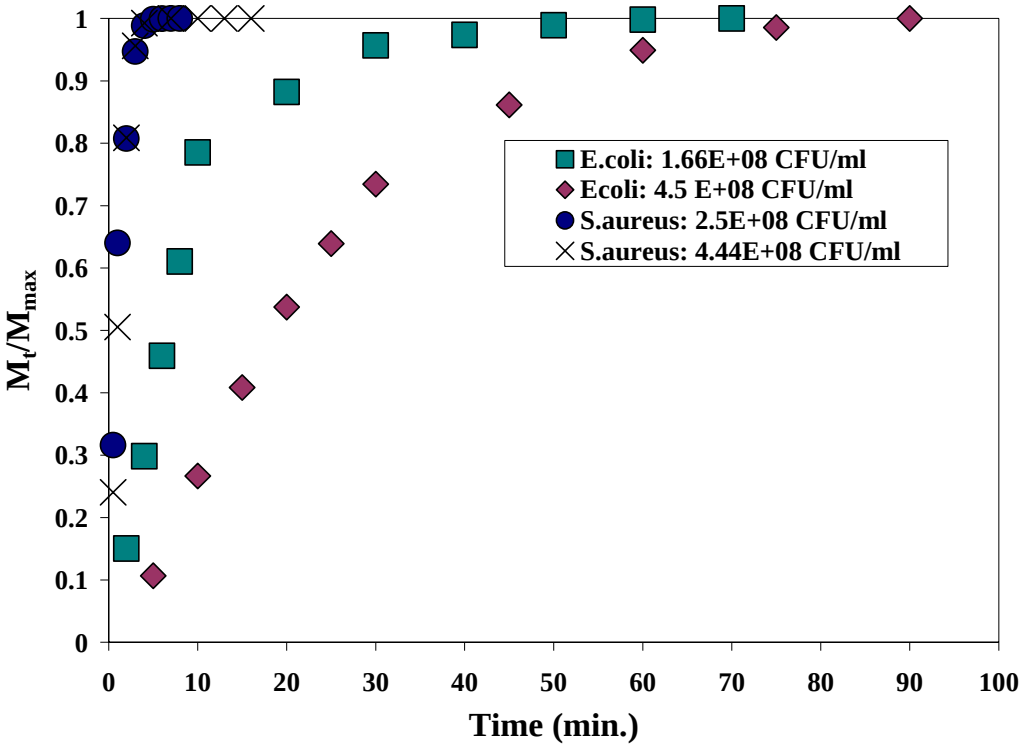

Figure 1.1 Fractional uptake curve of *E.coli* K12 and *S.aureus* SH 1000 on SWCNT aggregates

If the fractional uptake (M_t/M_{max})of the adsorbate (bacteria) on nanotubes is greater than 70% the effective diffusivity De (cm^2/s) of the bacteria in nanotubes can be estimated from a macropore diffusion model developed by Ruthven (1984).

$$\frac{M_t}{M_{\max}} \cong 1 - \frac{6}{\pi^2}\exp\left(-\frac{\pi^2 D_e t}{R_p^2}\right) \qquad (1)$$

This solution for the fractional adsorption uptake is used to correlate the adsorption kinetics data. A plot of ln (1-(M_t/M_{max})) vs. time is obtained for *E.coli* and *S.*aureus and is shown in Figure 1.2. The slope of the fitted straight line is $\left(\frac{-\pi^2 D_e}{R_p^2}\right)$ and the intercept of $Ln\left(\frac{6}{\pi^2}\right)$ from which the effective diffusivity D_e in macropores of carbon nanotubes can be calculated.

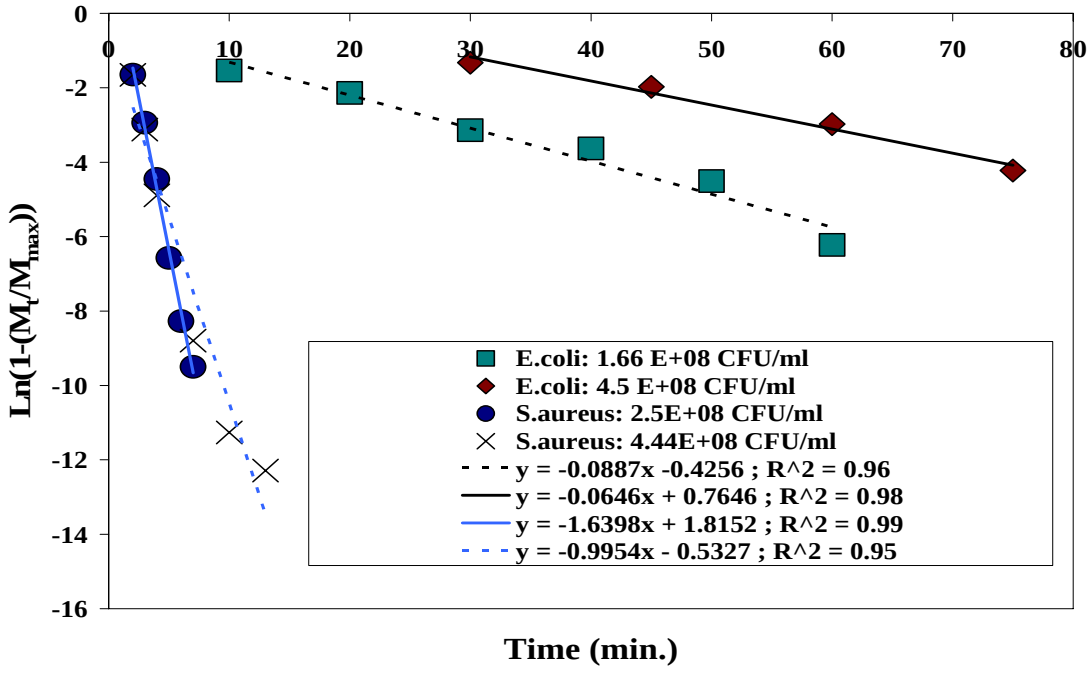

Figure 1.2 Correlation of adsorption kinetics data with diffusion model for *E.coli* K12 and *S.aureus* SH 1000 on SWCNT aggregates at 25°C

The diffusion time constant $\left(\frac{D_e}{R_p^2}\right)$ and corresponding diffusivities (D_e) obtained for *E.coli* and *S.aureus* at two different initial concentrations is calculated and reported in Table 1.1.

Table 1.1 Diffusion time constants and diffusivities of *E.coli* and *S.aureus* cells in single-walled carbon nanotube aggregates ate different initial concentrations.

Bacterium	**Initial Concentration (CFU/ml)**	**(D_e/R_P^2) (s^{-1})**	**D_e (cm^2/s)**
E.coli	$1.66x10^8$	$8.98x10^{-3}$	$8.98x10^{-9}$
E.coli	$4.5x10^8$	$6.54x10^{-3}$	$6.54x10^{-9}$
S.aureus	$2.5x10^8$	0.166	$1.66x10^{-7}$
S.aureus	$4.44x10^8$	0.100	$1.00x10^{-7}$

The results obtained in Table 1.1 indicate that diffusion of *S.aureus* in nanotubes is two orders of magnitude higher than *E.coli*. This could be due to the smaller size and shape of *S.aureus* cells because it is generally believed that adsorption of bacteria with size less than 1 μm is higher than that with size greater than 1 μm (Stevik et al. 2004). Typical bioparticle diffusivity in carbon nanotubes is 10^{-7} cm^2/s (Zhou et al. 2006). Our diffusivity data closely match this value.

TEM images of *E.coli* and *S.aureus* cells adsorbed on single wall carbon nanotube aggregates are given in Figures 1.3(A) and 1.4(A) and the corresponding ultramicrotomy images are given in Figures1.3 (B) and 1.4 (B).

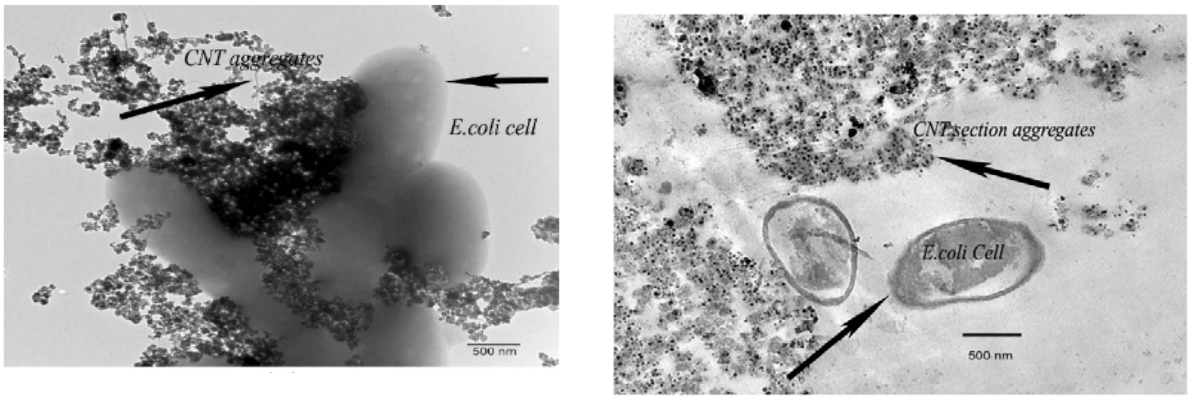

Figure 1.3(A) TEM image of *E.coli* adsorbed on SWCNT aggregates; **1.3(B)** Ultrathin image of *E.coli* adsorbed on SWCNT aggregates.

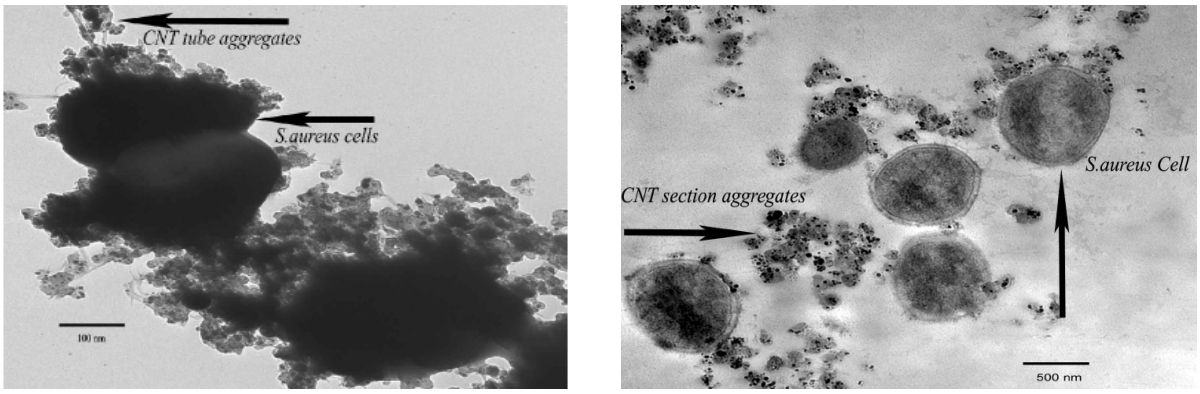

Figure 1.4(A) TEM image of *E.coli* adsorbed on SWCNT aggregates; **1.4(B)** Ultrathin image of *E.coli* adsorbed on SWCNT aggregates.

It appears from the TEM images (Figure 1.3(A)) that *E.coli* cells colonize in presence of carbon nanotubes. Although the colonization phenomena are observed with *S.aureus* cells, it can be seen that it is not as effective as it is in *E.coli* cells. One more observation is that the structural integrity of *E.coli* cells (Figure 1.3(A)) is still retained though the cell activity might have been lost. This is similar to what Lee et al. (2004) observed where the cell structure is intact but the viability of the cells is lost immediately after coming in contact with nanotubes. On the contrary, some other researchers (Kang et al. 2007; Narayan et al. 2005) depicted that the intracellular contents are released and the cell activity is completely disrupted when they are adsorbed on carbon nanotubes.

In conclusion, batch adsorption studies and microscopic studies show that *E.coli* and *S.aureus* in single-walled carbon nanotube aggregates has rapid diffusion kinetics. The diffusivity and kinetics of *S.aureus* two orders of magnitude higher than *E.coli*. This suggests that the diffusivity is concentration dependent and bacteria dependent. The association of adsorbed bacteria with carbon nanotubes is also a function of type of bacterial strain. These points are important in designing nanotube biosensor for pathogen detection because effective diffusion length in a carbon nanotube biosensor depends on a type of bioparticle (Zhou et al. 2006) and it appears that nanotube sensor can provide selective differentiation of bacterial strains.

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