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Research Letter

Single-Walled Carbon Nanotubes as Fluorescence Biosensors for Pathogen Recognition in Water Systems

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The possibility of using single-walled carbon nanotubes (SWCNTs) aggregates as fluorescence sensors for pathogen recognition in drinking water treatment applications has been studied. Batch adsorption study is conducted to adsorb large concentrations of *Staphylococcus aureus* aureus SH 1000 and *Escherichia coli* pKV-11 on single-walled carbon nanotubes. Subsequently the immobilized bacteria are detected with confocal microscopy by coating the nanotubes with fluorescence emitting antibodies. The Freundlich adsorption equilibrium constant (k) for *S.aureus* and *E.coli* determined from batch adsorption study was found to be 9×10^8 and 2×10^8 ml/g, respectively. The visualization of bacterial cells adsorbed on fluorescently modified carbon nanotubes is also clearly seen. The results indicate that hydrophobic single-walled carbon nanotubes have excellent bacterial adsorption capacity and fluorescent detection capability. This is an important advancement in designing fluorescence biosensors for pathogen recognition in water systems.

1. INTRODUCTION

Potential threats of contaminating drinking water sources with biothreat agents are on high rise in recent years. In US alone, in year 2003, more than 100 cases of actual, threatened, and disrupted plots were reported in which 20 incidents involved actual contamination of drinking water with biothreat agents [1]. Unfortunately, existing treatment techniques are not designed to treat and detect biothreat contaminants since most of these biothreat agents are colorless, odorless, tasteless, and have typical properties such as chlorine resistance and biofilm forming tendency [1–3]. This vulnerable situation has raised serious security concerns in many nations and needs immediate attention. A sensing system that can simultaneously concentrate and detect biothreat contaminants from water systems at a faster speed and greater accuracy has to be developed to overcome this situation.

Carbon nanotube technology has the potential to make important advancements in water security and protection from biothreat agents. Carbon nanotubes have high bacterial adsorption capacity [4, 5] and are able to concentrate different types of pathogens [4–6]. This exceptionally high adsorption capacity of nanotubes is due to its large surface to volume ratio and high aspect ratio that imparts high antimicrobial nature to the material [7, 8]. Another major advantage of using carbon nanotubes for water treatment applications is that nanotubes, if functionalized, have the ability to detect pathogens. There are multiple possibilities for surface modification [9–14] of carbon nanotubes, but functionalization with antibodies has gained significant importance in recent years. Antibodies not only provide the

required biocompatibility [11] but also alter the electrical and optical properties of nanotubes and make their surface sensitive to surroundings [11, 13, 14]. To the best of our knowledge, two research groups used antigen-antibody approach to detect pathogens using carbon nanotubes [11, 12]. Both groups have attempted to enhance the hydrophilicity of carbon nanotubes, since the bacterial binding capacity of hydrophilic nanotubes is higher than hydrophobic nanotubes [11, 12]. This is true but when it is desired to use carbon nanotube adsorbent media to simultaneously concentrate and detect pathogens from the flowing stream of water, it is desirable to have hydrophobic nanotubes from a practical standpoint.

The present study is initiated to evaluate the capability of hydrophobic carbon nanotubes to concentrate microorganisms and subsequently detect them when coated with fluorescent antibodies. The microorganisms chosen for this study are *S.aureus and E.coli*. In this report, we present the results of our batch adsorption study and confocal microscopy analysis of *S.aureus* and *E.coli* immobilization on single-walled carbon nanotubes aggregates.

2. MATERIALS AND METHODS

The single-walled carbon nanotubes samples (SWCNTs) (AP-Grade) purchased from Carbolex, Inc. (Lexington, KY, USA) were used for the study. These carbon nanotubes are 80% pure and have an average diameter of 1.4 nanometers and lengths of 2–5 μm. Bacterial cultures of *E.coli* pKV-11 and *S.aureus* SH1000 are obtained from Professor Nishiguchi and Professor Gustafson of Biology Department of NMSU. *E.coli* pKV-11 has intrinsic green fluorescent protein (GFP) and exhibits fluorescence under confocal microscopy. *S.aureus* strain is an opportunistic human pathogen and care is taken while handling.

2.1. Batch adsorption study

Shaker experiments are performed to determine the adsorption equilibrium concentration of *E.coli* and *S.aureus* bacteria on single-walled carbon nanotubes. For this experiment, fresh cultures of bacterial solutions of *E.coli* and *S.aureus* were prepared from their respective stock cultures. The cultures were grown overnight in tryptic soy broth at 37°C incubator. Then 1 mL aliquot of this solution is taken in 1.5 mL eppendorf tubes and centrifuged for 2 minutes. The supernatant broth solution is removed and the bacterial pellet at the bottom of the tubes is suspended in 0.85% saline solution and vortexed to obtain uniform suspension of the solution. The solution is again centrifuged and resuspended in distilled water. The bacterial solutions were now ready for shaker experiments.

100 mL of distilled and autoclaved water each are taken in four conical flasks (duplicate sets), and 0.1 grams of single walled carbon nanotubes is added to each flask. To this mixture, bacterial solutions of *S.aureus* are added to the two flasks, and *E.coli* is added to another two flasks. The initial concentration (C_0) of *S.aureus* was 2.8×10^8 and 2.7×10^8 CFU/mL and *E.coli* pKV-11 in the suspension was found to be 1.62×10^8 and 1.7×10^8 CFU/mL, respectively. The flasks are then placed on a mechanical shaker and are subjected to shaking at 6000 rpm under room temperature. The contents of the flask are shaken for 60 minutes and 1 mL of supernatant solution is drawn from each flask and collected in an eppendorf tube.

The supernatant solutions were then filtered via 3 μm polycarbonate filter paper (Millipore, Mass, USA). 100 μL of this filtrate are taken and used to prepare dilutions ranging from 10^{-1} to 10^{-5}. Then 20 μL of sample volume is drawn out from each dilution and inoculated on agar plates. McKonkey agar plates are used of *E.coli* pKV-11 cells and Mannitol salt agar for *S.aureus* cells, respectively. These two agar media selective grow the respective bacterial colonies and inhibit the growth of other bacteria. The number of colonies enumerated was counted according to the following equation:

$$\text{number of colonies}\left[\frac{\text{CFU}}{\text{mL}}\right] = \frac{\text{number of colonies for each dilution}}{(\text{dilution factor})/(\text{sample volume})}. \quad (1)$$

The values at each different dilution are averaged to obtain final concentration for two samples. The experiment is repeated four more times with four different initial concentrations. The initial concentrations used for *E.coli* were 2.0×10^7, 6.66×10^7, 3.33×10^8, and 4.44×10^8 CFU/mL. The initial concentrations used for S.auerus were 6×10^7, 1.44×10^8, 4.4×10^8, and $7.0*10^8$ CFU/mL respectively.

2.2. Confocal microscopy analysis

2.2.1. E.coli pKV-11

After running the shaker experiment for the first time, one mL of uniformly suspended solution (not the supernatant) is taken in 1.5 mL eppendorf tubs and allowed to settle for 15 minutes. After 15 minutes, the supernatant in the tube is slowly removed using glass pipette without disturbing the suspension at the bottom. Then 1 mL of 0.1 M phosphate buffer was added and the contents were spun in a microfuge for 30 minutes. The spinning step was succeeded by five times washing with 0.1 M phosphate buffer solution. After washing, 1 μL of Alexa 647 (Invitrogen, Calif, USA) fluorescent dye solution was prepared in the 200 μL of 0.1 M phosphate buffer solution and added to nanotube-bacteria mixture. The contents were refrigerated for one hour and removed, washed again with phosphate buffer to remove excess fluorescent dye and is subjected for analysis under confocal microscope. For actual imaging, the pellet was resuspended in buffer and 10 μL of sample were taken on a 25 mm glass slide (with cover slip placed) and is imaged under Olympus Fluoview FV1000 confocal microscope (Olympus America Inc, Pa, USA). Alexa dye will fluoresce nanotubes, while *E.coli* pKV-11 species already has intrinsic fluorescence protein.

2.2.2. *S.aureus SH-1000*

A procedure similar to the above was followed for confocal microscopic study of immobilization of *S.aureus* SH-1000 strain on nanotubes but with slight modifications. The supernatant from the eppendorf tube is removed using glass pipette and bacterial cells were fixed in 1% phosphate buffered formaldehyde solution, refrigerated for three hours and washed with 0.1 M phosphate buffer for five times. Then, *S.aureus* primary antibody solution in phosphate buffer (1 μL : 100 μL) is added to the cells so as to conjugate the primary antibody specific to the *S.aureus* cell wall surface. The sample was refrigerated and the cells were kept in suspension of primary antibody solution for about 16 hours. The sample was removed and 1 μL of FITC-Goat Anti Rabbit (Jackson Immuno Research Laboratories, Inc., Pa, USA) fluorescent dye solution is prepared in 100 μL of 0.1 M phosphate buffer (1 μL : 100 μL) and added to the cells. The sample was again refrigerated to allow the conjugation of secondary dye solution to the primary antibody associated with *S.aureus* cells. Finally to this mixture, Cy-5 secondary antibody solution (Invitrogen, Calif, USA) prepared in phosphate buffer was (1 μL : 100 μL) added. Cy-5 fluoresces single-walled carbon nanotubes at a different wavelength region other than *S.aureus* cells. For imaging, 10 μL of this sample was used for imaging under Zeiss LSM510META microscope (Carl Zeiss Micro Imaging Inc., NY, USA).

A control sample of pure single-walled carbon nanotubes labeled with Alexa 647 dye is also prepared and imaged under Olympus Fluoview FV1000 confocal microscope.

3. RESULTS AND DISCUSSION

The final bacterial concentration of the solution (C_e) measured after 60 minutes is treated as the adsorption equilibrium concentration at which the maximum adsorbed amount can be calculated according to the following equation. The equilibrium point was determined by adsorption kinetics data reported elsewhere [4]:

$$Q_{\max} = \frac{[C_0 V_F - C_e V_S]}{m}, \tag{2}$$

where $Q_{\max}$ is the maximum amount of bacteria adsorbed on a given adsorbent at C_e (CFU/g), C_0 is the initial concentration of the bacteria in the feed solution (CFU/mL), C_e is adsorption equilibrium concentration of bacteria in solution (CFU/mL), V_F is volume of the feed solution (mL), V_s is volume of supernatant solution (mL), and m is the mass of the adsorbent (g).

From the maximum adsorbed amount obtained ($Q_{\max}$) and adsorption equilibrium concentration (C_e), the Freundlich adsorption model (3) was used to correlate the adsorption equilibrium data:

$$Q_{\max} = kC_e^{1/n}, \tag{3}$$

where k (mL/g) is the Freundlich adsorption equilibrium constant that depends on temperature and pH of the feedwater, and n is a constant accounting for the potential energy uniformity of the adsorbent surface. Typically, the "k" value is related to adsorption affinity of the adsorbent to the adsorbate. A large k value suggests a higher adsorption affinity of the adsorbent toward the adsorbate. The adsorption isotherms for *S.aureus* SH1000 and *E.coli* pKV-11 on the single-walled carbon nanotubes are plotted in Figure 1. The Freundlich equation constants are $k = 9 \times 10^8$, $n = 1.85$ for *S.aureus* SH1000 and $k = 2 \times 10^8$, $n = 2.39$ *E.coli* pKV-11. The higher k value for *S.aureus* SH1000 than that for *E.coli* pKV-11 suggests a higher affinity for *S.aureus*. The high Freundlich adsorption equilibrium constants of these bacteria on single-walled carbon nanotubes might be due to their fibrous mesoporous structure that provides accessible external surface area for the adsorption. In addition, high antimicrobial nature of nanotubes also contributes toward high adsorption capacity. The ratio of k values can be used to calculate the adsorption selectivity. A high selectivity between *S.aureus* and *E.coli* was obtained on the single-walled carbon nanotubes.

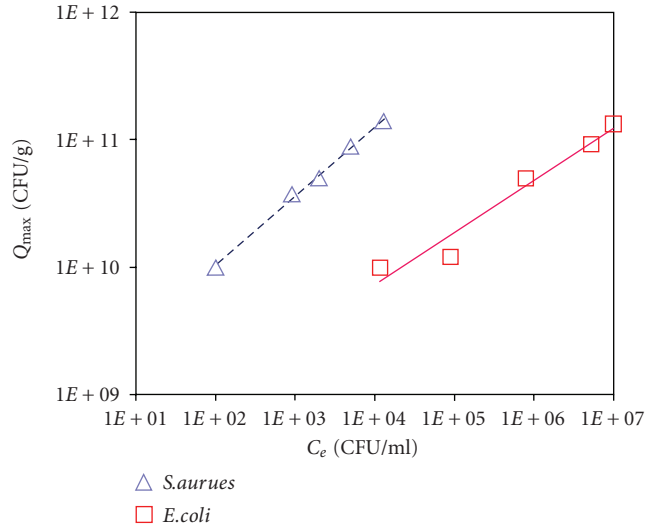

FIGURE 1: Adsorption isotherms of *S.aureus* and *E.coli* on carbon nanotubes.

S.aureus is a cocci-shaped bacterium with an average cell diameter being less than or equal to the mesopores of single-walled carbon nanotubes [4]. Due to this reason, the adsorption affinity of *S.aureus* is significantly higher than *E.coli*. Adsorption kinetics and equilibrium of *S.aureus* and *E.coli* on SWCNTs are reported in much detail in our previous study [4] in which it is clearly observed that *S.aureus* not only has high adsorption affinity at wide range of initial concentrations. The adsorption kinetics of *E.coli* is fast but the kinetics of *S.aureus* is faster even at high concentrations [4]. This is good from a biosensor standpoint because an effective design of biosensor depends on speed of concentration of biothreat agents and their selectivity, when multiple species are present. The confocal images confirm the adsorption affinity data discussed above.

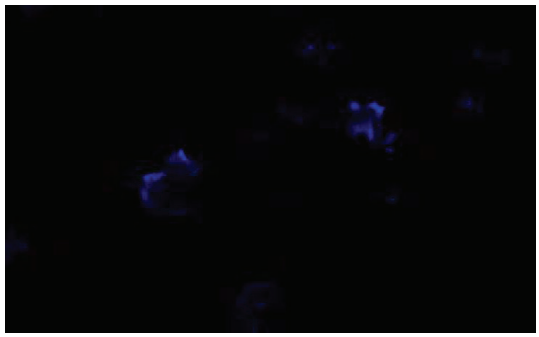

Figure 2: Confocal microscopy image of pure single-walled carbon nanotube aggregates.

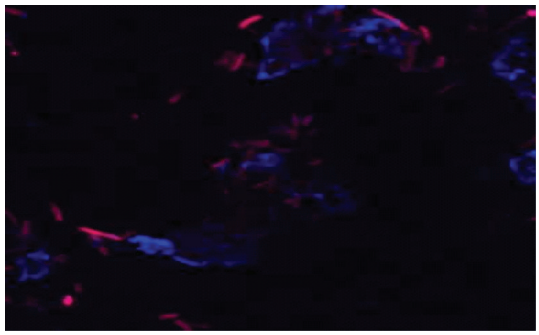

Figure 3: Confocal image of *E.coli* pKV-11 (violet rod shaped) cells immobilized on single-walled carbon nanotube aggregates (blue color).

The description about Figures 2, 3, and 4 is given in this paragraph. Although there appear some free cells, the association of bacteria with SWCNTs is visible. No functional groups are attached to enhance the hydrophilicty of carbon nanotubes. A small amount of fluorescent antibodies is made to immobilize nonspecifically on carbon nanotubes. Yet, the detection under confocal microscopy is clearly visible.

4. CONCLUSION

It was demonstrated that unmodified, hydrophobic single-walled carbon nanotubes have a very high adsorption affinity toward both *E.coli* and *S.aureus* bacterial cells. The adsorption capacity of *S.aureus* is significantly higher than that of *E.coli* on the single-walled carbon nanotubes. The Freundlich adsorption model can correlate the adsorption isotherm data well. The immobilization of the bacterial cells on single-walled carbon nanotubes is clearly seen in the confocal images, which confirms the adsorption equilibrium data obtained in the batch experiments. The high adsorption capacity and high selectivity of single-walled carbon nanotubes for *S.aureus* suggest that the carbon nanotubes are promising sensor materials.

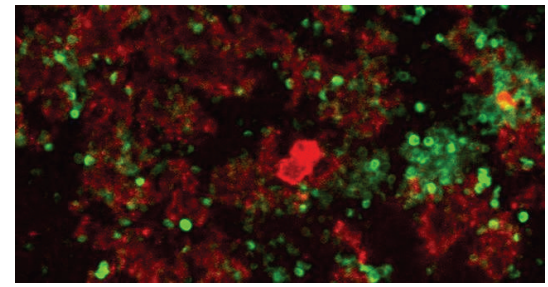

Figure 4: Confocal image of *S.aureus* (green dots in the image) cells immobilized on single-walled carbon nanotube aggregates (orange color in the image).

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