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Carbon Nanotube Cluster Based Micro-Fluidic System for Bacteria Capture, Concentration, and Separation

A thesis submitted in partial fulfillment
of the requirements for the degree of
Bachelor of Science with Honors in
Biological and Agricultural Engineering

By

Chris Nelson

April 2009
University of Arkansas

Faculty Mentor: **Dr. Jin-Woo Kim**, Associate Professor

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ABSTRACT

Carbon Nanotube Cluster Based Micro-Fluidic System for Bacteria Capture, Concentration, and Separation

Chris Nelson

Thesis Advisor: Dr. Jin-Woo Kim

Disease-causing pathogens continue presenting enormous global health problems, especially due to their easy transmittance to people via water supply systems. The detection, filtration, and purification of bacteria contaminated water samples are complex and subject to a great amount of error. Here we present a new and highly effective micro-fluidic system with carbon nanotube clusters for effective and efficient detection, filtration, and purification of bacteria contaminated medium. The developed system is based upon two unique properties of carbon nanotubes (CNTs): high bacterial affinity and magnetic susceptibility. The CNTs' high affinity to bacteria cells makes them a key candidate for the bacteria adsorption. Their magnetic susceptibility allows an effective way of separating as well as containing them in the system. In this study, we designed and tested a prototype CNT-cluster based micro-fluidic system by uniquely combining the two excellent properties of CNTs. The CNT-based micro-system consisted of a micro-channel, which positions CNT clusters evenly on the bottom surface using a strong Neodymium block (1" × 1" × 1") rare-earth magnet (surface magnetic field strength = 0.684 Tesla). When bacteria suspensions were introduced, the CNT clusters in the micro-fluidic system were shown to effectively serve as bacterial adsorbing centers, which led to spontaneous adsorption and concentration of bacteria to the clusters. This was shown to happen for both types of microorganisms, i.e., Gram-positive and Gram-negative bacteria. The results demonstrate the excellent potential of the CNT based micro-fluidic system for bacteria capture, concentration, and separation.

BACKGROUND

The presence of bacteria in water systems is a danger to any operation; hence, the detection of bacterial contaminations has become a very big issue. Current methods for determining the presence of bacteria include polymerase chain reaction (PCR), colony counting methods, and immunology-based methods, which have long waiting periods and are complicated processes [1]. Additionally, the problem still remains that bacteria in a dilute solution cannot be detected. In the following research, the beginnings of a method are suggested for determining the presence of particular pathogens and reducing the concentration requirements 10^7 – 10^4 CFU/ml to close to 81 CFU/mL [1].

The advent of nanotechnology has opened a lot of doors for sensing in the nano-scale. Nanotechnology has the ability to revolutionize molecular electronics, medical chemistry, and biomedical engineering [2]. Research has explored applications from electronics, composites, fuel cells, sensors, optics, and biomedicine [3]. More pertinent to the proposed research are environmental applications. Carbon nanotubes were first discovered in 1991 by Iijima and are cylindrical carbon rods with a diameter of 4 to 30 nm [4] and lengths up to millimeters in length [5]. The aspect ratio for such a material is enormously high 1:28,000,000 making the carbon nanotube a very unique material with peculiar and novel properties. Multi-Wall Carbon Nanotubes are similar in structure with additional walls located around the central wall.

Recently, high affinity binding interactions between carbon nanotubes (CNTs) and bacteria have been demonstrated [6,7,8] and exploited to use them as bacterial filters [7], photothermal, and photoacoustic antimicrobial contrast agents [8,9]. These studies demonstrate excellent potentials of CNTs as bacterial capturing agents. In addition, research by Brady-Estevez, et al. (2008) explored the use of single-walled-nanotubes (SWNT) as a size exclusion

filter with *Escherichia coli* K12 (a model bacterium for E. coli 0157:H7) [3]. They also demonstrated a method in which the SWNT system can be reused. Also, CNTs have been shown to exhibit para-magnetic and di-magnetic properties when under the presence of a strong magnetic field [10].

This previous research opens the door for the proposed project to continue exploring the frontier of nanotechnology by testing specifically the feasibility of a multi wall nanotube (MWNT) system for filtering and concentrating bacteria in water solutions by adsorption and magnetic retainment.

Parallel to the research being conducted on the applications of CNTs is the research that highlights the possible toxicity that they may possess. This potential drawback needs to be addressed. Warheit et al., (2003) reported that CNTs can be harmful to the pulmonary system of rats and, by extrapolation, humans [11]. If kept in solution, the CNTs will not cause pulmonary problems. In addition, Blaise et al., (2008) reported that certain nano-materials are toxic to aquatic organisms [12]. Their research emphasized that CNTs should be retained by any system that uses them. That is where the unique magnetic properties of CNTs can be used to increase the retainment of the CNTs inside the system. The system should incorporate this concept. The system has two very distinct potential applications. The first is bacteria "filtration", functioning by size exclusion or adsorption of the bacteria. This system would need to be reusable in order to make it cost effective. The second is bacteria "concentration". The concept is that the microbes in a dilute solution become concentrated inside a micro channel, which would provide a mechanism for more efficient bacteria detection, in particular pathogen detection. This application likely will have the greatest impact on rapid water source analysis.

The objectives of this research are to:

Build and Evaluate a MWNT based system for bacteria concentration and filtration.

This includes:

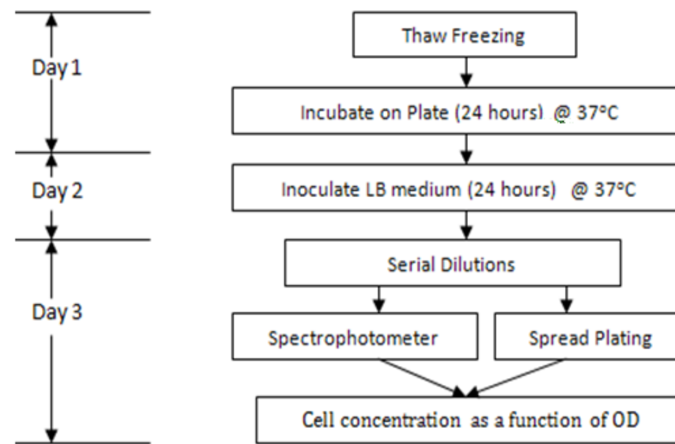
1) Designing and fabricating an apparatus to conduct tests
2) Determining optimal operating parameters including flow rate and MWNT concentration
3) Determining bacterial adsorption capacity of MWNT system
4) Imaging the system to determine the location and state of the bacteria cells

MATERIALS AND METHODS

BACTERIA EVALUATION

In the preliminary stage, research was done to select the best bacteria for testing. Non-pathogenic *E. coli* K12 was selected in this study because this strain has been widely used as a model bacterium for pathogenic *E. coli* 0157:H7 which represents a great risk to water sources and food processing when present. The preparation of a culture of *E. coli* is shown in **Figure 1**.

Figure 1 - Process of estimating cell concentration as a function of optical density (OD)



The process for preparing a liquid culture of *E. coli* is shown above. The frozen culture that was ordered was first incubated on a Luria Bertani (LB) plate with the streak method for 24 hours. A single colony was isolated from the streak plate and incubated in liquid medium for 24 hours. This culture can then be diluted and used for experiments.

Figure 1 shows the standard procedure used for bacteria growth and concentration determination. It is necessary to develop a relationship between OD and concentration for making accurate dilutions for later experiments. It is beneficial to know the approximate bacterial concentration before the experiment begins. The first step is to culture the bacteria from a frozen sample onto a fresh agar plate (Luria-Bertani (LB) medium), then cultivate in fresh medium (LB broth). For the quantitative analyses, the relationship between bacteria cell number

and optical density (OD) was established during cell growth. This was accomplished with a spectrophotometer and the spread plating method.

DEVICE DESIGN

After the relationship between bacteria concentration and OD was made, the next step was to design the device in which the holding capacity of MWNTs for *E. coli* could be measured. Previous attempts of designing a micro-channel in our laboratory used polydimethylsiloxane (PDMS) as a gasket and glass slides to form a channel which was the basis of the following design. The procedure for preparing a PDMS microchannel and the dimensions used are shown in **Figure 2**.

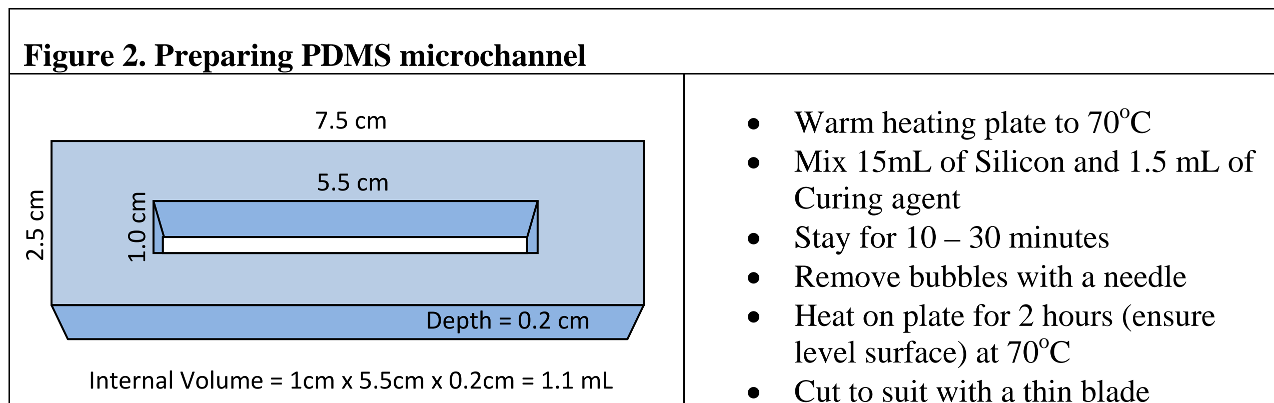
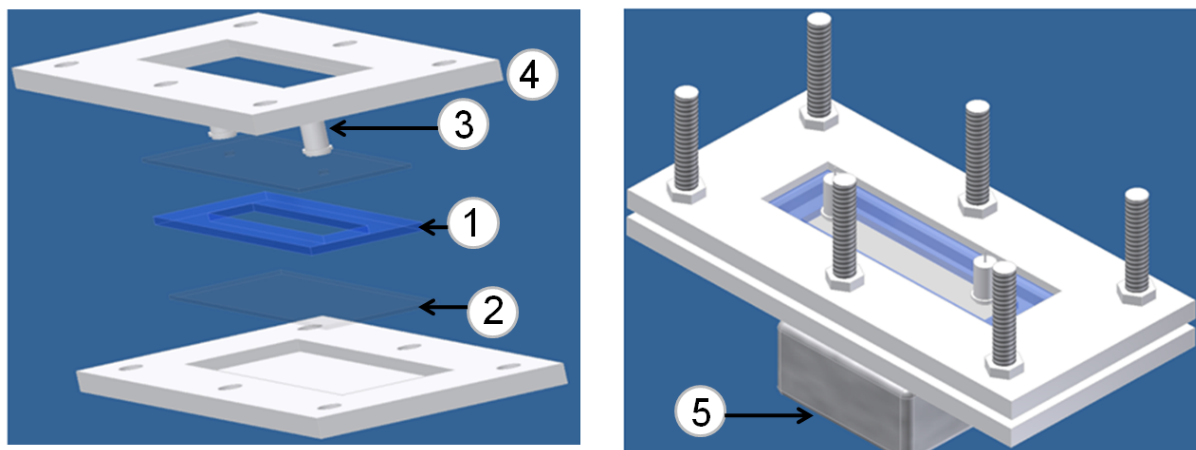


Figure 2. Preparing PDMS microchannel

The MWNTs were injected into the channel where a magnetic field from a permanent magnet (Neodymium block (1”x1”x1”) rare-earth magnet with a surface field strength = 0.684 Tesla) held them in place. The magnetic properties of all CNTs make it possible to separate them in solution and hold them in place. The glass slide allowed leaking when not clamped, however, too much force applied on the glass in a focused area broke the glass. This opened the possibility for the design of a device that applies adjustable steady pressure on the entire surface of the slides (both top and bottom). Because a strong magnetic field (0.684 Tesla) was applied to the system, it must be made of a non-magnetic, sterile material which narrowed the options to a

non-magnetic metal or plastic. The material of choice was ultra high molecular weight polyurethane (UHMW) and nylon. On the top slide, two 1 mm holes were drilled where a strong epoxy holds micro fluidic attachments in place. An inlet tube was attached which was connected to a syringe pump and an outlet tube was attached to take the effluent to a container for testing (centrifugal tube). The preliminary design of the device and the micro-channel is shown below (**Figure 3**). The nylon bolts and nuts can be adjusted to provide varying pressure evenly across the glass slides. A slot allows for the inside of the channel to be viewed. The channel itself was built the same as previous attempts with a PDMS gasket and glass slides. Once the device was built, it was tested for leaking and adjusted when necessary.

Figure 3 – Micro-channel system design



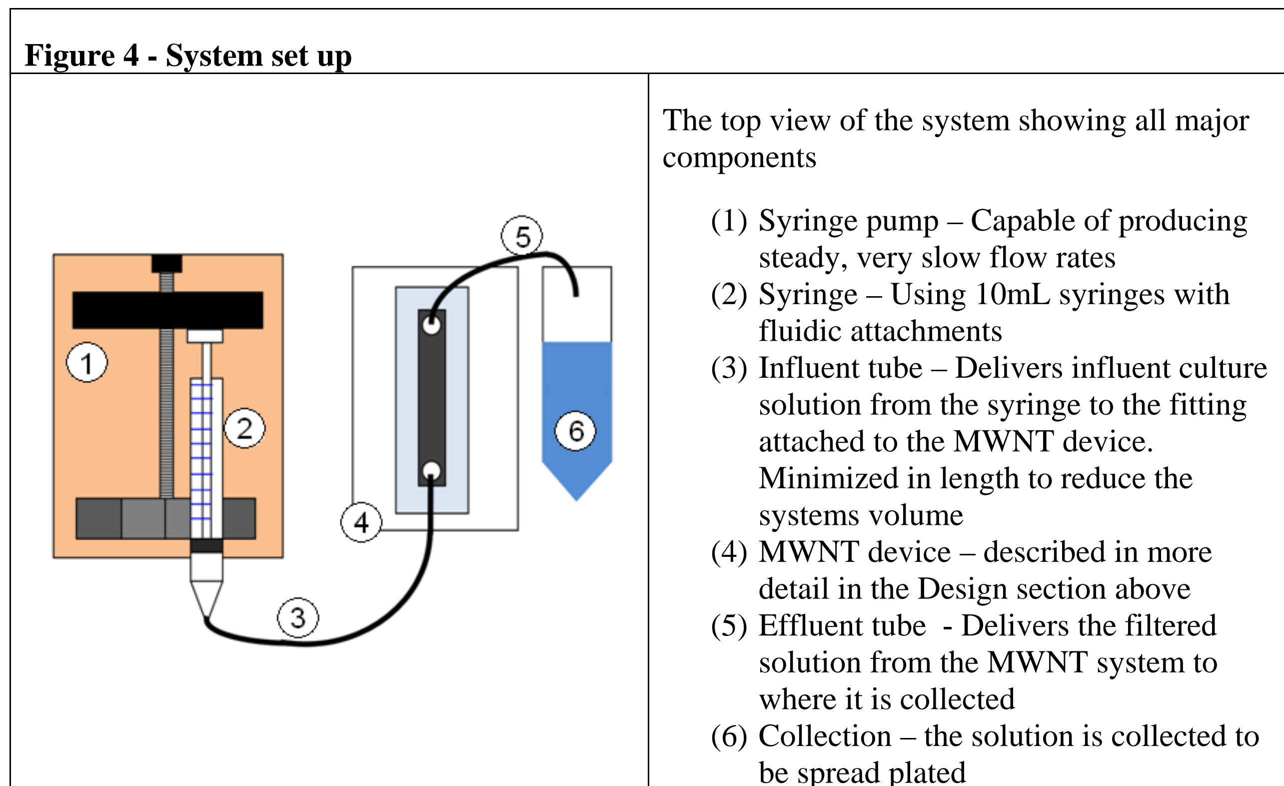
The left diagram shows an exploded view. The clamp(4), glass microscope slides(2), and PDMS are shown(1). Also, the pipe fittings(3) which are glued to the microscope slides are shown.

The right diagram shows the assembled view. The earth magnet (5) is placed where it will be in experiments. Bolts and nuts are used to provide adjustable pressure for the clamp.

MAXIMUM FLOW RATE DETERMINATION

The first step was to test the device with distilled water similar to the water that would be used in experiments. The system must not leak and the pressure inside the channel must not remove any components. Once it was confirmed that the device works with water, carbon nanotubes were injected into the channel. The first test with carbon nanotubes determined the maximum amount of MWNTs that could be placed inside the channel and the maximum flow rate at which water can move through the channel without dislodging the MWNTs. The magnet held the MWNTs in place up to a maximum concentration. As the MWNT solution was injected into the system, the magnetic field held the MWNTs in place and water was free to move. MWNTs were injected into the system until the system was saturated. The second determination was based on visual MWNT loss at various flow rates. **Figure 4** shows the setup used both in all flow rate experiments and the capacity experiments that follow.

Figure 4 - System set up



The top view of the system showing all major components

(1) Syringe pump – Capable of producing steady, very slow flow rates
(2) Syringe – Using 10mL syringes with fluidic attachments
(3) Influent tube – Delivers influent culture solution from the syringe to the fitting attached to the MWNT device. Minimized in length to reduce the systems volume
(4) MWNT device – described in more detail in the Design section above
(5) Effluent tube - Delivers the filtered solution from the MWNT system to where it is collected
(6) Collection – the solution is collected to be spread plated

TESTING – CAPACITY

To measure the capacity, a known concentration of *E. coli* was injected into the system. The effluent was collected and the concentration was measured to find the percent removal.

$$\%Removal = 1 - \frac{[E.coli]_{out}}{[E.coli]_{in}}$$

The following procedure was used in testing the capacity of the system.

Overall Procedure

- Prepare a bacteria culture in LB liquid medium
- Measure the Optical Density (OD) to estimate the concentration
- Dilute a bacteria solution to the concentration that will be tested
- Run one of the procedures listed below (Procedure 1 or 2)
- Complete spread plating for
    - Control 1: effluent from the system before injection
    - Control 2: original bacteria culture stock to be injected into the system (for an accurate concentration)
    - Samples 1-n: effluent from the system after bacteria injection
- Incubate plates for 24 hours
- Count colony forming units (CFUs)

As the experiment was conducted, two different procedures were used depending on the input concentration. The first experiments were conducted at low concentration. This process was complicated because the system has its own volume (1.1 mL). To adjust, the following procedure was used.

Procedure 1 – Low Concentration

- Rinse system with 30mL water (Collect the last few drops of effluent for the control)
- Inject cultures by:
    - Inject 1 mL of bacteria solution of a known concentration
    - Rinse with 2 mL of water (collect effluent)
    - Repeat for the desired number of experiments
- Rinse with 1 mL of water (collect effluent)
- When computing concentration, multiply by a factor of 3 for the effluent bacteria concentration because it was diluted with 2 mL of water

Procedure 2 – High Concentration

- Rinse system with 30mL water (Collect the last few drops of effluent for the control)
- Inject 1.2 mL of bacteria solution of a known concentration
- Inject water at intervals of 0.2 mL (collect effluent)
- When computing concentration, there is no need to multiply by a factor to find the concentration because it was not diluted

All experiments were conducted in a biosafety cabinet and proper aseptic technique was used throughout the tests.

CLEANING

After each test, the device was completely cleaned and reassembled. The MWNTs were removed and properly disposed and the device was filled with 70% ethanol for several hours. The device was then rinsed with a large quantity of autoclaved, distilled water in preparation for the next test. The assembly was completely sterile during each test and control samples were collected at the beginning of each test to ensure contaminates did not enter the system. The device must also not retain any ethanol that could interfere with or kill the *E. coli* introducing systematic error. The rinse with autoclaved distilled water should remove all ethanol solution.

IMAGING

To this point, the results indicate that living bacteria were removed from the system. The MWNT system may have (1) "filtered" using size exclusion or adsorption or (2) killed by the relative toxicity of the MWNT and did not "filter" the solution. The bacterial capturing capability of the MWNT based system was assessed using light as well as epi-fluorescence microscopy. For the epi-fluorescence microscopy, sample suspensions were stained using the LIVE/DEAD BacLight^{TM} Bacterial Viability Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications.

RESULTS

BACTERIA EVALUATION

To develop a relationship between optical density (OD) and the number of bacteria suspended in a solution, the test discussed above was completed. A bacteria culture was diluted and then plated to find the cell concentration. The results are shown in **Table 1**.

Table 1 – Spread plating results

Dilution	Number of Colonies	Concentration (CFU/uL)
10^{-4}	-	-
10^{-5}	902	9.02
10^{-6}	152	1.52
10^{-7}	11	0.11
10^{-8}	2	0.02

Typically, the most accurate plate count is between 30 and 300 so the value of 1.52×10^{-6} CFU/uL is accepted as the concentration of the culture. The same dilution was measured with the spectrophometer. Assuming that the concentration of cell culture measured above was accurate, the cell concentration can be filled into the OD measurement.

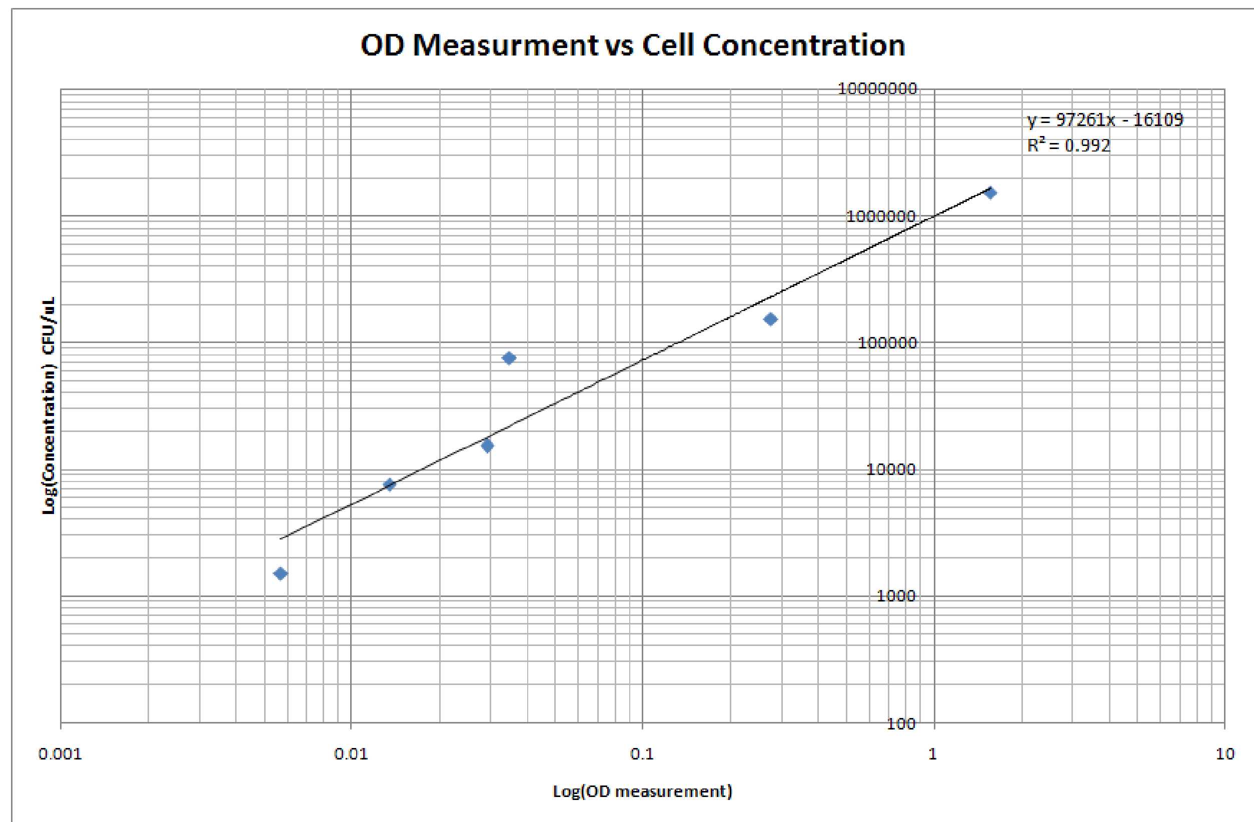
Table 2 – Spectrophotometry Results

Dilution	OD	Cell Concentration (CFU/uL)
10^{0}	1.5627	1520000
5×10^{-1}	No Data	760000
10^{-1}	0.2759	152000
5×10^{-2}	0.0348	76000
10^{-2}	0.0291	15200
5×10^{-3}	0.0134	7600
10^{-3}	0.0057	1520

The two sets of data were related in order to generate the following graph (**Figure 5**). The graph was plotted as the log of OD measurement vs. the log of the cell concentration. After a regression line was made, a relationship between OD and cell concentration was made. The R^2 value of 0.992 shows a good fit for the experiment. The following relation will be used throughout experiments as a way to approximate cell concentration with an optical density measurement.

$$Concentration\left(\frac{CFU}{\mu L}\right) = 97261(OD\ Measurment) - 16109$$

Figure 5 – OD measurement as a function of cell concentration



The graph above is a log-log plot of cell concentration as a function of OD measurement. The R^2 value shows a good fit. With this information, the concentration of a culture can be found instantly rather than having to wait 24 hours for a spread plates results.

DEVICE DESIGN

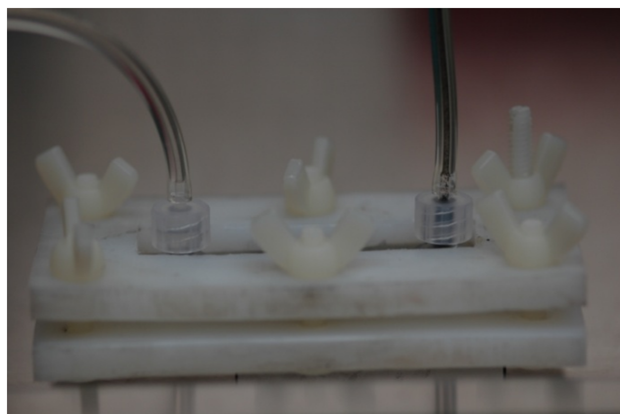
The device was built as specified in the methods above. Only small modifications were made based on experience. Wing nuts replaced the small hex-nuts in order to provide more even pressure. The device is shown in **Figure 6**. The device worked well for encapsulating the

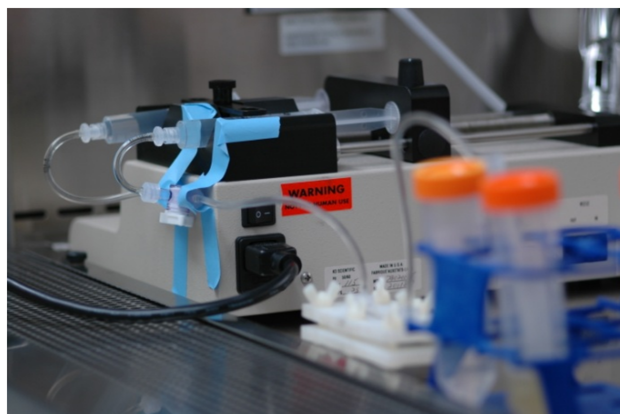
channel and providing a water-tight seal for the evaluation of MWNTs for bacteria filtration. Some problems that were addressed during experiments are:

- Failure of the epoxy between the pipe fittings and the microscope glass – High pressure could result in the glue coming off when the pipe fittings are not glued on properly. For the best results, the epoxy should be applied to the bottom of the pipe fittings and stuck quickly to the slide with a micro-pipette lodged to prevent the hole from clogging. The epoxy should be allowed to dry for much longer than the suggested 1 hour (~1 day).
- Leaking between the PDMS and the glass slides – The wing-nuts can be tightened to put more pressure but this could cause the glass slides to break. A fresh PDMS gasket is much less likely to leak. Making a new layer every few experiments will significantly decrease this type of mechanical failure.
- Sudden evacuation of MWNTs – With a sudden change in back pressure, the MWNTs may become dislodged from the channel. Great care should be taken to ensure that the back pressure remains constant. Problems can occur when
    - The syringe is detached and the now free tube is held up increasing the pressure head.
    - A valve fitting is turned without ensuring zero relative pressure in the tube that the valve is opening.

Recommendations are made in the discussion for a next generation of designs to further evaluate the system.

Figure 6 - The designed device and system





Left: A view of the plastic clamp with the channel enclosed. Wing nuts provide variable pressure to keep leaks from occurring.

Right: A view of the entire system. A syringe pump is used to create a consistent low flow rate. The effluent is connected to a 50 mL centrifugal tube which will be where the results are collected.

MAXIMUM FLOW RATE AND MWNT CONCENTRATION

The goal of this section was to determine optimal concentration of MWNT and flow rate for the system. A solution of 2mg/ml MWNT was injected under influence of the magnet (0.684 Tesla). When allowed to stand, it separated to fill ½ the volume of the channel. 4 mg/ml was attempted which saturated the system and a flow rate could pass without loss at some flow rates. There were several considerations in the flow rate through the device. The magnetic field is of limited strength and the MWNTs must remain in the system. The flow rate must not cause MWNT loss. Flow rate was varied, and the system was observed to check for macroscopic MWNT loss. From **Table 3**, values below 0.2mL/min are safe. The flow rate used throughout these experiments was 0.1ml/min.

Table 3 – MWNT loss for varying flow rates	
Flow Rate (mL/min)	**MWNT loss**
2.0	Loss Occurred
1.5	Some Loss
1.0	Some Loss
0.5	Slight Loss
0.2	No loss
0.1	No loss

CAPACITY

Data from the capacity experiment is presented below. The experiment was run over a variety of concentrations searching for the threshold concentration where the MWNTs lose their ability to filter *E. coli*. Specifically, the concentration that falls below 95% removal needs to be found. Many experiments were conducted to find the data below. The data show some important characteristics of the designed system. There was a strong correlation between capacity and concentration. This correlation shows that there was relative variance in the system, which may be due to several factors. Most important is the positioning of the MWNTs. The orientation of the MWNTs may have allowed streams of bacteria to flow unabated decreasing the removal efficacy. The accumulated data of all the tests is shown in **Table 4** and in **Figure 7** with a logarithmic scale (base 10) on the x-axis.

Table 4 – Results of MWNT system capacity					
#cells (X)	**%Removed (Y)**	**#cells (X)**	**%Removed (Y)**	**#cells (X)**	**%Removed (Y)**
20	100.0%	**9020**	99.7%	**40590**	99.0%
40	100.0%	**9450**	98%	**116000**	94%
60	100.0%	**12600**	97%	**200000**	100.0%
80	100.0%	**13533**	99.1%	**232000**	90%
100	100.0%	**15750**	97%	**300000**	57%
120	100.0%	**18040**	98.3%	**327881**	95.9%
140	100.0%	**18900**	96%	**348000**	90%
160	100.0%	**20000**	100.0%	**464000**	86%
200	100.0%	**22550**	98.7%	**580000**	80%
1840	100.0%	**27060**	98.6%	**600000**	57%
3150	99%	**29807**	94.3%	**696000**	71%
4510	99.3%	**31570**	98.8%	**900000**	56%
6300	99%	**36080**	98.9%	**1200000**	53%

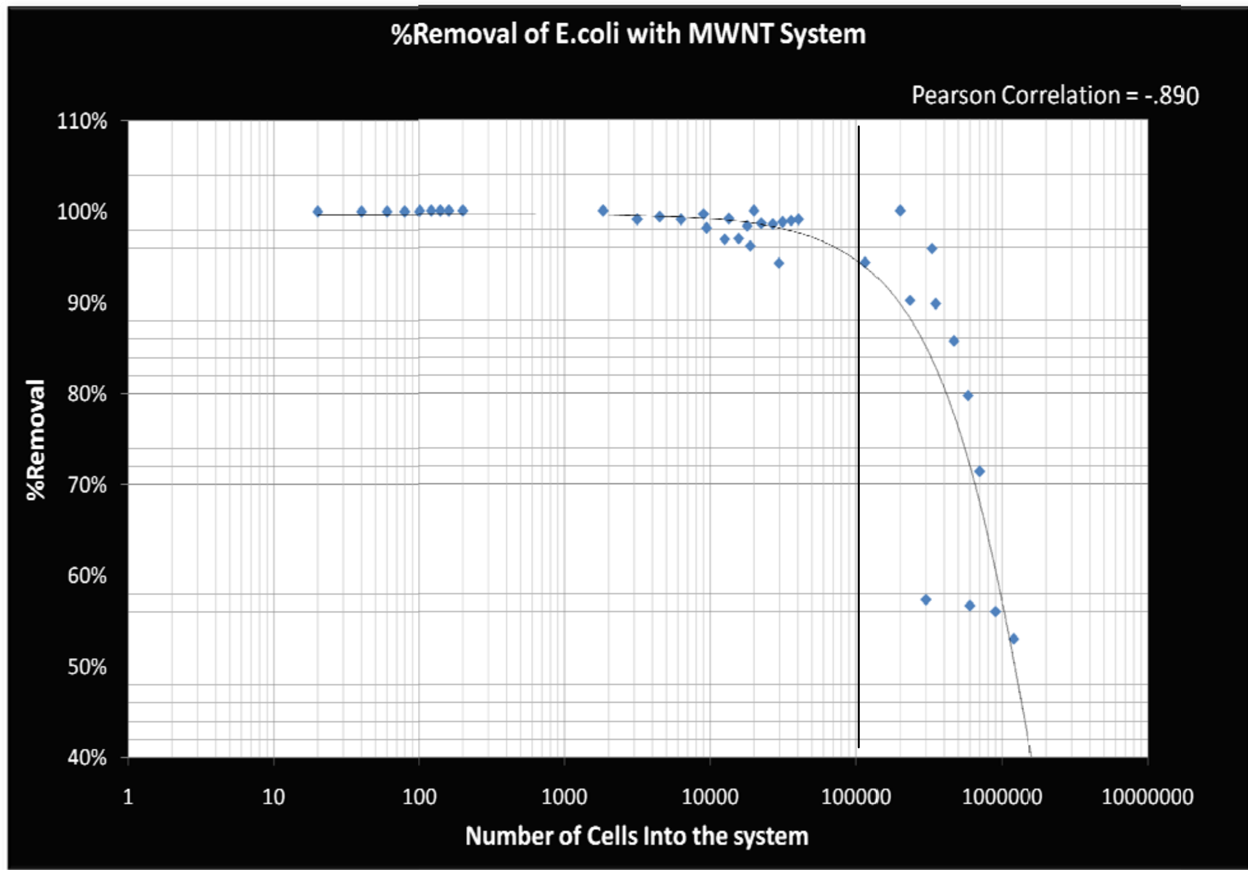
The Pearson Correlation can be completed on a set of data to determine how well related the two sets are. The Pearson Correlation was completed with Microsoft Excel and confirmed with the equation listed below. A strong Pearson correlation is considered to be above 0.8. Negative values represent negative sloping relationships (larger concentration causing small %Removal) and positive values represent positive relationships.

Pearson Correlation

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{N}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{N}\right)}} = -0.890$$

A Pearson Correlation of -0.890 suggests a strong negative relationship between the number of bacteria cells injected and the percentage removed.

Figure 7 graphs concentration against the %Removal. The x-axis (concentration) is shown in logarithmic form. The relationship is not linear. The graph shows a threshold where the system stops working (depicted by a vertical line). Because of the variance in the data, it is difficult to make a definite capacity for the MWNT system.

Figure 7 – The %Removal of *E.coli* as a function of the #cells entering the system

From the graph above, the threshold concentration is close to 100,000 cells.

An estimate for the threshold (change in slope) was 100,000 cells. Knowing this, the capacity of the MWNT system was found in terms of CFU/mgMWNT.

$$Capacity = \frac{Number\ of\ Cells}{Number\ of\ Carbon\ Nanotubes} = \frac{[E.coli]}{[MWNT]}$$

$$Capacity = \frac{\frac{100{,}000\ CFU}{1.2\ mL}}{4\frac{mgMWNT}{ml}} = 20833\frac{CFU}{mgMWNT}$$

$$Capacity\ \cong 20{,}000\frac{CFU}{mgMWNT}$$

The capacity of the system was approximately 20,000 cells per mgMWNT. This data was based on the assumption that the MWNT system was filtering the bacteria. It was equaally likely at this point that the MWNTs were toxic to the bacteria and that was why a removal was seen. The next experiment will validate the hypothesis that the *E. coli* are being filtered instead of killed.

IMAGING

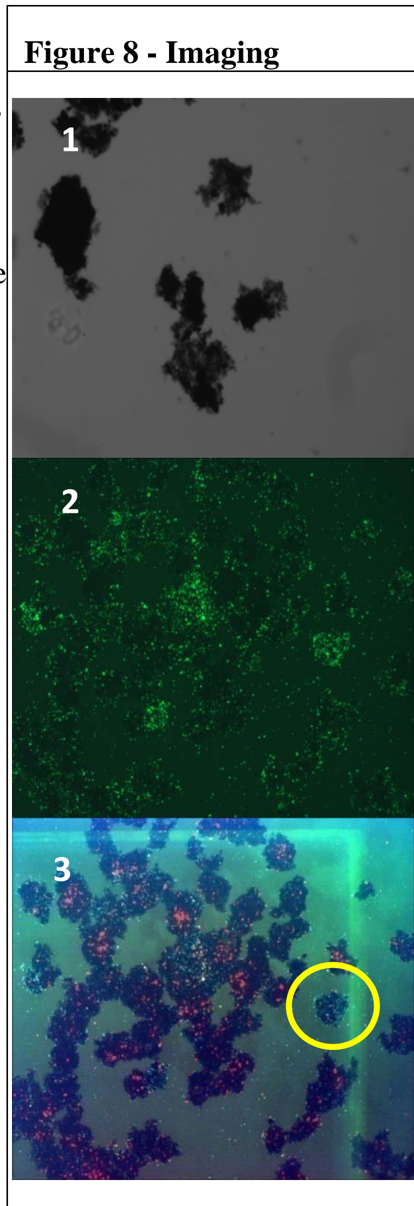
The images were taken using LIVE/DEAD® BacLight Bacterial Viability Kits. The kit contained two nucleic acid stains: green-florescent SYTO® 9 stain and a red-florescent propodium iodide stain. SYTO 9 (green) labeled both live and dead bacteria when used alone. Propodium iodide (red) penetrated bacteria with damaged membranes or dead bacteria. (http://ricfacility.byu.edu/baclightkit.pdf). First, bacteria were infused into the system using the capacity testing procedure. MWNT were isolated from the system, placed on a microscope slide, and images were taken as described below.

(1) MWNT only – The first image was taken using the light microscope at 40X optical zoom. Shows isolated MWNTs in solution.

(2) MWNT with epi-florescence (FITC) – Showing alive and dead cells as green. It is important to note that the cells are highly concentnrated around clusters of MWNTs

(3) MWNT with epi-florescence (FITC and PI hybrid image) showing live cells (blue-green) and dead cells (red). Again, the cells are concentnrated on the MWNT clusters. It appears that the number of dead bacteria out number the live. There is a MWNT cluster that shows live bacteria that appear to have been adsorbed (circled)

Figure 8 - Imaging



The microscope used was a Light Microscope(Axioskope 2 Plus, Carl Zeiss, Inc., Germany) equiped with a 12-bit Color MicroImager II cooled digital camera (Qlmaging, Burnaby, Canada). For the filtered images (2 and 3) a band pass filter 450 to 490 nm (FTIC) and 512 to 546 nm (PI) was used for exciters and 515 nm (FITC), 590 (PI) was used to acquire epi-florescence images for the stained cells and MWNTs (FITC, PI, and DAPI; Carl Zeiss).

The images showed that *E. coli* cells were adsorbed and retained by MWNT clusters. From the images, it appeared that the *E. coli* cells contacted MWNT clusters by diffusion or propelled by their own flagella movement.

DISCUSSION

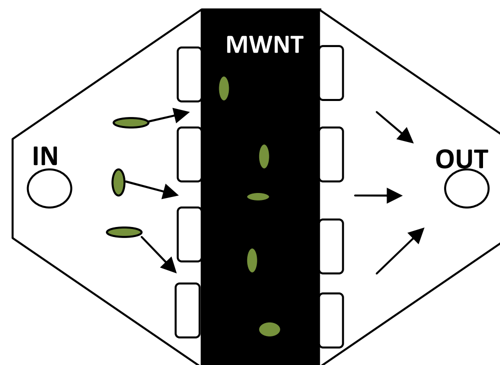
NEW SYSTEM

From the results above, a better system can be suggested. Major considerations for the new design are as follows

1) Leaking
2) Size and Cost
3) Bacteria concentration gradient

The proposed system for future research falls into the realm of BioMEMS (Micro electronic and mechanical system). The device will take advantage of the results of the preliminary system during this research (**Figure 9**). The device consists of a PDMS gasket made from a negative mold. The PDMS can then be plasma bonded to the glass. The cascading shape allows for a much higher flow rate through the system and an increased surface area over the MWNTs, which maximizes the capacity per weight of MWNT.

Figure 9 – Proposed MWNT system



An ideal system which would maximize the adsorption capacity of the MWNTs.

TOXICITY

The images show many dead bacteria attached to MWNT indicating that there may be relative toxicity either by chemical stress or mechanical stress to the *E. coli* cells. Scaning electron microscope pictures taken by other researchers in the lab show MWNTs penetrating the membrane of *E. coli* cells indicating mechanical stress may be the reason for death. Further research needs to be done to quantify the toxicity and amount of time that is available to detect the bacteria.

APPLICATIONS

1) Bacteria Filtration

   As a bacteria filter, the system functions well, but is certainly not ideal. A bacteria filter should filter more than 95% at high concentrations of bacteria. It is more feasible than SWNTs because MWNT are relatively cheap ($400 per kilogram compared to $37,500 per kilogram– www.cheaptubes.com). If the system used 4 mg, the MWNT cost would be 0.16 cents.

2) Bacteria Concentration

   For bacteria concentration, the system functions well, provided that the *E. coli* remain alive in the channel for enough time to detect them. A large volume with a low concentration of harmful pathogens could be concentrated. The system should be improved to increase the ideal flow rate through the device decreasing detection time.

3) Blood Infections

   CNTs can be functionalized to select for specific molecules giving them potential medical applications. A future application could be filtering blood for specific

molecules and pathogens and could be used to detect diseases by being made specific for proteins indicating the disease.

The Carbon Nanotube (CNT) including SWNT and MWNT is a very unique class of molecules with novel properties. Their applications should continue to be explored.

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