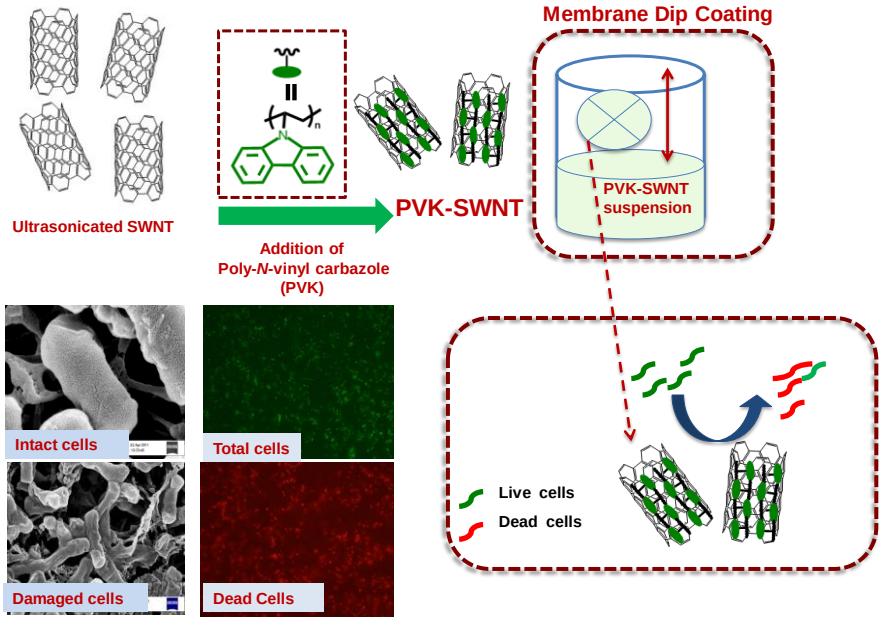
Highlights

Antimicrobial PVK:SWNT nanocomposite coated membrane for water purification: performance and toxicity testing

- Nitrocellulose membrane filters coated with PVK: SWNT (97:3 wt%) nanocomposite.
- High bacterial inactivation (~ 80-90%) on the filter surface was observed.
- High bacterial DNA concentration in the filtrate confirmed cell membrane damage.
- PVK-SWNT nanocomposites present minimal toxic effects to mammalian cells.

Graphical Abstract (for review)



Antimicrobial PVK:SWNT nanocomposite coated membrane for water purification: performance and toxicity testing

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Abstract

This study demonstrated that coated nitrocellulose membranes with a nanocomposite containing 97% (wt%) of polyvinyl-*N*-carbazole (PVK) and 3% (wt%) of single-walled carbon nanotubes (SWNTs) (97:3 wt% ratio PVK:SWNT) achieve similar or improved removal of bacteria when compared with 100% SWNTs coated membranes.Membranes coated with the nanocomposite exhibited significant antimicrobial activity towards Gram-positive and Gram-negative bacteria (~ 80–90%); and presented a virus removal efficiency of ~2.5 logs. Bacterial cell membrane damage was considered a possible mechanism of cellular inactivation since higher efflux of intracellular material (Deoxyribonucleic acid, DNA) was quantified in the filtrate of PVK-SWNT and SWNT membranes than in the filtrate of control membranes. To evaluate possible application of these membrane filters for drinking water treatment, toxicity of PVK-SWNT was tested against fibroblast cells. The results demonstrated that PVK-SWNT was non toxic to fibroblast cells as opposed to pure SWNT (100%). These results suggest that it is possible to synthesize antimicrobial nitrocellulose membranes coated with SWNT based nanocomposites for drinking water treatment. Furthermore, membrane filters coated with the nanocomposite PVK-SWNT (97:3 wt% ratio PVK:SWNT) will produce more suitable coated membranes for drinking water than pure SWNTs coated membranes (100%), since the reduced load of SWNT in the nanocomposite will reduce the use of costly and toxic SWNT nanomaterial on the membranes.

Keyword: Nitrocellulose membrane, Single-walled carbon nanotubes, nanocomposite, bacteria, DNA, Cytotoxicty.

1. Introduction

Membrane separation systems are used for drinking water treatment because of their potential to remove microorganisms (Hilal et al. 2004). The major issue of membrane operations is that they are often affected by biofouling phenomena (*e.g.,* bacterial adhesion) on the membrane surface (Khulbe et al. 2000). Membrane biofouling is initiated by bacterial adhesion and growth on the membrane surface, which can eventually form a biofilm (Hilal et al. 2004). Hence, developing membranes with anti-bacterial property is certainly an attractive solution. The most common approach for developing anti-microbial membranes is the modification of commercial membrane surfaces with polymeric materials containing silver nanoparticles (Aslan et al. 2010, Liu et al. 2010). Similarly, carbon based nanomaterials, like single-walled carbon nanotubes (SWNTs), have been reported to significantly reduce the bacterial and viral load in water due to its anti-microbial properties (Kang et al. 2008, Kang et al. 2007, Kang et al. 2009, Brady-Estévez et al. 2008). Apart from anti-microbial properties, incorporation of SWNT into membranes has also been reported to improve membrane strength, thermal stability, and water flux (Peng et al. 2007). The use of SWNTs to coat membranes is, however, still limited by high cost and poor dispersibility in aqueous solutions (Aslan et al. 2010, Arias and Yang 2009, Upadhyayula and Gadhamshetty 2010). Though polymer nanocomposites have been used for surface modification of membranes, SWNT incorporated in the polyvinyl-*N*-carbazole (PVK) polymer has not yet been investigated for its application in water treatment. In our recent study with PVK-SWNT nanocomposite in suspension and immobilized on solid surfaces, we demonstrated significant antimicrobial effects against both Gram-positive and Gram-negative bacteria (Ahmed et al. 2012). PVK was selected as a base polymer because of its multiple aromatic groups that facilitate π-π interaction with carbon-based nanomaterials, making it a more compatible polymer with

SWNT. Besides, PVK possesses excellent thermal, mechanical, and biocompatible properties and is easy and economical to prepare (Guimard et al. 2007, Ahuja et al. 2007). Furthermore, the ratio of SWNT in the PVK-SWNT (97:3 wt% ratio PVK: SWNT) nanocomposite reduces the use of costly SWNT in the membrane preparation while showing excellent dispersion of SWNT in aqueous solution in the presence of PVK (Cui et al. 2011).

In this study, we investigated the antimicrobial properties of nitrocellulose membrane filters coated with PVK-SWNT (97:3 wt% ratios, PVK: SWNT). Highly purified and well characterized SWNT was used to synthesize the PVK-SWNT nanocomposite. Membrane surfaces were dip coated in PVK-SWNT suspension to create a film of PVK with a 3 wt% SWNT load. Antibacterial and virus removal properties of the membranes coated with PVK-SWNT was investigated with Gram-positive (*Bacillus subtilis*), Gram-negative (*Escherichia coli*) bacteria and the model virus MS2. Cytotoxicity of PVK-SWNT nanocomposite was investigated with mammalian fibroblast cells to assess suitability of this nanocomposite for drinking water treatment.

2. Materials and Methods

2.1 Single-walled carbon nanotubes preparation

Single-walled carbon nanotubes (SWNTs) were purchased from Cheap Tubes Inc. (Vermont, US). The results of the characterization of the SWNT are provided in the Supporting Information (Table S1-S2, Figure S1). Prior to use, further purification of SWNT was done by heat treatment at 200^0C for 6 hours. The SWNT suspension was prepared in deionized water (DI) (1 mg/ml) according to previously published methods (Rodrigues and Elimelech 2010). Briefly, SWNT was dispersed in DI water through 3 cycles of sonication for 1 h.

2.2. Preparation of PVK and PVK-SWNT nanocomposite

The PVK-SWNT (97:3 wt% ratio PVK:SWNT) was prepared according to previously reported procedure (Ahmed et al. 2012, Cui et al. 2011). Briefly, separate suspensions of 1mg/ml of SWNT and PVK (Sigma Aldrich Chemicals, USA) were prepared by ultrasonication with N-cyclohexyl-2-pyrrolidone (CHP). Later, 0.15 ml of the CHP-SWNT solution was added to 4.85 ml of CHP-PVK solution. The mixture was centrifuged (4400 rpm, 1h) and the pellet of PVK-SWNT was washed with DI water and resuspended by Ultrasonication, which furnished a well dispersed suspension of PVK-SWNT (1 mg/ml) in DI water. This procedure yielded a 97:3 weight ratio of well-dispersed PVK-SWNT mixture. The 97:3 (wt%) ratio of PVK and SWNT was selected for this nanocomposite based on our previous study, which demonstrated that at this particular ratio SWNT remains highly dispersed and stable for an extended period of time (Cui et al. 2011). A PVK only suspension was prepared by dissolving PVK in water (1mg/ml) followed by 1 h ultrasonication.

2.3. Coating of the filter membranes

Nitrocellulose membrane filters (0.45 μm, Milipore USA) were dip-coated with PVK (1mg/ml), SWNT (1mg/ml) and PVK-SWNT (1 mg/ml) suspensions in DI water. Briefly, each filter membrane was placed into a small petri dish containing 5 ml of the sample suspension to cover the filter membrane completely. After 30 minutes of impregnation, the filter membranes were carefully removed and dried overnight in a vacuum oven. Bare nitocellulose membranes were used as controls. Characterization of PVK, SWNT, and PVK-SWNT suspensions and coated membrane filters were conducted according to procedure described in our previous work (Ahmed et al. 2012, Cui et al. 2011).

2.4. Bacterial Culture

Freshly prepared suspensions of *Escherichia coli* MG 1655 (*E. coli*) and *Bacillus subtilis*-102 (*B. subtilis*) were used for all experiments in this study. In order to prepare fresh suspensions, single isolated colonies of *E. coli* and *B. subtilis* were inoculated in 5 ml of Tryptic Soya Broth (TSB) (Oxoid, England) and incubated overnight at 35 ^{0}C and 200 rpm (INNOVA 44, New Brunswick Scientific Co, USA). The bacterial culture was centrifuged at 10,000 rpm for 10 minutes. To remove any residual growth medium, cells were washed twice and re-suspended in phosphate buffer solution (PBS, 0.01M, pH=7.4) (Fisher Scientific, USA). The bacterial suspension was adjusted to give an optical density (OD) of 0.5 at 600 nm, which corresponds to a cell concentration of ~10^{7} colony forming units (CFU)/ml.

2.5. Bacterial Cell Filtration

All the filtration apparatus were sterilized prior to use. The prepared membrane filters were washed for 15 min with ethanol (70%) and air dried for 24 h in a biological safety cabinet (LABGARD, NuAire Inc, USA) under laminar flow to evaporate any residual ethanol. All the filtration assays were conducted under a constant permeation rate (~57 L m^{-2} h^{-1}) using a peristaltic pump (Cole-Parmer, USA). The filtration experiment set up consisted of all glass filtration apparatus with 47 mm stainless steel screen (Milipore, USA). The mode of filtrations was dead-end. Prior to each filtration experiment, the filter surfaces were preconditioned by passing 10 ml of sterile PBS. For each membrane filter type (i.e., coated with SWNT, PVK-SWNT and non-coated), 2 ml of bacterial suspension in PBS at OD= 0.5 was passed through the membrane filter. Each membrane filter was tested at least in duplicate.

2.6. Bacterial Viability Assay

This test was performed to determine the percentage of inactivated bacterial cells retained on the surface of the membranes. The bacterial viability assay was performed using the LIVE/DEAD Baclight kit (Invitrogen, USA) to quantify the number of live and dead cells on the filter surfaces (Kang et al. 2008). Immediately after the filtrations, the filter surfaces were stained with the LIVE/DEAD BacLight Bacterial Viability kit and were observed with a fluorescence microscope (OLYMPUS, Japan). SYTO 9 dye was used to stain the total number of cells, while propidium iodide (PI) was used to stain cells with compromised membranes. Five representative images at 40x magnification were taken for each sample and all the samples were tested in triplicate. Total cells and dead cells were counted with the Image-Pro Plus software (MediaCybernetics, USA). The percent of inactivated cells was determined from the ratio of the number of cells stained with PI divided by the number of cells stained with SYTO-9 plus PI. The results were averaged out and the standard deviations were calculated.

2.7. Bacterial quantification in the filtrate

The plate count method was used to enumerate viable bacteria in the filtrate (Hilal et al. 2004). The filtrates were collected and diluted in PBS through serial dilution. The dilutions were plated on Tryptic Soya Agar (TSA) (Oxoid, England) media and incubated overnight at 37^0 C. The total number of colony forming units (CFU) was enumerated. Each filtrate sample was plated in duplicate and standard deviations were calculated from the results.

2.8. Filter Agar test

Viability and re-growth potential of the retained bacterial cells on the membrane surfaces were tested using a previously described method (Hilal et al. 2004). Immediately after filtration, the filter surfaces were flipped on a TSA plate facing down and incubated overnight at 37^0 C.

157 Bacterial growth on the membrane perimeter was measured with a Mitutoyo 500-196-20 Digital
158 micrometer Caliper (MSI Viking Gage, USA). Averages and standard deviations were calculated
159 from triplicates.

160 *2.9. Scanning electron microscopy (SEM) Imaging*

161 SEM sample preparation and imaging was performed as previously described (Kang et al.
162 2007). Briefly, bacterial cells on the filter surfaces were fixed with 2% gluteraldehyde in 0.05M
163 cacodyle buffer solution (Fisher Scientific, USA). The fixed cells were subsequently stained with
164 1% osmium tetraoxide (Sigma Aldrich Chemicals, USA) and dehydrated with increasing
165 concentrations of ethanol (25%, 50%, 75%, 95% and 100%). SEM images were acquired using a
166 LEO Gemini 1500 series microscope at 10 keV. Prior to imaging, the samples were mounted on
167 carbon tape and coated with Au/Pd using a Denton Vacuum Desk II sputter coater.

168 *2.10. Deoxyribonucleic acid (DNA) quantification assay*

169 This assay was performed to quantify the DNA concentration (ng/µl) in the filtrate that was
170 released from damaged bacterial cells after filtration. The experimental procedure was adapted
171 from a previously described method (Kang et al. 2008). Briefly, immediately after filtration, 2 µl
172 of the filtrates were placed in a Take 3 Plate (for DNA quantification) in the Synergy MX
173 (BioTek, USA). Sterile PBS without bacteria and DNA were used as blanks. Average DNA
174 concentrations and standard deviations were calculated from duplicate filtrate samples.

175 *2.11. Viral culture and quantification*

176 MS2 bacteriophage was selected to test viral removal by these new membranes. MS2
177 bacteriophage and its host *E. coli* 15597 were obtained from the American Tissue Culture
178 Collection (ATCC). The stock solution of MS2 was prepared as previously described(Brady-

179 Estévez et al. 2010). Stock solutions of MS2 in PBS ($4.5x10^{11}$ Plaque Forming Units (PFU)/ml)
180 were used for the filtration experiments. The concentrations of bacteriophages were determined
181 before and after each filtration experiment. The membrane filters were prepared and
182 preconditioned as described in *Section 2.5* "Bacteria Cell Filtration". A solution of 2 ml of the
183 MS2 was filtered through the membrane filter at constant permeation rate (~57 L m^{-2} h^{-1}). Filtrate
184 was collected on sterile 2 ml tubes and viral concentrations were determined with the PFU
185 method (Brady-Estévez et al. 2010). Briefly, each serial dilution in PBS of the filtrate was mixed
186 with the *E. coli* host and molten soft agar (0.7% TSA), then poured on TSA plates. The plates
187 were incubated overnight at 35 °C and the plaque forming units were quantified. All the virus
188 experiments were done in a biological safety cabinet. Each filter was tested in duplicate and the
189 standard deviations were calculated.
190
191 *2.12. Cytotoxicity Evaluation*
192 Cytotoxicity test of PVK-SWNT, SWNT, and PVK solutions were performed against NIH
193 3T3 Fibroblast cells using CellTiter 96 AQ_{ueous} (Promega) as previously described (Tria et al.
194 2010). The NIH 3T3 Fibroblasts were a gift from Dr. Albee Messing of the University of
195 Wisconsin-Madison and were cultured at 37 °C in a growth media containing 86% of Dulbecco's
196 modified Eagle's medium, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% 4-(2-
197 hydroxyethyl)-1-piperazineethanesulfonic acid in 1 M HEPES, 1% L-glutamine, and 1%
198 minimum essential medium (MEM) in 10 mM nonessential amino acids solution (100×;
199 GibcoBRL). Fibroblast cells of passages 129 and 132 were harvested from culture flasks by 10-
200 12 min incubation with 0.25% trypsin and were resuspended in the growth media. Assay kits
201 containing 3- (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2*H*-
202 tetrazolium, inner salt (MTS) and an electron coupling reagent, phenazine methosulfate (PMS)

were used. Briefly, cells were seeded onto a 96-well plate with a seeding density of $2.5x10^4$ cells/ 100 µL and incubated at 37° C and 5% CO_2 in humidified air for 24 h. The cell culture medium was then aspirated from the wells and the plates containing cells were gently rinsed with Dulbecco's Modified Eagle Medium (DMEM) to remove any non-adherent cells. Next, 100 µl of nanomaterials (PVK, SWNT and PVK-SWNT) were added onto each well containing cells and incubated for 24 h at 37° C with 5% CO_2. After the incubation, the nanomaterials dispersed in solutions were aspirated and the wells were rinsed 3 times with DMEM. The adherent cells were evaluated for their viability using MTS assay as described by the manufacturer (Promega, Madison, WI, USA). Briefly, MTS and PMS detection reagents were mixed, using a ratio of MTS/PMS 20:1. This procedure was done immediately before to addition to the cell culture media (DMEM) in which a 1:5 ratio of detection reagents to cell culture medium was used. Then the aspirated wells containing the samples were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. A well containing only the culture medium (i.e. DMEM) was used as a "medium only" control. The untreated cell suspension was used as a negative control. For the positive control, 4% paraformaldehyde in PBS buffer was added to the cells grown on the plate. The absorbance of the formazan was read using a Synergy MX Microtiter plate reader (BioTek, VT) at 495 nm.

3. Results and Discussions

3.1. Filter membrane Characterization

Prior to membrane fabrication, the PVK-SWNT and PVK sample solutions were characterized using UV-Vis. Figure 1 shows the UV-visible spectra for the pure SWNTs. As expected no absorption peaks at the visible region of the electro-magnetic spectrum were observed. However for the pure PVK solution, main signature bands occurring at 331 and 345

nm were observed. These peaks are attributed to the π-π* and n- π* optical transitions in pendant carbazole moieties of PVK (Fulghum et al. 2008). It can be seen from the spectra that the main absorption peaks for pure PVK still prominent in the PVK-SWNT nanocomposite, but the intensity was slightly reduced due to the presence of the SWNT. FTIR was also used to determine the functional groups present on the modified filter surfaces. Figure 2 shows the IR spectra of the nitrocellulose filter membrane with the following peak assignments: 832 cm^{-1} (NO stretch), 1651 cm^{-1} (asymmetric NO_2 stretch), 1282 cm^{-1} (symmetric NO_2 stretch), 1060 cm^{-1} (asymmetric CO stretch) and a weak band at 1746 cm-1 (CO stretch) (Sloane 1963). Similar peaks were observed for the PVK-SWNT membranes corresponding to olefinic C-H bending (822-837 cm^{-1}), C-N stretching (1012-1273 cm^{-1}) and C=C stretching (1635-1645 cm^{-1}), except for the weak band at 1746 cm^{-1} found in bare nitrocellulose filter (inset). The disappearance of this band as well as the significant increase of absorbance intensity in modified membranes suggests successful coating of the filter with PVK-SWNT.

The successful modification of the filter was characterized using XPS (Figure 3). Narrow scan in the N 1s region of the unmodified filter membrane showed an intense peak at ~408 eV coming from N=O of the nitrocellulose membrane. Upon addition of PVK-SWNT, a new peak centered at ~399 eV appeared, indicative of the N-C coming from the carbazole moieties of PVK. Furthermore, the C 1s scan of the PVK-SWNT showed higher peak intensity in the C-C region (284.5 eV) as compared to the PVK and unmodified membranes due to the incorporation of C-C containing SWNT. To estimate the amount of SWNT loaded on the filter membrane, the peak area ratios from the N-C and C-C peaks of the PVK-SWNT and PVK were used. Using this method, the amount of SWNT was estimated to be ~3 wt %. This value is similar to the solution mixture ratios of PVK-SWNT used to prepare the modified filter. The morphology of all the

membranes were evaluated using SEM. Figure 4a depicts the SEM image of the unmodified nitrocellulose membrane, which revealed a layered and mat-like porous surface. The SWNT and PVK-SWNT-modified filters, on the other hand, formed a denser coating on the surfaces that were seen over several layers. Furthermore, the uniform aspect of the surfaces throughout the membranes demonstrates successful and homogeneous coating of the nitrocellulose membrane surfaces with SWNT and PVK-SWNT (Figure 4, b & c).

3.2. Antibacterial property of the coated membranes

The LIVE/DEAD assay was performed to determine bacterial viability after interaction with the nanomaterials during filtration. Fluorescence microscopy was used to assess the loss of bacterial viability. SYTO 9 dye (green dye) was used to stain both live and dead cells while propidium iodide (PI) (red dye) was used stain the cells with compromised membranes. Figure 5 (a, b) shows representative fluorescence images of the *E. coli* and *B. subtilis* cells on the filter surfaces. Results show that in the absence of the nanomaterials (control), bacterial inactivation was <10% (Figure 5, c). While, ~90 % and ~81 % of the *E. coli* cells were inactivated after being retained on the PVK-SWNT and SWNT coated membranes, respectively. Similarly, ~90 % and ~40 % of the *B. subtilis* cells were inactivated after retained on PVK-SWNT and SWNT coated membranes, respectively. In similar studies with SWNT coated membranes, 80-90 % *E coli* inactivation was observed (Kang et al. 2007, Brady-Estévez et al. 2008). No noticeable toxicity effects (inactivation < 10%) of PVK coated membranes were observed on either *E. coli* or *B. subtilis*. This suggests that toxicity observed on PVK-SWNT coated membranes were either due to the presence of SWNT or synergistic effects of PVK-SWNT, but not due to the presence of PVK. In the case of 100% SWNT coated membranes (Figure 5, c), the toxic effect of these membranes on *B. subtilis* were considerably smaller than on *E. coli*. However, these findings are

similar to many other studies where *E. coli* and *B. subtilis* exhibited different tolerance levels towards SWNT. These finds were explained as differences in cell wall structure, the protective effect of the outer membrane surface properties, ability to form spores and/or unique repair mechanisms of different microorganisms (Aslan et al. 2010, Kang et al. 2009, Lyon and Alvarez 2008, Lyon et al. 2005).

The results demonstrated that PVK-SWNT nanocomposite with only 3% SWNT content achieved similar or better cell inactivation than 100% SWNT coated membranes. These results can be explained by the better dispersion and debundling of SWNT in the presence of PVK, which would increase the probability of SWNT to be in contact with bacterial cells(Kang et al. 2007). Though the exact mechanism of SWNT-bacterial interaction has not been completely elucidated yet, several studies suggested physical disruption of bacterial membrane and oxidative stress as the major mechanisms (Aslan et al. 2010, Kang et al. 2007, Rodrigues and Elimelech 2010, Schiffman and Elimelech 2011). Therefore, the cells in contact with this nanomaterial were probably inactivated by one or both mechanisms.

3.3. Intracellular DNA Release

Although the membrane damage test (LIVE-DEAD) is a strong indicator of cell damage, not all damaged membranes will lead to bacterial cell death. Current literature describes that the release of large quantities of intracellular material from cells, only occurs when bacterial cell walls and cellular membranes suffer irreparable damages (Kang et al. 2008). In many SWNT cytotoxicity studies, cell membrane damage has been reported as one of the mechanisms for bacterial toxicity. This mechanism is verified by measuring the efflux of cytoplasmic material (e.g. DNA) in the filtrate. In Figure 6, the filtration of both *E. coli* and *B. subtilis* yielded higher DNA concentrations in the filtrate of SWNT and PVK-SWNT coated membranes than uncoated

and PVK coated membranes. In the case of SWNT coated membranes, ~2 fold and ~1 fold increase in DNA efflux compared to the control were observed for *E. coli* and *B. subtilis,* respectively. While for PVK-SWNT coated membranes, ~4 fold and ~2.5 fold increase in DNA efflux were observed for *E. coli* and *B. subtilis,* respectively. In similar studies, release of intercellular DNA was observed to be as high as 5 fold for *E. coli* as a result of the bacterial interaction with SWNT and subsequent membrane damage (Kang et al. 2008, Kang et al. 2007). In the case of PVK-SWNT coated membranes, the SWNTs were highly dispersed, which increased the chances of cell interaction with the open ends of nanotubes and led to cellular damage. Similar results were also described on studies with coated surfaces with SWNTs and other polymers (Aslan et al. 2010, Ahmed et al. 2012, Schiffman and Elimelech 2011).

We believe that the measured DNA concentration in the filtrate of SWNT and PVK-SWNT coated membranes should have been much higher than what we are reporting, since DNA tends to adsorb to SWNT surfaces (Kang et al. 2008). This high efflux of DNA suggests considerable bacterial cell membrane damage and potential cell death.

In Figure 7, the DNA efflux from *B. subtili*s cells (Gram-positive) was lower than from *E. coli* cells (Gram-negative), which could be explained by the thicker peptidoglycan cell-wall found in Gram-positive bacteria. This thick peptidoglycan cell-wall would make it harder for nanomaterials to cause considerable cell membrane damage (Pratt and Kolter 1998, Yang et al. 2010).

3.4. Filter Agar printing test

Recent studies have shown that *E. coli* can endure and repair low to moderately damaged cell membranes (Kang et al. 2008, García et al. 2006). The main goal of this test was to confirm the results from the DNA release assay and determine at what extent the damaged bacterial cells

retained on the membrane filter could recover from the cellular damage and grow after the exposure to the nanomaterial. In the results of the LIVE/DEAD and DNA release assays, the control and PVK coated membrane filters presented very few bacterial cells with compromised cellular membranes as opposed to SWNT and PVK-SWNT coated membrane filters. Similarly, in the agar printing assay (Figure 7), much higher bacterial growth was observed on the control and PVK-coated membranes than on the other coated membranes. SWNT membranes presented ~73% and ~66% growth inhibition for *E. coli* and *B. subtilis*, respectively, when compared to the control. Similar inhibition was also observed for PVK-SWNT membranes (~70% for *E. coli* and ~65% for *B. subtilis*). These results suggest that bacteria retained on the membrane filters significantly lose their potential for re-growth. Bacterial re-growth and biofilm formation have been demonstrated in many studies to cause great problems in membrane operations (Zator et al. 2007); these results show that this new coating has the potential to solve such problems.

3.5. Bacterial morphology on the filters

The SEM images of *E. coli* (Figure 8) showed bacterial cells disrupted and shrunk on both SWNT and PVK-SWNT filter surfaces. This result corroborates our results of the Live/Dead assay, intracellular DNA release, and the filter agar printing test. Similar bacterial damage was also observed for *B. subtillis* after filtration on the PVK-SWNT and SWNT modified membranes.

3.6. Bacterial Removal Property

In theory, membrane filters with smaller pore size than the size of bacterial cells are expected to retain all cells by a sieving mechanism. Studies, however, have shown that bacterial cells can entrain through membrane pores due to high filtration rates, solution chemistry, and lack of membrane surface uniformity (Brady-Estévez et al. 2010, Tufenkji and Elimelech 2003).

Our study demonstrated that both SWNT and PVK-SWNT coated filters had ~ 4 log higher bacterial removal than the control filters (Figure 9). This bacterial removal might be a combined effect of cell retention and inactivation by SWNT while passing through the membranes. The small pore size (0.45 μm) of the nitrocellulose membranes, the added thickness of SWNT and PVK-SWNT layers to the membrane, and the strong affinity of the bacterial cells to SWNT surfaces increased the efficiency of the filters by cell inactivation, sieving, and depth filtration mechanisms (Brady-Estévez et al. 2008, Brady-Estévez et al. 2010, Brady-Estévez et al. 2010). The similar log removal of 100% SWNT coated membranes and PVK-SWNT coated membranes with only 3% SWNT load could be attributed to a more homogeneous dispersion of SWNTs on the membrane surface in the presence of PVK, and hence more SWNT open ends to inactivate bacterial cells.

3.7. Virus removal property

MS2 bacteriophage was used to investigate the removal efficiency of nanometer-sized viral particles. The bacteriophages were filtered through bare membranes (control), and through PVK, SWNT, and PVK-SWNT coated membranes. As expected, the log removal of MS2 for both control and PVK coated membranes were very poor (<1 log), due to the very small size of the virus (~27-34 nm) compared to the membrane pore size (0.45 μm) (Figure 10). On the other hand, the log viral removal was ~3 and ~2.2 for SWNT and PVK-SWNT coated membranes, respectively. The mechanism of virus removal on SWNT coated membranes has been demonstrated to be by depth filtration (Brady-Estévez et al. 2010). A higher virus removal (~3 logs) by 100% SWNT coated membranes than PVK-SWNT (with 3% of SWNT) coated membranes can be attributed to a larger amount of SWNT in the 100% SWNT membranes. The higher the SWNT concentration on the membrane, the larger will be the surface area available

for the virus particles to adsorb. Studies have shown that SWNT loads of 0.5 mg/cm^2 achieve more than 4 log virus removal. Furthermore, a linear relationship was established between the effluent virus concentration and SWNT load on the membrane surface (Brady-Estévez et al. 2010).

3.8. Cell cytotoxicity Test

During the filtration process, some SWNT particles may detach from the membrane surface and end up in the drinking water. Certain concentrations of SWNTs have been shown to be toxic to humans and other mammalians. For instance, pure SWNT/MWNT was described to damage the plasma membrane of mammalian cells and to induce considerable toxicity (Cheng et al. 2011, Chen and Schluesener 2010). Therefore, it is essential to investigate the cytoxicity of the SWNT concentrations used in the filters in this study to assess their suitability for drinking water treatment. Fibroblast cells are part of connective tissues and play an important role in wound healing; therefore they are often used in *in vitro* studies. These cells can easily get exposed by SWNT entering the mammalian cells through physical contact or ingestion (Tian et al. 2006).

The exposure of fibroblast cells to SWNTs showed that the concentration of SWNT plays an important role in the toxic behavior of SWNT towards mammalian cells (Figure 11). In SWNT samples, with a concentration of 1mg/ml, ~75% cytotoxicity was observed in the mammalian cells. In the PVK-SWNT suspension with a SWNT concentration of 0.03 mg/ml was observed only ~20% toxicity. PVK, on the other hand, that functions as dispersant, displayed minimal cytotoxic effects (~10%). Previous studies with other mammalian cells, such as the human umbilical vein endothelial cells, demonstrated that a SWNT mixture with the phosphorycholine polymer exhibited a cytotoxicity of only ~8-10%, while SWNT/MWNT suspended with polyethylene glycol (PEG) showed no considerable toxicity to mammalian cells (Yang et al.

2010, Cheng et al. 2011, Xu et al. 2008). The PEG and other polymers mixed with SWNT/MWNT were reported to be able to penetrate mammalian cells without damaging the plasma membrane, and their accumulation did not show significant toxic effect on cell cycle (Chen and Schluesener 2010) . Hence, it seems that in the event of some leaching from the PVK-SWNT coated membranes, the SWNT used in this study will not exhibit considerable toxicity towards fibroblast cells. However, the effects of SWNT exposure to other mammalian cell types and effects of chronic exposure need to be further investigated before such material can be widely used for water treatment.

4. Conclusion

"In this study, we demonstrated that membrane (nitrocellulose) coated with SWNT and PVK-SWNT nanocomposite can effectively remove and inactivate bacterial cells during filtration. The PVK-SWNT (97:3 wt% ratio PVK: SWNT) coated membranes achieved similar or improved bactericidal effects than 100% SWNT coated membrane. Log removal of MS2 virus was lower in PVK-SWNT filters compared to 100% SWNT filters due to smaller loads of SWNT in the nanocomposite, which reduced adsorption sites for the virus. The log removal efficiency (both bacteria and virus) could be further investigated by increasing the thickness of PVK:SWNT and SWNT coating. Again, substantial reduction of bacterial re-growth of the retained bacteria on the new membranes and the high concentrations of intracellular material efflux (DNA, ng/µL) in the filtrate are suggestive of irreversible bacterial cell membrane damage and bacterial death as a possible mechanism of bacterial inactivation by both SWNT and PVK-SWNT membranes. These observations are promising in terms of controlling biofouling problem during membrane filter operations. Cytotoxicity tests on fibroblast cells demonstrated that PVK-SWNT (97:3 wt% ratio PVK: SWNT) are considerably less toxic than pure SWNT (100%). Although SWNT and

412 PVK-SWNT membranes tested in this study showed impressive removal of pure bacterial
413 culture, further study should be conducted with natural water where many other factors like
414 natural organic material (NOM), complex microbial communities and solution chemistry can
415 influence the membrane filter performance."

416
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418
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426 **Supporting Information**

427 Additional data associated with this manuscript is provided in the Supporting Information
428 section.

429 **References**

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517

Figure File

(Antimicrobial PVK:SWNT nanocomposite coated membrane for water purification: performance and toxicity testing)

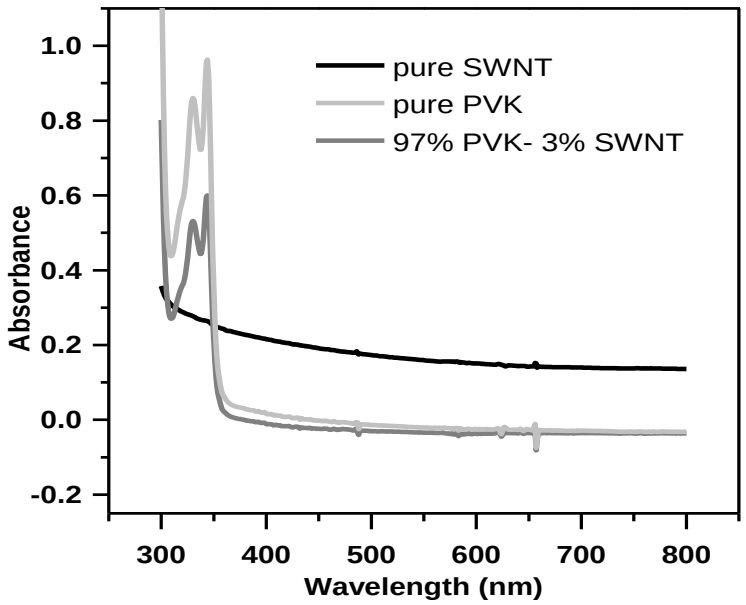

Figure 1. UV-vis spectra of the pure SWNT, PVK and PVK-SWNT nanocomposite solutions.

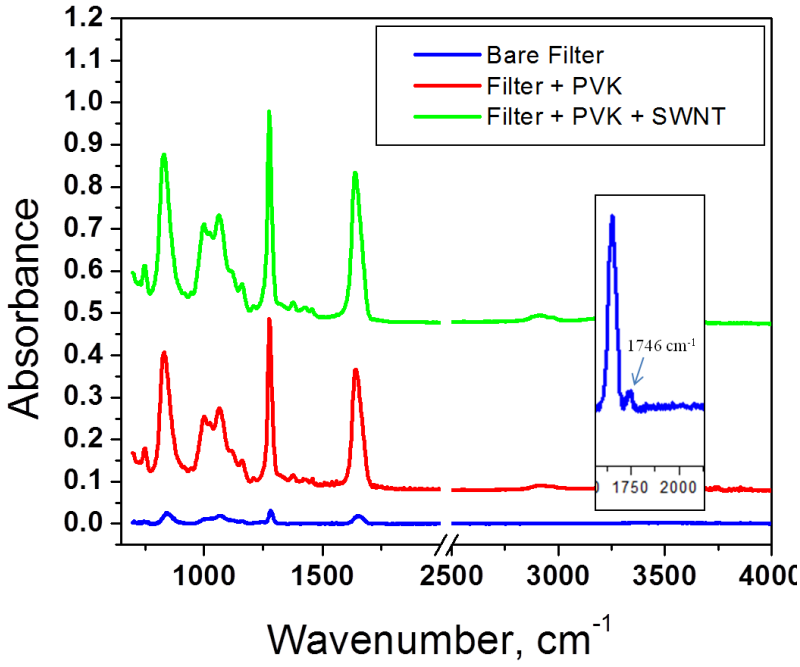

Figure 2. FTIR spectra of the unmodified, PVK-modified and PVK-SWNT modified nitrocellulose membranes.

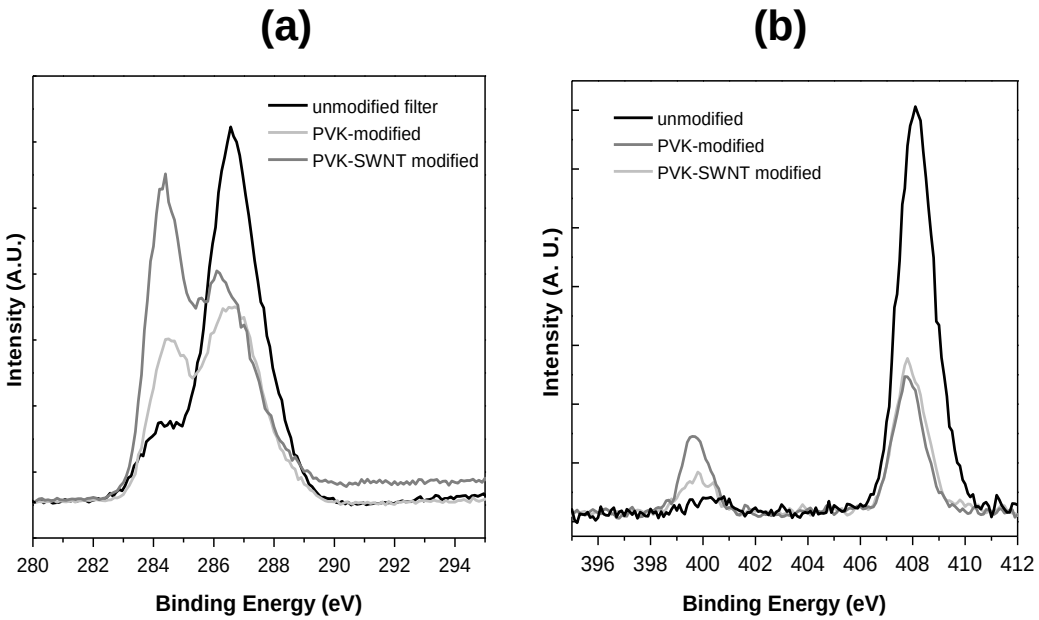

Figure 3. XPS spectra of the unmodified, PVK-modified, and PVK-SWNT modified nitrocellulose membrane. (a) C 1s and (b) N 1s regions.

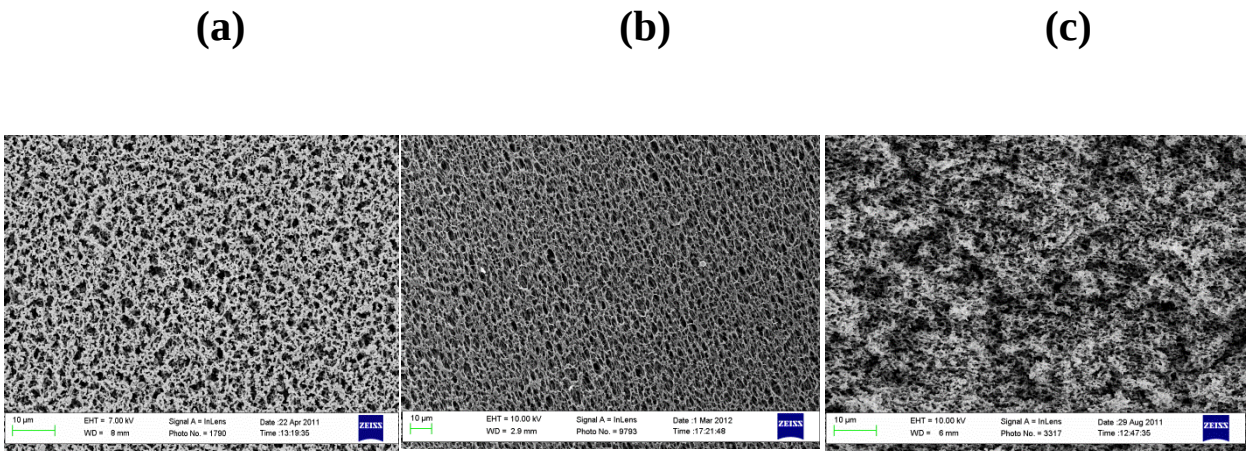

Figure 4. SEM images of membrane morphologies: (a) bare nitrocellulose membrane; (b) SWNT coated membrane; (c) PVK-SWNT coated membrane. Scale: 10 µm

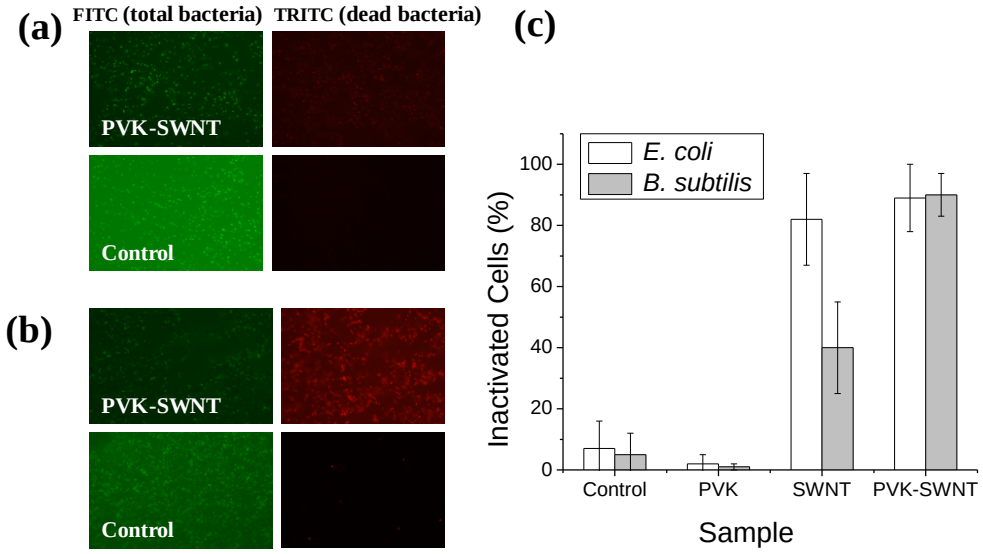

Figure 5. Viability assay for bacteria retained on membrane filters: (a) representative digital images after live and dead cell staining of *E. coli* retained on PVK-SWNT coated filter and on bare filter (control); (b) representative digital images after live and dead cell staining of *B.*

subtilis retained on PVK-SWNT coated filter and on bare filter (control). (c) Correlation of the % of non-viable *E. coli* and *B. subtilis* (percent inactivated cells) after retention on PVK-SWNT, SWNT, PVK and bare filters.

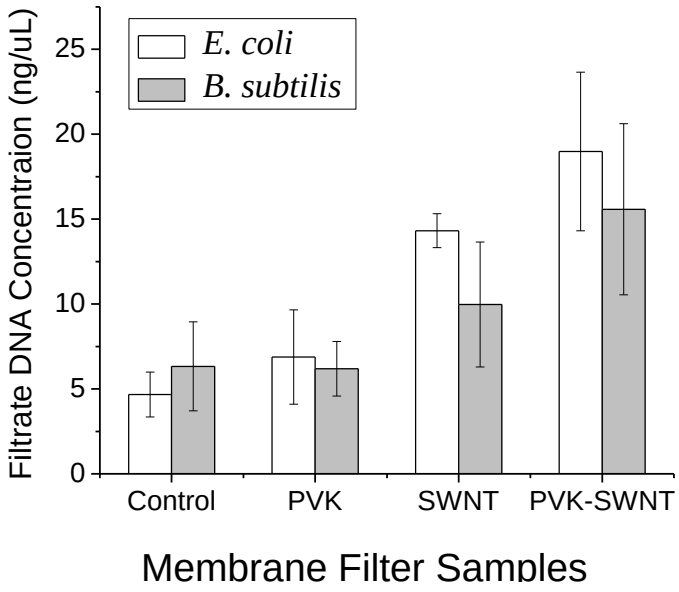

Figure 6. Efflux of cytoplasmic material (DNA, ng/µL) in the filtrate after filtration of *E. coli* and *B. subtilis* through PVK, SWNT and PVK-SWNT coated membrane filter and non-coated filter (control).

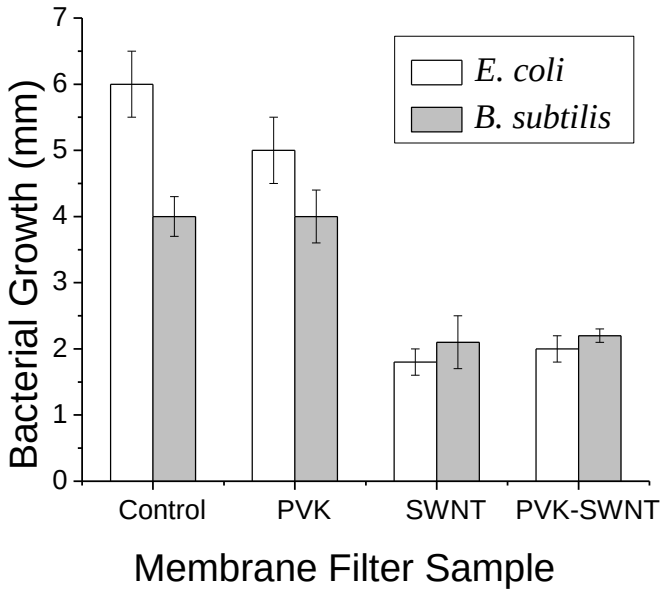

Figure 7. Agar printing assay to determine the growth behavior of bacteria retained on membrane coated with PVK, SWNT, PVK-SWNT and bare membrane (Control).

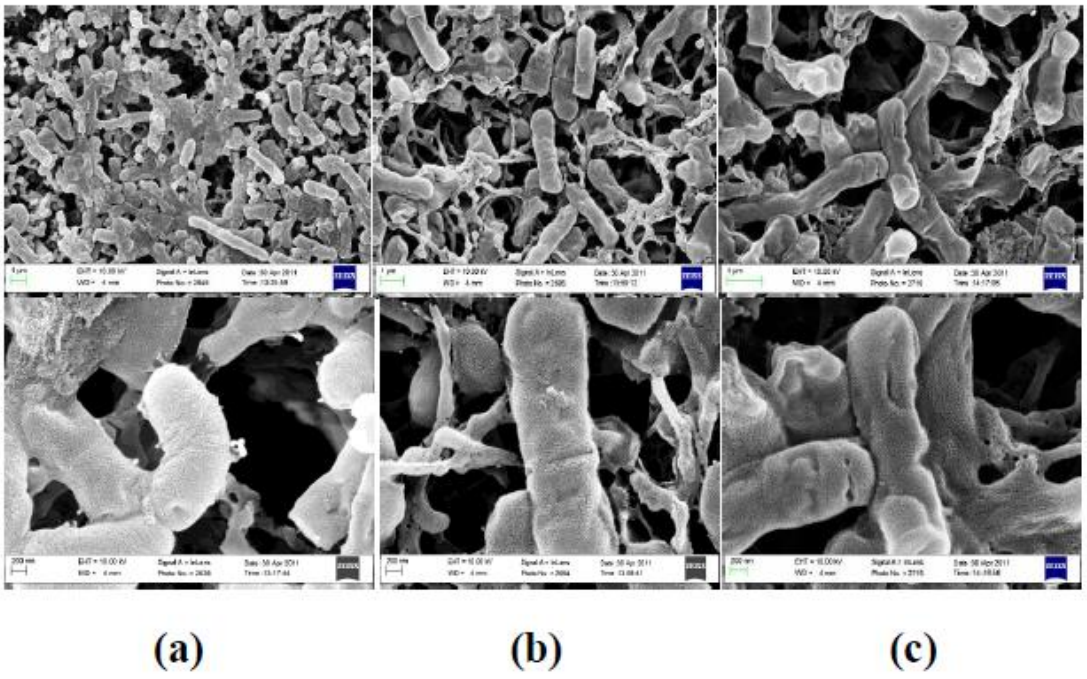

Figure 8. SEM images of membrane filter with retained bacterial (*E. coli*) cells on (a) unmodified membrane (control), (b) SWNT coated membrane, and (c) PVK-SWNT coated membrane. Scale: 1 µm (top), 200 µm (bottom).

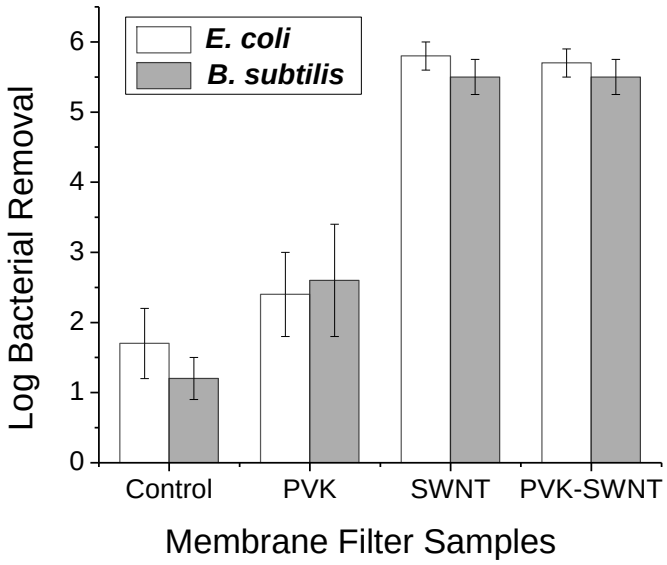

Figure 9. *E. coli* and *B. subtilis* (10^7 CFU/ml) log removal after filtration at constant permeation rate through bare membrane (control) and through PVK, SWNT and PVK-SWNT coated membranes.

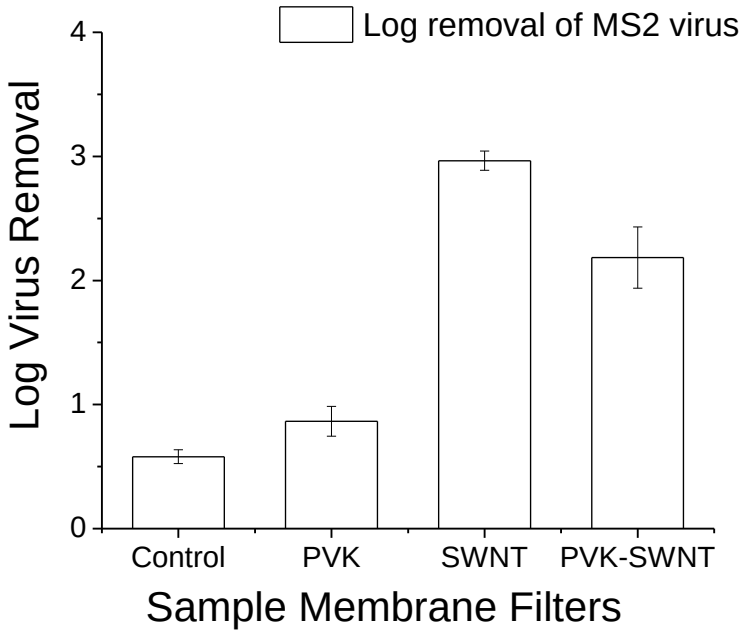

Figure 10. Log removal of MS2 virus ($4.5x10^{11}$ PFU/ml) after filtration at constant permeation rate through bare membrane (control) and through PVK, SWNT, and PVK-SWNT coated membranes.

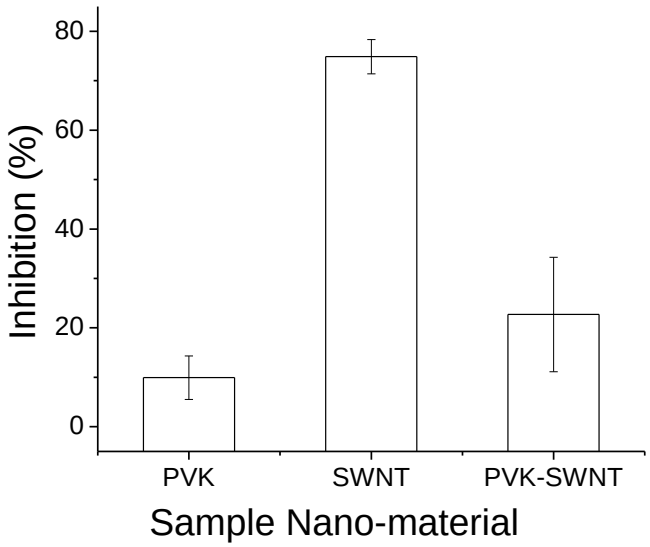

Figure 11. Cytotoxicity of the PVK-SWNT (1000 μg/ml), SWNT (1000 μg/ml), and PVK (1000 μg/ml) solutions against NIH-3T3 Fibroblasts.

Figure file (Supporting Information)

(a) (b)

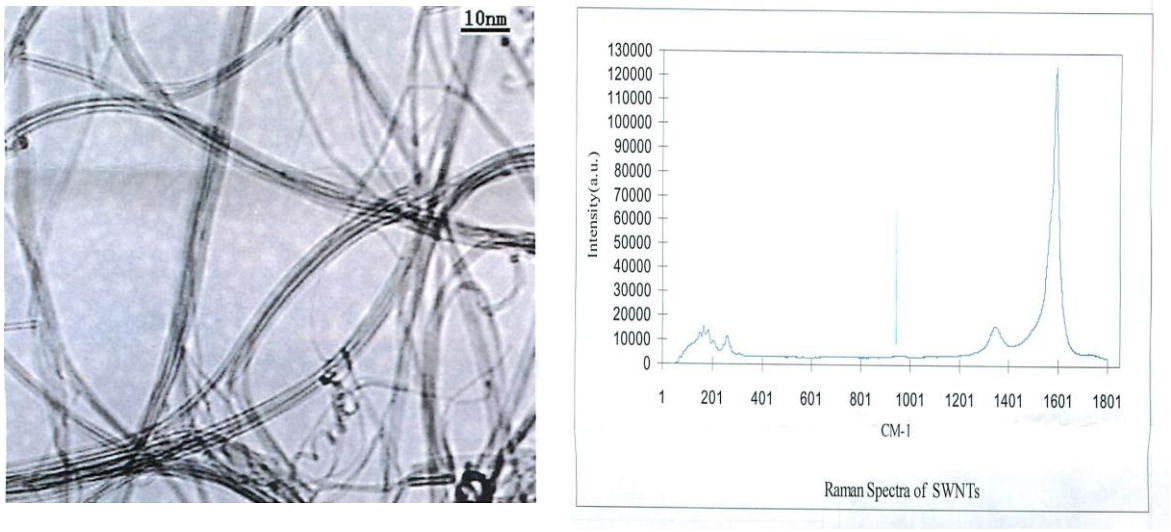

Figure S1. (a) Transmission Electron Microscopy (TEM) image (b) Raman spectra of SWNT supplied by the manufacturer (Cheap Tubes, VT)

Electronic Supplementary Material (for online publication only)

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