Toxicity of Nickel-Silica Nanoparticles and Carbon Nanotubes Against Pseudomonas aeruginosa

B.S Bioengineering

Serena Ogie Evah Omo-Lamai

ABSTRACT

We investigate the antibacterial properties of carbon nanotube adsorbed silk membranes and antibiotic vancomycin loaded nickel-silica nanoparticles. Metal nanoparticles and carbon nanotubes have been shown to produce reactive oxygen species which cause oxidative stress in bacterial cells and lead to cell death. Nickel-silica nanoparticles were synthesized using a one-pot, multi-step reverse microemulsion technique. Carbon nanotubes were synthesized using degummed silk from silkworm cocoons. The antibacterial properties of carbon nanotube membranes were tested by incubating with Pseudomonas aeruginosa and performing fluorescence microscopy. UV Vis spectrophotometry was used to measure vancomycin loading of nickel silica nanoparticles. The results showed that the carbon nanotube membranes had a larger proportion of dead bacterial cells than the pure silk membranes which proves that they are antibacterial. It was also shown that drug loading of nickel silica nanoparticles is dependent on pH and is lowest at alkaline values.

Index Terms – Carbon nanotubes, Nickel-silica Nanoparticles, Silk membranes, Antibacterial

EXECUTIVE SUMMARY

One objective of this project is to investigate whether core-shell nickel-silica nanoparticles loaded with vancomycin will yield an enhanced antimicrobial effect compared to nanoparticles or antibiotics alone and to determine the effect of pH on drug release. The silica forms the shell because it is biocompatible and non-toxic. Another objective of this project is to determine whether adsorbing carbon nanotubes onto a silk membrane will inhibit bacterial growth.

Nickel-silica nanoparticles were synthesized using a one-pot, multi-step reverse microemulsion technique. Using spectrophotometry, the calibration curve of concentration against absorbance for vancomycin were generated. This was accomplished by measuring the optical densities of different vancomycin solutions with varying concentrations. An optimum concentration of vancomycin to ensure maximum loading of the nanoparticles was calculated. The optical density of the vancomycin solutions with different pH values were also measured using spectrophotometry. The corresponding vancomycin concentrations were obtained using the calibration curve. This determined whether there was binding between the nanoparticles and the drug and if the level of binding and drug release was affected by pH. Growth inhibition experiments of *Pseudomonas aeruginosa* bacteria were carried out to test the effects of vancomycin loaded Ni@SiO_2 nanoparticle concentration on bacterial growth inhibition. Nanoparticles were serially diluted in a 96-well plate and exponential growth phase colony forming units were added. The plate was then shaken and the optical density at 600nm was measured at hourly intervals from 0 hours to 8 hours. The interactions of the nanoparticles with bacteria were also analyzed with scanning electron microscopy. Carbon nanotube silk membranes were synthesized using degummed silk from silkworm cocoons. *Pseudomonas aeruginosa* bacterial biofilms were grown on both silk and carbon nanotube silk membranes and then incubated. The membranes were both stained with Propidium Iodide and SYTO 9 and a live/dead cell viability assay was carried out with fluorescence imaging. UV Vis spectrophotometry

showed that the vancomycin loading of nickel-silica nanoparticles is pH dependent. Drug loading is higher at neutral pH and lower at alkaline pH values.

In conclusion, fluorescent imaging of CNT adsorbed silk and pure silk membranes incubated with *Pseudomonas aeruginosa* for different time intervals showed that there is substantial killing caused by the carbon nanotube adsorbed silk membranes as compared with the pure silk membranes. UV Vis spectrophotometry showed that the vancomycin loading of nickel-silica nanoparticles is pH dependent. Drug loading is higher at neutral pH and lower at alkaline pH values. This research project is a step towards tackling antibiotic resistance. By binding antibiotics to nanoparticles, this project aims to develop a more potent method of killing bacteria. It also aims to reduce bacterial infections relating to wound dressings by investing carbon nanotube silk membranes as potential wound dressing candidates. The long-term impact of this research work is a reduction in antibiotic-resistant infections.

TABLE OF CONTENTS

Toxicity of Nickel-Silica Nanoparticles and Carbon Nanotubes Against *Pseudomonas aeruginosa*

Serena Omo-Lamai[1], *Tagbo Niepa*[2]

[1]Biomedical and Chemical Engineering Department, Syracuse University, Syracuse, NY, 13244

[2]Department of Chemical and Petroleum Engineering, University of Pittsburgh, Pittsburgh, PA, 15213

INTRODUCTION

Antibiotic resistance is a phenomenon that occurs when bacteria develop the ability to resist the effects of an antibiotic. In this process, bacteria change their cell structures to make them less permeable to drugs or other chemicals intended to treat or prevent infections. Antibiotic resistance is a huge threat to global health today because infections such as pneumonia and tuberculosis are becoming increasingly harder to treat due to a decrease in the efficacy of antibiotics (WHO, 2018). Antibiotic bacteria strains affect approximately 2 million people each year in the United States and this has led to at least 23,000 deaths annually (CDC, 2018). Additionally, the number of novel antibiotics being developed has plummeted in recent years as shown in Figure 1 (CDC, 2018). Increasing antibiotic-resistant infections and declining antibiotic discovery make it necessary to develop alternative antimicrobial agents.

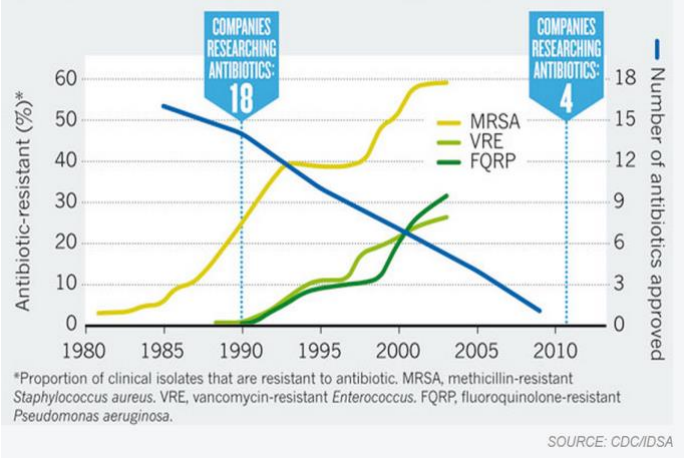


FIGURE 1: GRAPH SHOWING COMPANIES RESEARCHING ANTIBIOTICS, PERCENTAGE OF RESISTANT BACTERIA STRAINS AND NUMBER OF

Nanoparticles and thus have high surface area to volume ratios (Fu et al, 2014). In addition, metal nanoparticles possess the ability to release reactive oxygen species and free radicals such as superoxide radicals, hydroxyl radicals and hydrogen peroxide (Fu et al, 2014). These characteristics allow nanoparticles to damage the structure of bacterial cell membranes and destroy proteins and DNA. Previous studies have demonstrated the antimicrobial properties of gold colloid nanoparticles and silver nanoparticles (Burygin et al, 2009 and Hwang et al, 2012). However, to our knowledge, no previous studies have investigated the antimicrobial effects of core shell nickel-silica nanoparticles combined with antibiotics. The use of nanoparticles has great potential to combat antibiotic resistance when used in conjunction with antibiotics (Pinto- Alphandary et al, 2000). Core-shell nickel-silica nanoparticles are especially attractive because of their biocompatible outer silica shells and antibacterial inner nickel cores.

Antibiotic resistance has also affected the area of wound and burn healing. Wounds usually provide a conducive environment for the growth of microbes, slowing down healing and leading to infections. There is increasing concern that conventional wound dressings containing silver could generate silver resistant bacteria (Percival et al, 2004). Therefore, improved antimicrobial wound dressings are needed to facilitate wound healing. Carbon nanotube adsorbed silk membranes provide a good alternative to Ag-containing wound dressings due to the superior biocompatibility and unique mechanical properties of silk (Kang et al, 2007 and Wang et al, 2016). Preceding research works have demonstrated the cytotoxicity of carbon nanotubes against bacterial cells (Vecitis et al, 2010).

The objective of this study is to develop carbon nanotube adsorbed silk membranes and test their antibacterial effects against *Pseudomonas aeruginosa* bacteria. Another objective is to investigate the combined antibacterial effects of antibiotic vancomycin loaded nickel-silica nanoparticles against *Pseudomonas aeruginosa* bacteria.

This study hypothesizes that antibiotic loaded nanoparticles will have a more potent effect against bacteria. It also predicts that carbon nanotube adsorbed silk membranes also have antibacterial effects.

METHODS

Synthesis and Preparation of Nickel-Silica Nanoparticles

Nickel-silica nanoparticles ($Ni@SiO_2$ NPs) were synthesized using a one-pot, multi-step reverse microemulsion technique as reported previously (Mahoney et al, 2016). The prepared nanoparticles were then sterilized at 200°C in a vacuum oven overnight. Immediately preceding use, $Ni@SiO_2$ nanoparticles were dispersed in ultra-pure water at a concentration of 1mg/ml and probe sonicated for ten minutes at 90% pulsing and mid-level power.

Synthesis of Carbon Nanotube Membranes

The carbon nanotube (CNT) adsorbed silk membranes used in this experiment were synthesized and supplied by the Nano Product Lab at the University of Pittsburgh. The CNT silk membranes were synthesized via a degumming, dissolving, dialysis and filtration technique as outlined in Figure 2. Solid carbon nanotubes were then added to the regenerated silk fibroin solution. The thickness of the membranes ranged from 30-40μm. The membranes were synthesized with 4g of 5% or 5.1% silk and different concentrations of carbon nanotubes.

Bacteria and Medium

The Pseudomonas *aeruginosa* strain used in this experiment was from a frozen suspension of cells obtained from the University of Pittsburgh and was grown in Luria Bertani broth. The day before an

experiment, single colonies of P. aeruginosa stored at -80°C were transferred into 25ml of LB medium and incubated for 14-18 hours at 37°C in an orbital shaker at 212rpm.

Imaging

Live/dead cell viability assays were carried out on pure silk and carbon nanotube adsorbed silk membranes. Samples of each membrane were first incubated with PAO1 bacteria in 3ml of LB medium for 3 hours, 6 hours, 12 hours and 24 hours. The membranes were then stained in individual solutions containing 1ml of 154mM NaCl, 1.5μL of Propidium Iodide dye and 1.5μL of SYTO 9 dye (Thermo Scientific Lite Technologies Corporation) for 15 minutes. A Zeiss fluorescence microscope was then used to view the stained samples.

Cell Viability Experiments

The effects of NH_2 functionalized carbon nanotubes on stationary phase cell viability were measured after 1 hour, 3 hour and 24 hour exposures. Solid NH_2 functionalized carbon nanotubes were dispersed in ultra-pure water at 1mg/ml and probe sonicated for 10 minutes at 90% pulsing and mid-level power. After 14-18 hours of inoculation, an overnight culture was washed once in 30ml of 154mM NaCl and then resuspended in 20ml NaCl. Spectrophotometry was used to measure the optical density at 600nm of the sample and the solution was diluted to obtain an optical density value between 0.6 and 0.7. The bacterial solution was exposed to carbon nanotubes at different concentrations of 1, 2, 5, 10, and 100μg/ml. This experiment was repeated for exposure times of 1 hour, 3 hours and 24 hours. After exposure to respective CNT concentrations for different time intervals, the treated bacterial solutions were suspended within 96-well plates and six replications of each condition were made. Twenty microliters from each well were serially diluted six times in 154mM NaCl before being drop plated on LB agar plates and incubated at 37°C overnight. Colony forming units were counted 19-20 hours later.

Quantifying Vancomycin Concentrations with Absorbance

Vancomycin stock solutions were prepared with concentrations of 50mg/ml and were 0.22-μm filter sterilized. Vancomycin calibration curves of concentration against absorbance were generated by serially diluting a 1ml solution of vancomycin at 250μg/ml and using UV Vis spectrophotometry to measure the absorbance at 286nm for each resulting solution. Calibration curves were also generated at pH 3.188 and pH 12.724. To measure the effect of pH on the optical density of vancomycin, eight solutions were prepared with concentrations of 250μg/ml. The pH values were then varied using 1M NaCl and 1M NaOH and measured with a pH probe (Orion ROSS Ultra Semi-Micro pH) to obtain pH values ranging from 1.38 – 12.31. A UV Vis spectrophotometer (Evolution 260 Bio) was then used to measure the optical density of the prepared solutions. The effect of pH on the vancomycin loading of nickel silica nanoparticles was also investigated. Nine 1ml nanoparticle solutions were prepared and a pH probe was used to obtain pH values ranging from 0.78 to 12.53. Vancomycin was added in each solution at 250μg/ml and the solutions were vortexed and incubated at 37°C for an hour. The solutions were centrifuged at 6000rpm for 10 minutes to remove the nanoparticles from suspension. Absorbance values were then measured with a spectrophotometer.

RESULTS

The prepared nickel-silica nanoparticles used in this experiment are shown in Figure 3a. An annular dark field scanning transmission electron microscopy image taken of the nanoparticle sample as seen in Figure 3b shows that the Ni nanoparticles are mostly encapsulated by the SiO_2 nanoparticles. As shown in Figure 4a, the 4g 5.1% silk membranes were transparent. Figure 4c shows that the COOH and NH_2 functionalized carbon nanotube membranes are darker in color. The 0.1% carbon nanotube membranes were the darkest

in color since they were the most concentrated as seen in Figure 4b. The vancomycin calibration curve generated in this experiment at pH 6.8 is shown in Figure 5a and it has an R_2 value of 0.99535. Figures 5b and 5c show the calibration curves of vancomycin in acidic conditions at pH 3.19 and basic conditions at pH 12.72. The R_2 values of these graphs are 0.99856 and 0.98168 respectively. Absorbance values are higher in alkaline conditions.

UV Vis spectrophotometry experiments showed that as the pH was increased from 1.38 to 12.34, the absorbance of vancomycin also increased as seen in Figure 6. It was also shown that an increase in pH generally led to a decrease in vancomycin loading by nickel silica nanoparticles. Figure 7 illustrates that there was a rapid increase in drug loading when the pH value was 2.38.

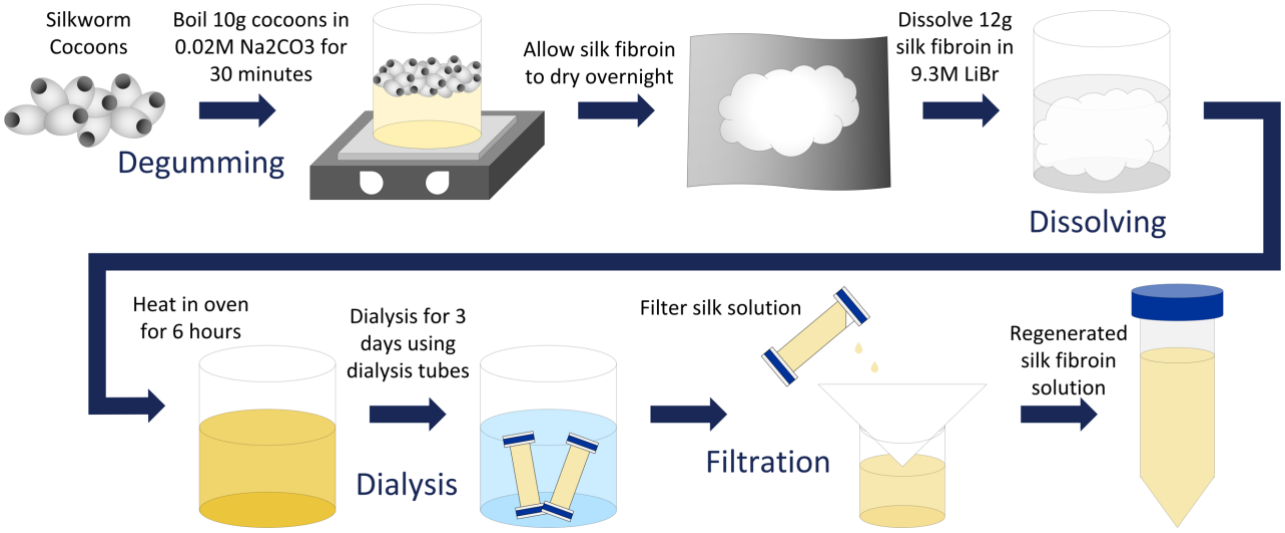


FIGURE. 3 PROCEDURE FOR SYNTHESIS OF CNT ADSORBED SILK MEMBRANES

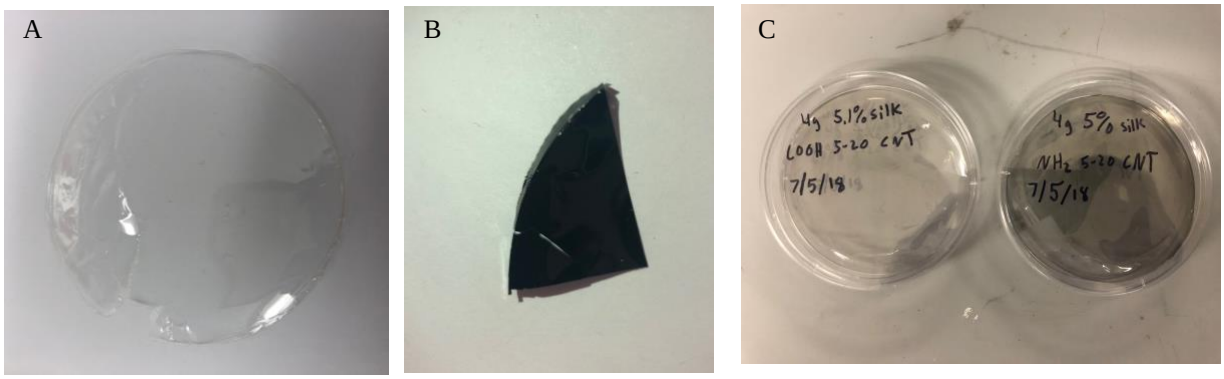
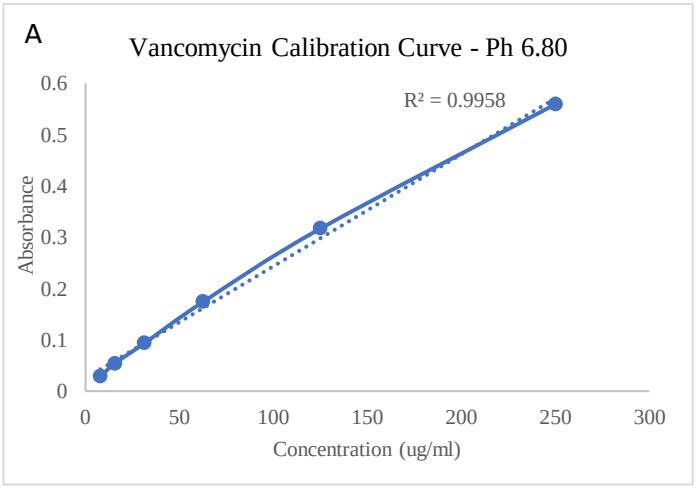
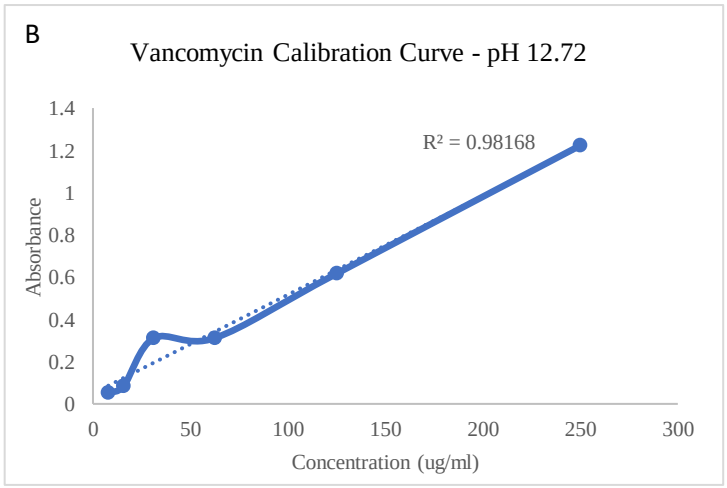


FIGURE. 4 OPTICAL PICTURES OF (A) PURE SILK MEMBRANE (B) 0.1 CNT ADSORBED SILK MEMBRANE (C) NH_2 AND COOH





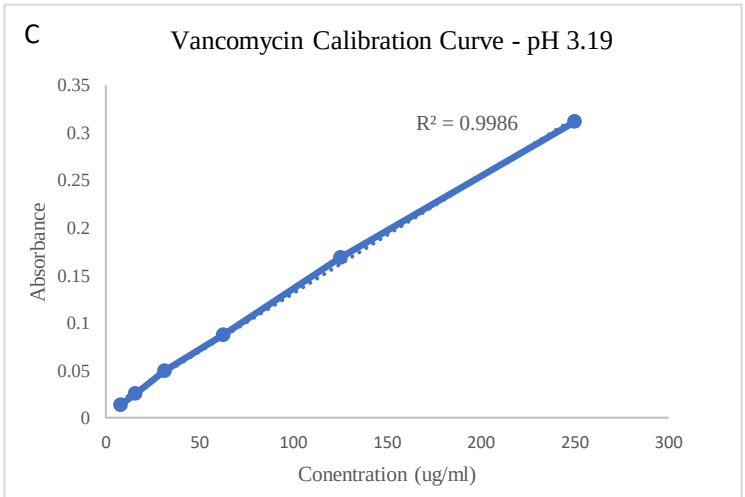


FIGURE. 5 VANCOMYCIN CALIBRATION CURVE AT (A) PH 6.8 (B) PH 12.72 (C) PH 3.188

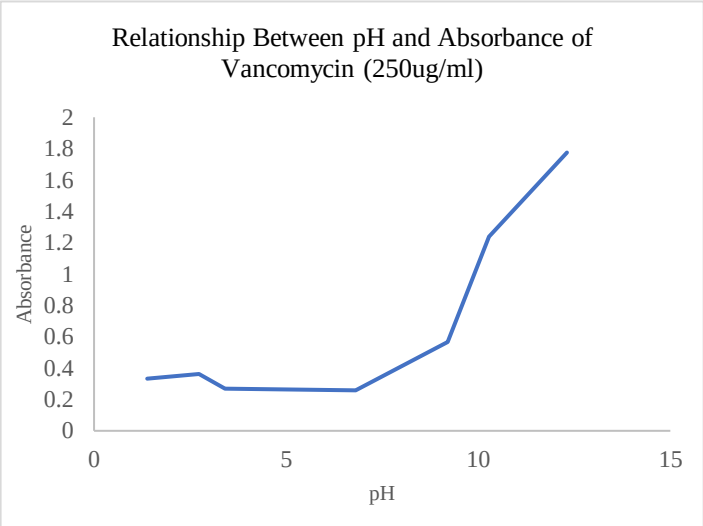


FIGURE. 6 RELATIONSHIP BETWEEN PH AND VANCOMYCIN ABSORBANCE

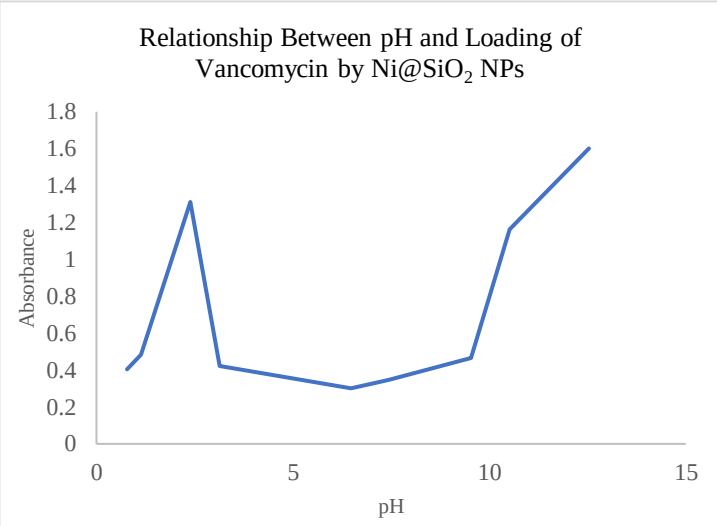


FIGURE. 7 RELATIONSHIP BETWEEN PH AND DRUG LOADING OF NI@SIO2 NPS

Figures 8 a-d show the fluorescence images taken of the CNT and pure silk membranes after incubation for set time intervals and staining with Propidium Iodide and SYTO 9. The images were then split into their live and dead components to compare the fraction of live cells to that of dead cells.

DISCUSSION

Annular dark field scanning transmission electron microscopy images shown in Figure 10 show that the nickel-silica nanoparticles are hollow in nature since the nickel nanoparticles particles are contained within the silica dioxide nanoparticles. This is beneficial because silicon dioxide is non-toxic and biocompatible. Thus, silicon dioxide nanoparticles create a layer around the toxic, antibacterial nickel nanoparticles. Therefore, Ni@SiO_2 nanoparticles can safely come into contact with the human body without the release of harmful substances. This makes them suitable for biomedical applications.

The calibration curves of vancomycin at pH values of 3.19, 6.80, and 12.72 were between 0.98168 and 0.99856. This indicates a that the calibration curves had high accuracy levels. The results also demonstrate that the loading of vancomycin by nickel silica nanoparticles is pH dependent. Absorbance is highest at alkaline pH values approaching 12 and is lowest at neutral pH values approaching 7. This means that under very alkaline conditions, there is more vancomycin in solution and thus there is less vancomycin being loaded into the nickel silica nanoparticles. Under neutral conditions, there is less vancomycin in solution which means that there is maximum vancomycin being loaded into the nickel silica nanoparticles. Therefore, drug loading of nickel silica nanoparticles is optimum at pH 6.48.

In this experiment, fluorescent staining was carried out using Propidium Iodide and SYTO 9 dyes. Propidium Iodide is a red fluorescent dye. It is unable to cross live cell membranes and it binds to double stranded DNA. SYTO 9 is a green fluorescent dye that is permeant to both prokaryotic and eukaryotic cell membranes. Carrying out a live/dead cell viability assay with these dyes is an effective method of detecting the amount of cell killing caused by the CNT adsorbed silk membranes because the fraction of live and dead cells after incubation with bacteria can be quantified. The fluorescence images in Figures 8 a-d demonstrate that after 3h, 6h, and 24h of incubation with *Pseudomonas aeruginosa*, the pure silk membranes contain mostly live bacterial cells which indicates very little cell killing. However, after 3h, 6h, and 24h of incubation, the CNT adsorbed membranes demonstrate substantial cell killing. The 3D image in Figure 11 represents a cross section of the CNT adsorbed membrane after 24 hours of incubation. It illustrates that most of the cell killing occurs on the bottommost layer closest to the CNT membrane. This indicates that bacterial cell killing is due to the release of carbon nanotubes from the CNT adsorbed silk membrane. There appears to be optimum bacterial cell killing at 6h. This could be because at 24 hours of incubation, bacterial biofilms begin to grow over the CNT membrane and this could form a barrier over

the carbon nanotubes released. At 3 hours of incubation, the exposure of the bacterial cells to the antibacterial carbon nanotubes has not been maximized.

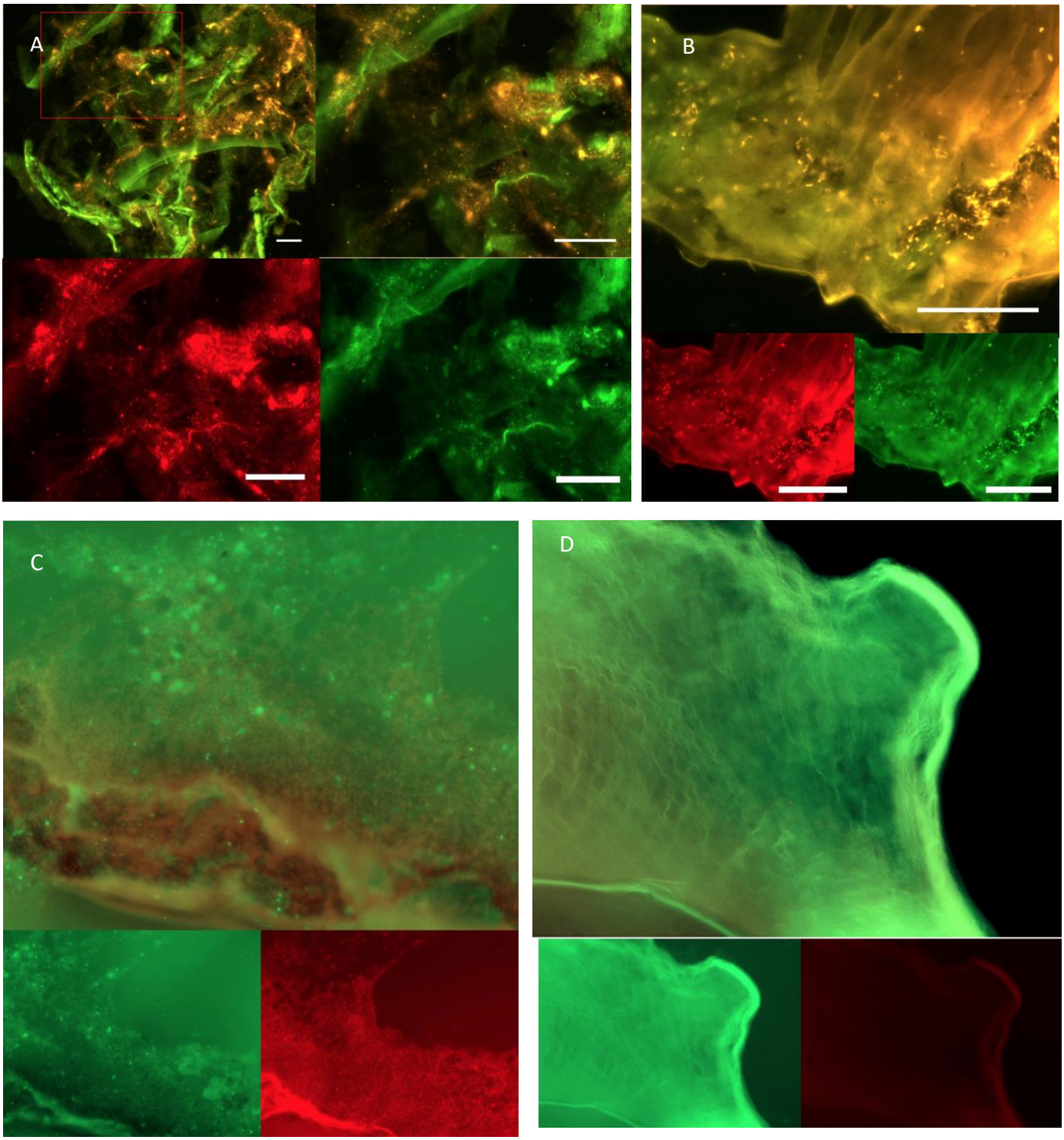


FIGURE. 8 FLUORESCENCE IMAGES OF (A) CNT MEMBRANES AFTER 3H INCUBATION (B) CNT MEMBRANES AFTER 6H INCUBATION (C) CNT MEMBRANES AFTER 24H INCUBATION AND (D)PURE SILK MEMBRANE AFTER 24H INCUBATION

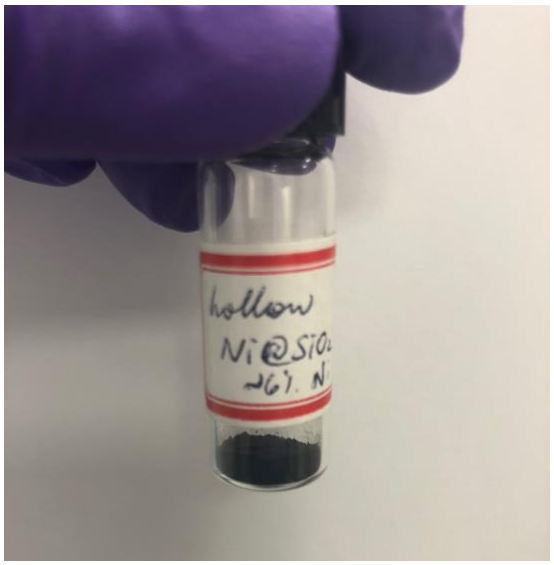


FIGURE. 9 SYNTHESIZED HOLLOW NI@SiO_2 NPS

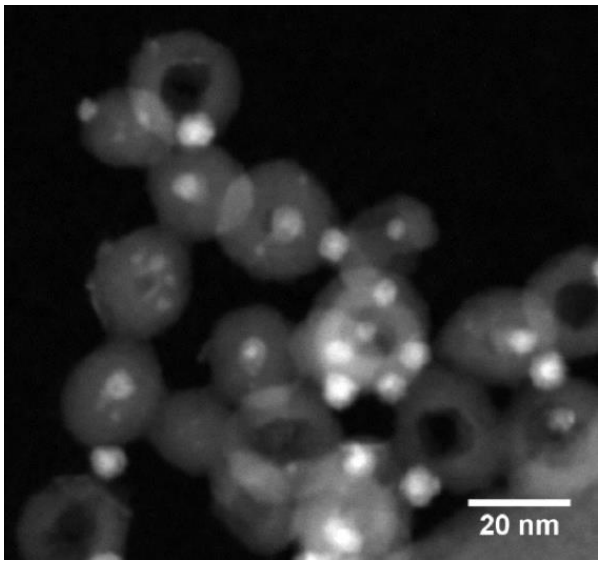


FIGURE. 10 ADF-STEM IMAGING OF SYNTHESIZED

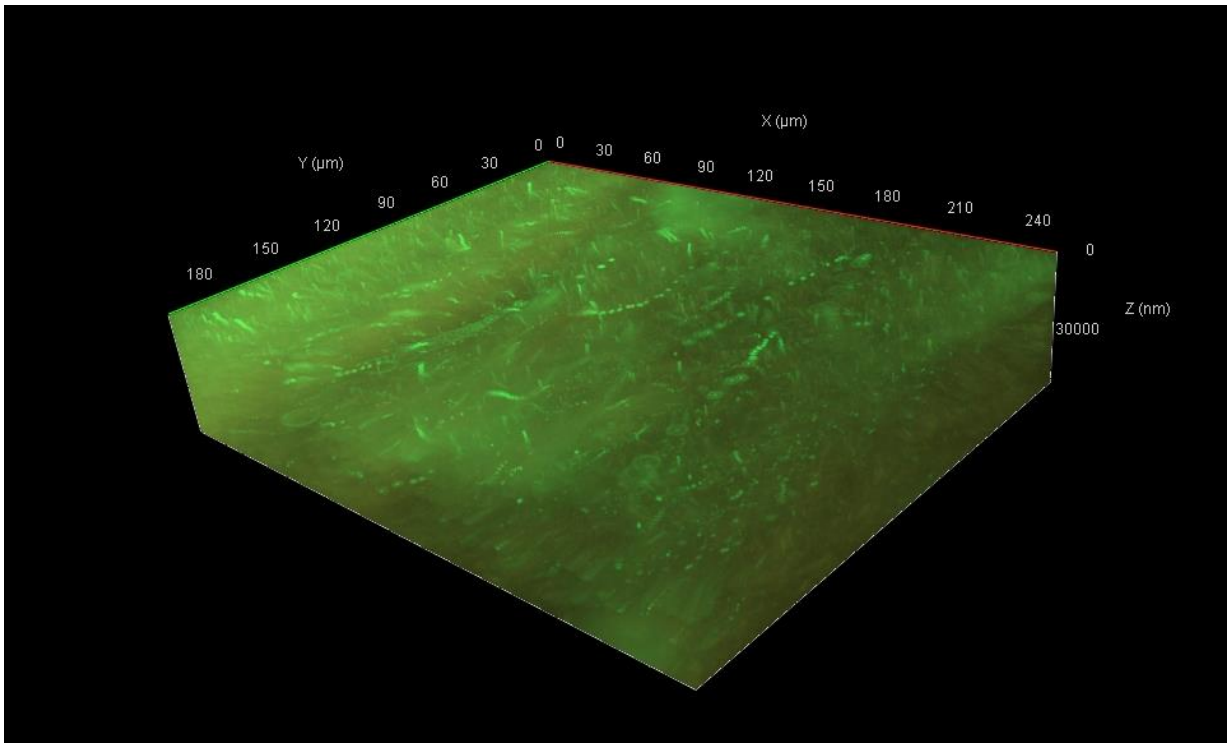


FIGURE 11: 3D IMAGE OF CNT SILK MEMBRANE AFTER 24H INCUBATION

CONCLUSION

In this experiment, we have been able to synthesize hollow nickel nanoparticles using a one-pot multi step reverse microemulsion technique. Carbon nanotube adsorbed silk membranes were also synthesized from degummed silkworm cocoons. UV Vis spectrophotometry showed that the vancomycin loading of nickel-silica nanoparticles is pH dependent. Drug loading is higher at neutral pH and lower at alkaline pH values.

Fluorescent imaging of CNT adsorbed silk and pure silk membranes incubated with *Pseudomonas aeruginosa* for different time intervals showed that there is substantial killing caused by the carbon nanotube adsorbed silk membranes as compared with the pure silk membranes. Analyzng 3D imaging showed that cell killing was highest on the bottommost layer closest to the CNT membrane which proves that cell killing was due to the release of carbon nanotubes. Thus, carbon nanotube silk membranes and nickel silica nanoparticles can have a number of antibacterial biomedical applications such as wound dressings due to their bacterial cell toxicity and the biocompatibility resulting from the presence of silk and silica. For further testing, cell viability assays using colony forming unit experiments need to be conducted for both the nickel silica nanoparticles and the carbon nanotube adsorbed silk.

REFERENCES

Burygin, G., Khlebtsov, B., Shantrokha, A., Dykman, L., Bogatyrev, V. and Khlebtsov, N. (2009). On the Enhanced Antibacterial Activity of Antibiotics Mixed with Gold Nanoparticles. Nanoscale Research Letters, 4(8), pp.794-801.

Cdc.gov. (2018). Antibiotic / Antimicrobial Resistance [online] Available at: https://www.cdc.gov/drugresistance/index.html [Accessed 12 Jun. 2018].

Cooper, M. and Shlaes, D. (2011). Fix the antibiotics pipeline. *Nature*, 472(7341), pp.32-32.

Costerton, J. (1995). Mechanism of Electrical Enhancement of Efficacy of Antibiotics in Killing Biofilm Bacteria. The Pediatric Infectious Disease Journal, 14(6), p.556.

Fu, P., Xia, Q., Hwang, H., Ray, P. and Yu, H. (2014). Mechanisms of nanotoxicity: Generation of reactive oxygen species. Journal of Food and Drug Analysis, 22(1), pp.64-75.

Hwang, I., Hwang, J., Choi, H., Kim, K. and Lee, D. (2012). Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved. Journal of Medical Microbiology, 61(Pt_12), pp.1719-1726

Jung, R., Kim, H., Kim, Y., Kwon, S., Lee, H. and Jin, H. (2008). Electrically conductive transparent papers using multiwalled carbon nanotubes. Journal of Polymer Science Part B: Polymer Physics, 46(12), pp.1235-1242

Kang, M. and Jin, H. (2007). Electrically conducting electrospun silk membranes fabricated by adsorption of carbon nanotubes. Colloid and Polymer Science, 285(10), pp.1163-1167.

Kim, H., Kang, M., Jin, H., Chin, I., Choi, H. and Joo, J. (2007). Carbon Nanotube-Organized Polymeric Fibers and Measurement of Their Electrical Conductivity. Molecular Crystals and Liquid Crystals, 464(1), pp.15/[597]-21/[603].

Mahoney, S., Najera, M., Bai, Q., Burton, E. and Veser, G. (2016). The Developmental Toxicity of Complex Silica-Embedded Nickel Nanoparticles Is Determined by Their Physicochemical Properties. *PLOS ONE*, 11(3), p.e0152010.

Percival, S. and Bowler, P. (2004). Bacterial Resistance to Silver in Wound Care. Journal of Wound, Ostomy and Continence Nursing, 31(Supplement), p.S29

Pinto-Alphandary, H., Andremont, A. and Couvreur, P. (2000). Targeted delivery of antibiotics using liposomes and nanoparticles: research and applications. International Journal of Antimicrobial Agents, 13(3), pp.155-168.

Vecitis, C., Zodrow, K., Kang, S. and Elimelech, M. (2010). Electronic-Structure-Dependent Bacterial Cytotoxicity of Single-Walled Carbon Nanotubes. ACS Nano, 4(9), pp.5471-5479.

World Health Organization. (2018). Antibiotic resistance. [online] Available at:http://www.who.int/newsroom/factsheets/detail/antibiotic-resistance [Accessed 12 Jun. 2018].

Wang, Q., Wang, C., Zhang, M., Jian, M. and Zhang, Y. (2016). Feeding Single-Walled Carbon Nanotubes or Graphene to Silkworms for Reinforced Silk Fibers. Nano Letters, 16(10), pp.6695-6700.