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Uptake of Nanoparticles by *Vibrio Gazogenes*

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UPTAKE OF NANOPARTICLES BY *VIBRIO GAZOGENES*

by

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ABSTRACT

Vibrio gazogenes, a Gram-negative species of marine bacteria, was capable of transporting 20 nm (diameter) surface-carboxylated, polystyrene, fluorescent FluoSpheres® microspheres (excitation/emission = 505/515) through the outer membrane, which was indicated by a detectable decrease in the fluorescence intensity of the nanoparticles in the culture medium. A mechanism of transport was investigated involving the ATP-binding cassette (ABC) protein transporters that traverse the outer membrane. Inhibition of the ABC transporters did not prevent the entry of the nanoparticles into the cell, suggesting there was an alternate mechanism of transport. The addition of nanoparticles to the culture medium also did not provide any growth benefits for *Vibrio gazogenes*.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
Glu.....	Glucose
h.....	hours
KCl.....	Potassium Chloride
MB	Marine Broth
mL.....	Milliliter
mM.....	Millimolar
nm	Nanometer
OD.....	Optical Density
rpm	Revolutions per Minute
secs.....	Seconds
Suc.....	Sucrose
μ L.....	Microliter

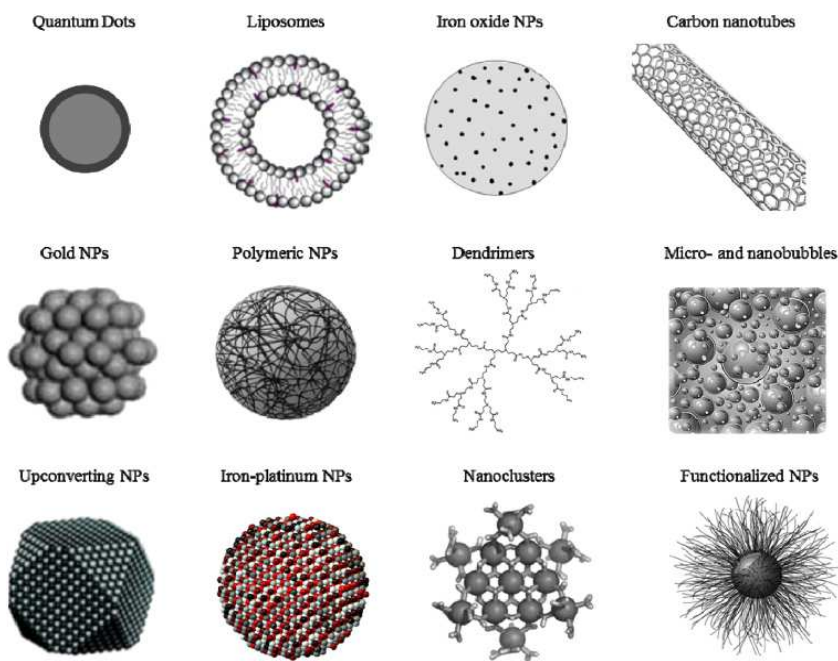
CHAPTER 1

INTRODUCTION

Nanomedicine

Nanotechnology is a rapidly developing field with diverse applications and has emerged as a hot topic in the scientific community. Emphasis within this field is placed on engineering and utilizing nanoscale materials to function in more-efficient and -effective capacities than macroscale materials. One discipline exploring the possibilities and potential of nanotechnology is nanomedicine. Nanomedicine combines traditional therapeutic concepts with current nanotechnological advancements to create novel approaches for the screening, diagnosis, and treatment of infections and diseases. The novelty of nanomedicine lies in the unique ability to modify the physicochemical properties of the nanomaterials (nanoparticles). Nanoparticles have traditionally been defined as being 1 to 100 nm in size in at least one dimension (ASTM 2006); however, newer definitions are inclusive of materials up to 1,000 nm in size (Wagner et al 2006). Complex engineering can impart specific optical, magnetic, electronic, and biological properties to the nanoparticles, as well as, certain shapes, sizes, surface chemistries, and chemical compositions (Kim et al 2010) (Figure 1.1). Modifiable characteristics allow the nanoparticles to be finely tuned to target specific components of biological systems at the atomic and molecular level. It is believed that nanoparticle-based therapies will have a

significant impact on healthcare in the future by providing effective diagnostics and treatments that will improve the quality of life, extend life expectancies, and ultimately lower the overall cost of healthcare worldwide (Pautler and Brenner 2010). One area where the innovation provided by nanomedicine is desperately needed is in the treatment of antibiotic-resistant bacterial infections, which are responsible for the largest number of deaths worldwide for adults under the age of 60 (Fauci et al 2005).



Re F, Moresco R, Masserini M. 2012. Nanoparticles for neuroimaging. *J Phys D: Appl Phys* 45(7): 073001 (12 pp). ©IOP Publishing. Reproduced with permission. All rights reserved.

Figure 1.1 Different types of Nanoparticles

Creation of Superbugs

An estimated 4 to 5 billion dollars is spent yearly treating infections caused by antibiotic-resistant bacteria (Fauci et al 2005). These ‘superbugs’ have developed highly evolved resistance mechanisms over time in response to their overexposure to large

quantities of natural and synthetic antibiotics. The two major factors attributed to the creation of superbugs are antibiotic overuse and misuse. According to the CDC, three billion prescriptions for antibiotics are written annually by clinicians to treat infections, and at least fifty percent of these are incorrectly prescribed (CDC 2010). In addition, many patients are non-compliant when being treated with antibiotics and may even use previously prescribed antibiotics to treat subsequent illnesses (Reed et al 2002). In developing countries, lack of regulation allows people to purchase antibiotics to treat self-diagnosed illnesses (Vento and Cainelli 2010). Another major issue is the feeding of massive quantities of antibiotics to livestock by animal husbandry companies in order to promote growth, exposing consumers to antibiotics on a regular basis (Singer et al 2003). Due to these factors, antibiotic-resistant bacteria traditionally seen in clinical settings are now migrating into the global community, creating a major public health crisis (Cars et al 2011).

Biofilm Formation

Many multi-drug resistant bacterial strains are difficult to treat due to their ability to form biofilms, which confer strong antibiotic resistance properties to the community of bacterial cells within the biofilm. The formation of the biofilm state occurs after exposure to environmental stressors that trigger a series of physiological changes facilitating bacterial growth and survival in hostile environments (Costerton et al 1995) (Figure 1.2). Once the bacteria transition to the biofilm state, they become 10-1,000 times more resistant to the effects of antimicrobials (Morton et al 1998).

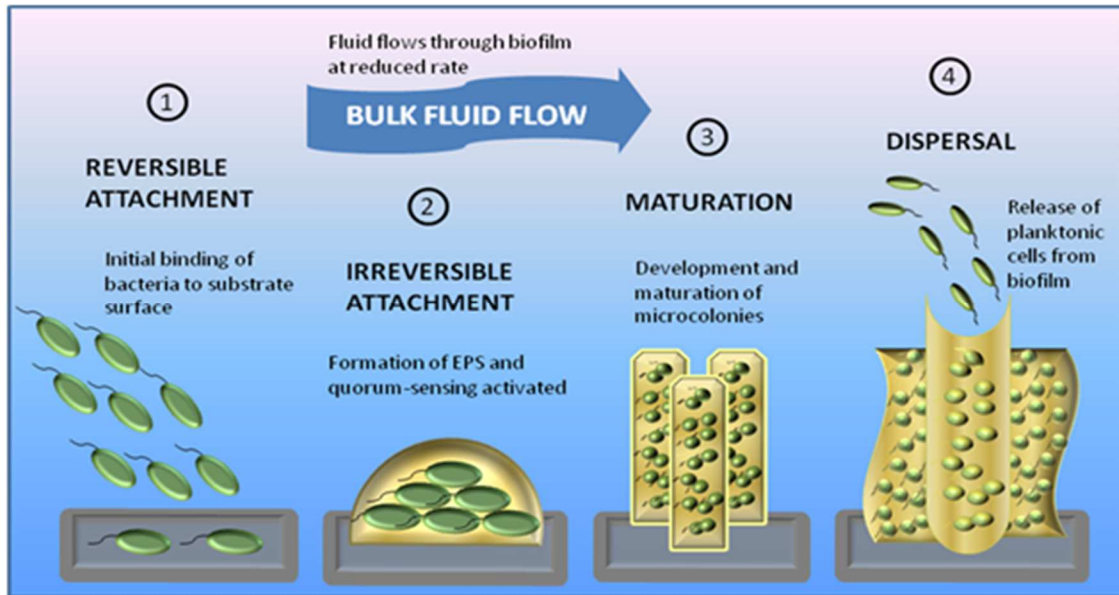


Figure 1.2 Biofilm Formation

Antibiotic Resistance in Biofilm

Increased antibiotic resistance in the biofilm state is mainly the result of the following factors: restricted antimicrobial penetration, spatial and population heterogeneity, quorum-sensing systems, enhanced exchange of resistance genes, and multi-drug efflux pumps. Restricted antimicrobial penetration is an effect generated by the formation of an exopolysaccharide matrix and reduced fluid flow (diffusion limitation) within the biofilms (Morton et al 1998). The exopolysaccharide matrix either prevents or significantly reduces the entry of antimicrobials into the biofilms, while diffusion limitation lowers their rate of transport through the biofilms (Costerton et al 1995). Spatial and population heterogeneity describe the variation in concentrations of metabolic substrates and by-products as well as differences in metabolic activity among the bacteria (Morton et al 1998). Bacterial cells in slow growing or inactive states (persisters) are able to resist treatment because antibiotics tend to target actively growing cells (Singh et al 2009). Persisters can become active after treatment, leading to reinfection, long-term colonization, and persistent infections.

Quorum-sensing systems allow the bacteria to communicate with each other through chemical signaling once a certain cell concentration is reached during biofilm formation (Nadell et al 2008). After the quorum has been achieved, the bacteria are able to coordinate their activities and alter their gene expression in response to any perceived threats, making them less susceptible to antimicrobials. The enhanced exchange of resistance genes amongst bacteria within the biofilm helps to provide antibiotic resistance to the biofilm as a whole. Multi-drug efflux pumps facilitate the export of antibiotics from the interior of the biofilm cells (McKeegan et al 2002). The combination of these resistance factors within the biofilm have rendered several potent antibiotics useless thereby creating the need for the development of new treatment methods that will increase the efficacy of currently available antibiotics by allowing them to travel efficiently through the biofilm and overcome the resistance mechanisms.

Antibiotic Discovery and Development

A significant issue in combating the problem of antibiotic-resistance is the lack of progress in antibiotic discovery and development. The majority of antibiotics in use today were discovered during the “golden age,” which spanned from the 1940s to the 1960s (Fabbretti et al 2011). Since this period, one antibiotic (doripenem) has been introduced and only three new compounds are in advanced clinical development (Boucher et al 2009). Even more discouraging is the fact that only 5 of the largest pharmaceutical companies currently have active antibiotic discovery programs (Boucher et al 2009). Smaller biotechnology companies capable of doing the research are unable to continue funding the development projects because they rely heavily on unstable venture capital to support their work (Fabbretti et al 2011). FDA approval of new antibiotics was also reduced by 75%

from 1983 to 2007, and the numbers continue to decline (Boucher et al 2009). This innovation gap led the Infectious Diseases Society of America to propose the 10 x '20 Initiative in 2009, which asked U.S. and European Union companies to develop ten new antibiotics within the next 10 years (IDSA 2010).

Nanomedicinal Therapies

The development of nanomedicinal therapies to assist in the fight against antibiotic-resistant strains is rapidly gaining momentum. Many papers have shown the ability of silver and gold nanoparticles as well as liposomes to treat drug-resistant bacteria (Couvreur et al 1991; Lara et al 2010; Ray et al 2013). Nanoparticles are capable of binding to bacterial membrane receptors and overcoming resistance mechanisms in planktonic cells and biofilms, making them exceptional carriers for drug delivery (Kumar et al 201; Bae et al 2011; Feng et al 2000; Rai et al 2009; Taylor and Webster 2009; Raghupathi et al 2011). The unique ability of nanoparticles to be physically- and chemically-modified gives researchers the ability to design nanoparticles with functional groups attached to the surface that act as ligands capable of bonding thousands of antibiotic molecules, effectively concentrating the dosage (Taylor and Webster 2011; Wang 2015). The delivery of massive doses of antibiotics to the cell surface could potentially saturate the multi-drug efflux pumps and other counter-mechanisms, rendering them useless (Wang 2014). The large surface-to-volume ratio conferred by the small size of the nanoparticles allows them to be highly diffusive, and therefore, capable of infiltrating biofilms and reaching previously inaccessible targets (Pinto-Alphandary et al 2000). Nanoparticles also behave as catalysts by driving chemical reactions at an elevated rate, which could result in antibiotic-nanoparticle conjugates that are adept at treating planktonic cells or biofilms at a faster

rate, consequently reducing treatment times. While nanomedicinal therapies seem very promising and a favorable alternative to traditional therapies, there is much that is unknown about how the nanoparticles interact with the bacterial cells, especially within bacterial biofilms (Fabrega et al 2009; 2011; Nevius et al 2012). For example, it is unknown whether all bacteria are capable of nanoparticle transport into cells or if the transport mechanisms vary depending on the species. In order to initiate the development of potentially life-saving and ground-breaking therapies, a thorough understanding of nanoparticle uptake and transport mechanisms must be acquired, with the aim of designing methodologies for the effectual delivery of nanoparticles to their intended destinations.

Elucidation of Nanoparticle Transport Systems

For many years, it was believed that particle uptake was solely a eukaryotic process, so the elucidation of nanoparticle transport systems is a relatively new area of study. Of the studies showing the ability of bacteria to transport various types of nanoparticles with diameters less than 80 nm, only a few have actually proposed a transport mechanism (Kumar et al 2011; Stojak et al 2011; Xu et al 2004; Murat et al 2010; Reith et al 2006; Kloepfer et al 2005; Lonhienne et al 2010). These studies also specifically focused on studying the interactions between Gram-negative bacteria and nanoparticles because of the difficulty in treating resistant, Gram-negative bacteria, which is due to the possession of a hydrophobic outer membrane that acts as a barrier against many bactericidal agents and the large number of multi-drug efflux pumps located within their plasma membranes (Morton et al 1998). The information known about basic molecular transport in Gram-negative bacteria is that it generally occurs either through passive or active transport systems;

therefore it is important to determine which of these systems play a role in nanoparticle transport.

Passive transport

Passive transport is a diffusive process; therefore, molecules travel across the semi-permeable plasma membranes by moving down their concentration gradient. This requires no ATP energy from the cell. In some cases, the cell requires a carrier protein to escort the molecules through the plasma membranes (facilitated diffusion) because they are either too large for simple diffusion or not water-soluble. Even though the carrier protein assists in the diffusive process, it still requires no energy input from the cell. Passive diffusion in Gram-negative bacteria, whether simple or facilitated, requires molecules be able to fit through porins located in the outer membrane. Porins are wide, water-filled channels with diameters varying between 0.6-2.3 nm that act as molecular sieves by exhibiting selectivity for molecules of certain sizes and molecular charge (Hancock and Bell 1988; Benz et al 1985). Due to the size-exclusion limits of the porins, it is widely accepted that nanoparticle transport occurs through an active transport system in Gram-negative bacteria (Stojak et al 2011; Mohammed et al 2011).

Active Transport

Active transport requires ATP as an energy source and is the movement of molecules across the semi-permeable plasma membranes against their concentration gradient. Large objects needing to travel over long distances in the cell also require active transport mechanisms to assist in their movement (Mignot and Shaevitz 2008). At the cell surface, active transport involves the use of carrier proteins powered by the generation of ATP energy to pump the molecules into the cell. A major class of active transport proteins that

are found in highly-conserved numbers in Gram-negative bacteria are ATP-binding cassette (ABC) transporters, which are integral transmembrane proteins responsible for transporting chemically-diverse substrates into the cell (Ponte Sucre 2007). ABC transporters are anchored to the membrane through which they traverse by transmembrane domains (Ponte Sucre 2007). These domains also form a pore that is responsible for allowing the passage of transported solutes (Ponte Sucre 2007). As an ATPase, ABC transporters contain ATP-binding domains that hydrolyze ATP to supply energy for the transport of ions and large polypeptides or polysaccharides (Holland and Blight 1999). The extraordinary features of ABC systems are their ability to transport a wide range of compounds and their ability to act as an importer or exporter (Holland and Blight 1999). Due to these abilities and their invariable linkage to a transport protein or protein domain (Holland and Blight 1999), it is reasonable to assume ABC transporters play a potential role in the active transport of nanoparticles for Gram-negative bacteria.

Growth Benefits from Nanoparticles

With the introduction of nanoparticles as a potential alternative treatment for antibiotic-resistant bacteria, it is important to determine how these nanomaterials interact with the bacterial cell during treatment. The phenomenon of increased growth after the addition of nanoparticles to a bacterial culture has been previously documented. In the paper by Ghalamboran and colleagues (Ghalamboran et al 2009), they observed that the addition of magnetite nanoparticles to a culture of *Bradyrhizobium japonicum* resulted in an increase in the growth rate of the cells within the culture. The authors postulated that the increased growth was due to a pH buffering effect imposed by the magnetite nanoparticles, which helped to keep the pH within a favorable neutral range (Ghalamboran 2009). They also

proposed that the nanoparticles were binding and inactivating oxygen scavengers within the media, which increased the oxygen availability during the growth phase (Ghalamboran 2009). Other papers by Merceda et al 2006 and Flores et al 2004 suggest the increase in growth is due to their large specific surface area and capacity to release electrons. Liu et al 2013 postulate that the electrons released by the nanoparticles augment the enzymatic functioning of bacterial outer membrane proteins and may accelerate electron transport chain function, thereby facilitating cell metabolism. Another plausible theory is key nutrients in the culture medium are non-specifically binding to the nanoparticles, causing the cells to take up more nutrients and allowing the augmentation of their growth rate. Several papers have shown the surface of various nanomaterials become covered by different proteins upon entry into biological media, leading to the production of a protein “corona” (Lundqvist et al 2008; Rahman et al 2013; Tenzer et al 2013; Lynch and Dawson 2008; Sahneh et al 2013). The protein corona develops as proteins in the biological medium adsorb onto the surface of the nanoparticle and form an outside coating with specific physicochemical properties, which strongly affects how the nanoparticle interacts with cells in the medium (del Pino et al 2014; Treuel 2014). The corona consists of two layers: a primary, high affinity protein layer (hard corona) and a secondary, low affinity protein layer (soft corona) (Milani et al 2012). The hard corona proteins are tightly bound and interact directly with the nanomaterial surface, while the soft corona proteins are loosely bound and adsorb to the outside of the hard corona (Rahman et al 2013). In the paper by Saptarshi et al 2013, the authors state the protein corona, physicochemical properties of the nanoparticle, and the properties of interacting cells all have an influence on the uptake of nanoparticles in media (Walczyk et al 2010; Monopoli et al 2012). It is

important to document these types of positive interactions between the nanoparticles and bacterial cells when developing new methodologies for treatment to ensure the nanomaterials being used do not increase the fitness of the bacteria in a way that interferes with the ability of the nanoparticles to properly treat the infection.

Purpose of Study

In this study, the ability of ABC transporters was investigated as an energy-dependent mechanism to allow the entry of 20 nm fluorescent, carboxylate polystyrene nanoparticles into the cytoplasm of planktonic *Vibrio gazogenes* cells. Although *Vibrio gazogenes* is non-pathogenic to humans, it is a Gram-negative bacteria, and the information gained from this study can be applied in principle to other pathogenic species. The reasoning behind this is many resistance mechanisms in Gram-negative species, whether non-pathogenic or pathogenic, are highly conserved; therefore, it is plausible nanoparticle transport occurs in similar ways among different species. The *hypothesis* proposed is that:

Ho; Transport of 20 nm fluorescent, carboxylate polystyrene nanoparticles into Gram-negative bacterial cells is an active process, which requires the assistance of ABC transporters.

If ABC transporters play an important role in nanoparticle transport, they can be used to develop ground-breaking, receptor-targeting therapies that take advantage of this pathway to treat multi-drug resistant bacteria.

In addition to determining the role of ABC transporters in nanoparticle transport, it will also be determined if the 20 nm, fluorescent carboxylate nanoparticles have a

positive effect on the growth of *Vibrio gazogenes*. This is important because it will add more information to the discourse of the many ways in which nanoparticles interact with bacterial cells. It is well known that some nanoparticles, like silver and gold, have inhibitory effects on bacterial growth, but few studies have addressed the beneficial effects nanoparticles may have on the growth capabilities of Gram-negative species. It is crucial to understand not only the mechanisms of nanoparticle transport but also if their transport leads to interactions which have the potential to increase the fitness of the cells. All of this information must be taken into consideration when designing nanomedicinal therapies for the successful treatment of antibiotic-resistant Gram-negative bacteria.

CHAPTER 2

MATERIALS AND METHODS

Organism

The organism observed in the following experiments was a marine species of bacteria known as *Vibrio gazogenes*. Colonies of *V. gazogenes* were isolated and purified from estuarine water samples that were collected from a location in Georgetown, South Carolina. *V. gazogenes* is a motile, rod-shaped, Gram-negative, marine bacterium recognizable by its small, dry, red to reddish-orange colonies when isolated on culture plates (Harwood 1978) (Farmer 1988). The red color stems from the production of a pigment (prodigiosin) during the growth cycle. *V. gazogenes* is also oxidase-negative and does not reduce nitrate to nitrite, making it unique amongst *Vibrio* species (Farmer 1988). To ensure the purification of *V. gazogenes* from the estuarine water samples was successful, 16S ribosomal RNA gene sequencing was performed on the isolated bacteria to confirm the identity. The results showed a 98.7% sequence similarity to the reference strain of *V. gazogenes* listed in GenBank (accession number D11253).

Nanoparticles

The nanoparticles used to assay nanoparticle transport were 20 nm carboxylate-modified, fluorescent, polystyrene FluoSpheres® microspheres (excitation/emission = 505/515 nm) from Molecular Probes. Carboxylate-modified FluoSpheres® have carboxylic acid groups attached to the surface, resulting in highly negatively charged and

relatively hydrophilic microspheres (Molecular Probes 2004). The nanoparticles were stored as a stock suspension in 2 mM sodium azide (2% solids). Prior to sampling, sonication was performed to disperse any aggregated nanoparticles in the storage solution.

Nanoparticle Uptake Assay

A working culture was prepared by pipetting 20 μ L of a stock culture of *V. gazogenes* stored at -80°C in 50% glycerol into a sterile culture tube containing 2 mL of DifcoTM Marine Broth (MB). DifcoTM MB has a high salt content, simulating sea water, and this makes it an ideal growth medium for marine organisms (BD Diagnostics 2009). The working culture was placed overnight in an incubator-shaker set at 28°C and 180 rpm. After growing overnight, the working culture was used to make an inoculum with an OD_{600} of 0.082 in a sterile tube containing 1 mL of DifcoTM MB. The OD_{600} readings were obtained by a Shimadzu UV-2401 PC UV-VIS Recording Spectrophotometer.

Treatment samples were prepared by pipetting 50 μ L of the inoculum, 49 μ L of DifcoTM MB, and 1 μ L of a diluted nanoparticle suspension (1 μ L of the stock suspension in 20 μ L of DifcoTM MB) into four wells on a sterile 96-well, black, flat bottom polystyrene microplate. Control samples were prepared by adding 50 μ L of the inoculum and 50 μ L of DifcoTM MB into four additional wells on the 96-well microplate. The final volume for the treatment wells and the sample wells (8 wells total) was 100 μ L per well. The 96-well microplate was covered with an ethanol-sterilized VWR Advanced Adhesive Film for Microplates and placed into a Perkin Elmer 2030 Multilabel Fluorescence Plate Reader. The reader was set at 28°C and fluorescence measurements were taken every 30 min at 490 nm for 13 hours.

Metabolic Inhibition Assay of ABC Transporters

An overnight culture of *V. gazogenes* grown in Difco™ MB was used to prepare an inoculum diluted to an OD₆₀₀ of 0.080 in a sterile culture tube containing 4 mL of Difco™ MB. Potassium chloride (KCl), glucose and sucrose were used as inhibitors of the ABC transporters in this experiment. The concentrations of each inhibitor tested were 200 mM and 400 mM. The treatment group containing 200 mM of the KCl inhibitor was prepared by first pipetting 50 µL of the inoculum into four wells of a sterile 96-well, black, flat bottom polystyrene microplate. Next, 24 µL of sterile Difco™ MB and 25 µL of an 800 mM stock solution of the inhibitor prepared in Difco™ MB were added to the wells. Then, in a sterile Eppendorf tube, 2 µL of the stock suspension of carboxylate-modified, fluorescent, polystyrene FluoSpheres® were pipetted into 40 µL of sterile Difco™ MB and vortexed for 10 secs. Lastly, 1 µL of the diluted FluoSpheres® suspension in the Eppendorf tube was pipetted into the treatment wells. The same procedure was repeated for the 200 mM glucose and sucrose treatment groups. Each treatment well had a final volume of 100 µL. The 400 mM concentration of the inhibitors were prepared in similar fashion by pipetting 50 µL of the inoculum, 49 µL of the inhibitor, and 1 µL of the diluted FluoSpheres® suspension into four treatment wells on the microplate. A final treatment group containing 50 µL of the inoculum, 49 µL of Difco™ MB, and 1 µL of the diluted FluoSpheres® suspension were prepared on the plate. This treatment group served as a positive control for the experiment to show the uptake of nanoparticles in the absence of any inhibitors. The control groups for the 200 mM concentration of the inhibitors were prepared by adding 50 µL of inoculum, 25 µL of sterile Difco™ MB, and 25 µL of the 800 mM stock solution of the inhibitor to four wells. The 400 mM concentration control groups

were prepared by pipetting 50 μL of the inoculum, 49 μL of the inhibitor, and 1 μL of Difco™ MB into four wells. The 200 mM and 400 mM concentration control groups were used to determine if any of the inhibitors had a significant effect on the fluorescence reading of the wells in the absence of nanoparticles. The last control group prepared on the microplate contained 50 μL of the inoculum and 50 μL of the Difco™ MB pipetted into four wells and served as a negative control for the experiment. The 96-well microplate was covered with VWR Advanced Adhesive Film for Microplates and placed into a Perkin Elmer 2030 Multilabel Fluorescence Plate Reader. The reader was set at 28°C and fluorescence measurements were taken every 30 minutes at 490 nm for 13 hours.

Growth Curve

An overnight culture of *V. gazogenes* grown in Difco™ MB was used to prepare an inoculum diluted to an OD₆₀₀ of 0.100 in a sterile Eppendorf tube containing 1 mL of Difco™ MB. To prepare the control group, one hundred microliters of the inoculum was pipetted into three culture flasks containing 99.9 mL of sterile Difco™ MB, and the inoculum was distributed throughout the broth by manually shaking the flasks. For the treatment group, one hundred microliters of the inoculum was pipetted into three culture flasks containing 99.8 mL of sterile Difco™ MB. Then, one hundred microliters of the stock suspension of carboxylate-modified, fluorescent, polystyrene FluoSpheres® were pipetted into the same three culture flasks, and the flasks were manually shaken to distribute the inoculum and nanoparticles.

The control and treatment flasks were placed in an incubator-shaker set at 28°C and 180 rpm. One milliliter of the culture medium was taken from each flask at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 24 h and analyzed using a Shimadzu UV-2401 PC UV-VIS

Recording Spectrophotometer to determine the optical density of the cultures at 600 nm.

Appropriate controls were used to blank the spectrophotometer prior to each reading.

CHAPTER 3

RESULTS AND DISCUSSION

Nanoparticle Uptake Assay

Fluorescence intensity was measured at 490 nm (emiss.) for each of the four control wells and the four treatment wells every 30 minutes over the course of 13 hours. The measurements obtained for the control wells and treatment wells at each time point were averaged, adjusted to account for background fluorescence from media components, and plotted on a graph (Figure 3.1).

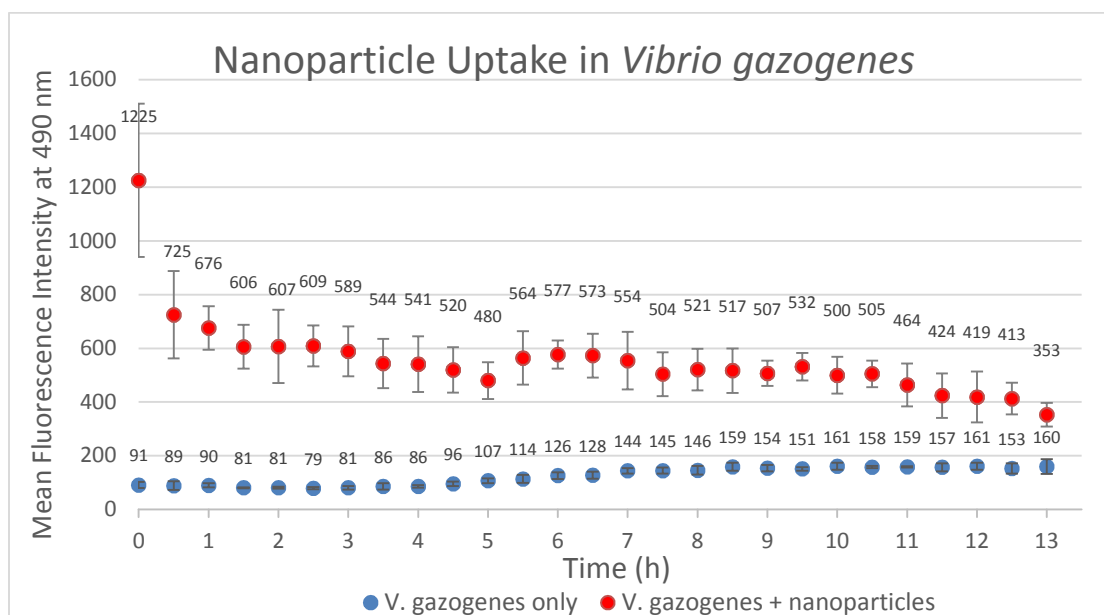


Figure 3.1. Mean Fluorescence Intensity of Nanoparticles after Addition to Culture of *Vibrio gazogenes* over 13 h. A decrease in fluorescence intensity correlates with uptake by cells. Values represent means \pm std. dev.; (n=3).

The average fluorescence intensity of the control wells remained relatively low until the 6 h time point where an increase of 38.46% in fluorescence was observed. Although minor fluctuations in the average fluorescence intensity of the control wells were observed over time, there was an overall increase of 75.82% in the fluorescence from 0 h to 13 h. The increase in the average fluorescence intensity from the 6 h time point forward was attributed to the production of prodigiosin by *V. gazogenes*. Prodigiosin is a red, water-insoluble pigment with no defined physiological function that is produced as a secondary metabolite during the growth cycle of *V. gazogenes*, with maximal pigment levels being reached by 10 h (Allen, Reichelt, and Gray 1983). Although its exact purpose is unknown, prodigiosin possesses antibacterial, antifungal, antiprotozoal, anticancer, and immunosuppressive properties which help to increase the fitness of *V. gazogenes* in competitive environments (Saha, Purkayastha, Saha 2012; Casullo de Araújo, Fukushima, Takaki 2010). The absorption maximum of prodigiosin is pH-dependent, so two different absorbance maximums have been noted in experiments where prodigiosin is extracted under acidified and neutral conditions (Andreyeva and Orgorodnikova 2015). Under neutral conditions, like the ones seen in this experiment, the absorbance maximum of prodigiosin was between 460 and 470 nm, which was close to the absorbance of 490 nm being measured in this experiment. Since there were no other detectable fluorescent components in the media or added to the control wells that would account for the increase in the average fluorescence intensity over time, it was fairly certain that the spectrophotometer was measuring absorbance by the prodigiosin being secreted into the media by *V. gazogenes*. This conclusion was also supported by the visible change in color of the Difco™ MB from yellowish-brown to a reddish color and the leveling off of the

average fluorescence intensity by 10 h, showing the maximal pigment level had been reached.

The addition of nanoparticles to the treatment wells produced a high average fluorescence intensity at T=0 h. To ensure the fluorescence signal being measured was attributed to the nanoparticles only, the average fluorescence intensity was adjusted by subtracting the fluorescence generated by the media, bacterial cell, and prodigiosin from the measurements obtained at each time point. Within 30 minutes, there was a decrease of 40.82% in the average fluorescence intensity, suggesting the nanoparticles were binding and being transported into the interior of the cell, thereby quenching their fluorescent signal. There was a continual decrease in the average fluorescence intensity until the 5 h mark. After 5 h, the intensity increased and then began to decrease again at the 7 h mark. Although this pattern of decreasing and increasing continued throughout the 13 h period, there was an overall decrease in the average fluorescence intensity of 71.18% from 0 h to 13 h. The flux of nanoparticles in the media, which was responsible for the increase and decrease in the average fluorescence intensity over time, was probably due to the nanoparticles being imported into the cell and then being pumped back out through an exportation mechanism. It was plausible that components in the media were binding to the exterior of the nanoparticles, creating a nutrient-rich protein outer shell that attracted the bacterial cell to import the nanoparticle.

Metabolic Inhibition Assay

Metabolic inhibitors block specific chemical reactions by binding (reversibly or irreversibly) to a target molecule, preventing the intended reaction from occurring (Winzler

1949). In a paper by Fox et al 2006, they observed the inhibition of ABC transporters in a wide range of Gram-negative organisms after the addition of different solutes that caused the bacteria to experience hyperosmotic shock (osmotic upshift). Hyperosmotic shock occurs when extracellular fluid osmolality is greater than intracellular fluid osmolality, which creates an imbalance in cellular homeostasis and exerts osmotic pressure and stress on the cell (Brocker et al 2012). Fox and colleagues (2006) also noted this type of inhibition was specific to ABC transport systems and osmotic upshift had no observed effect on non-ABC transport systems. In order to determine if the ABC transport systems in *V. gazogenes* were responsible for transporting the nanoparticles into the cell, an experiment was performed to inhibit these systems using osmotic upshift.

In this experiment, two concentrations (200 mM and 400 mM) of KCl, glucose (Glu), and sucrose (Suc) were added to the media of *V. gazogenes* growing in the presence of nanoparticles to determine if there was a significant increase in the average fluorescence intensity of the media after the addition of the inhibitors. If the metabolic inhibitors were able to effectively inhibit nanoparticle transport through osmotic upshift, there would be large increases in the mean percent fluorescence intensities for the treatment samples because blocking transport would increase the mean number of nanoparticles present, thereby increasing the fluorescence of the media. At 0 h, there was a 10.56% increase in the mean fluorescence intensity of the samples treated with 200 mM KCl, a 2.49% increase for the 200 mM Glu samples, and a 19.22% increase for the Suc samples when compared to the control samples containing no inhibitors (Figure 3.2). For the 400 mM samples at 0 h, there was an 18.23% increase in the average fluorescence intensity for the KCl samples, a 4.98% increase for the Glu samples, and a 4.28% increase for the Suc

samples. The 400 mM Suc samples were the only ones that actually showed a decrease in the average fluorescence intensity with an increase in the concentration of the inhibitor at 0 h.

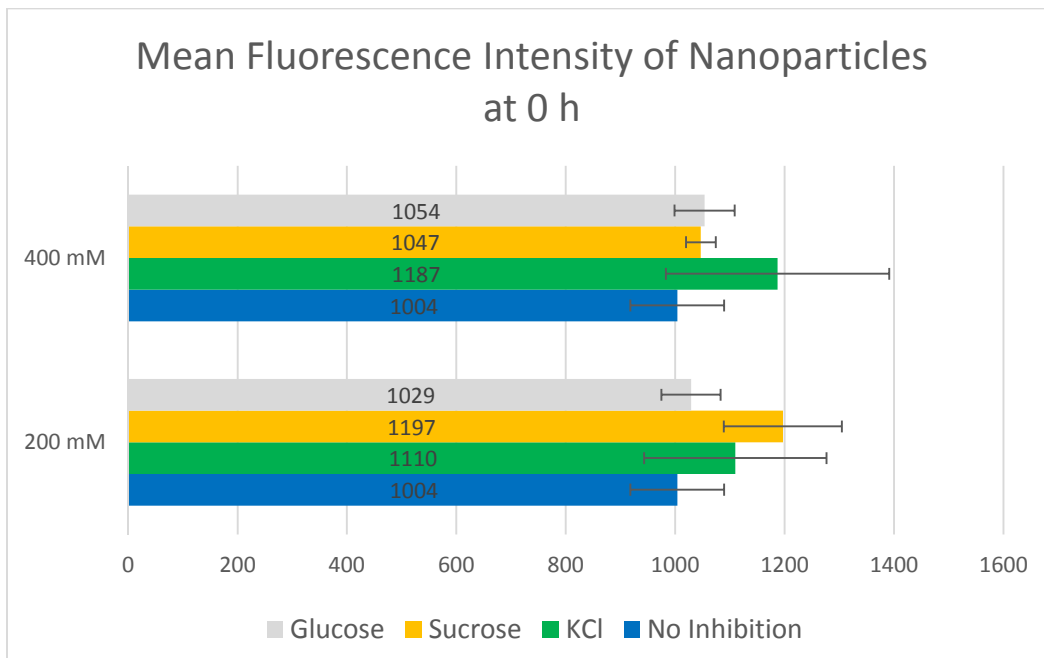


Figure 3.2. Mean Fluorescence Intensities of Nanoparticles after the Addition of Metabolic Inhibitors at 0 h. Values represent mean \pm std dev; (n=3).

The small increases in the percentage of average fluorescence intensity for the 200 mM and 400 mM KCl, Glu, and Suc samples at 0 h indicate there was not a significant difference in the mean fluorescence intensity for the control and treatment samples. To ensure the inhibitors did not have a delayed effect on nanoparticle transport, the control and treatment samples were measured again at 0.5 h. Figure 3.3 shows a 44% or greater decrease in the average fluorescence intensity of each inhibitor at the 200 mM and 400 mM concentrations when compared to the average fluorescence intensity of the inhibitors at 0

h, which suggested the nanoparticles were being transported into the interior of *V. gazogenes* without difficulty in spite of the extended exposure to the metabolic inhibitors.

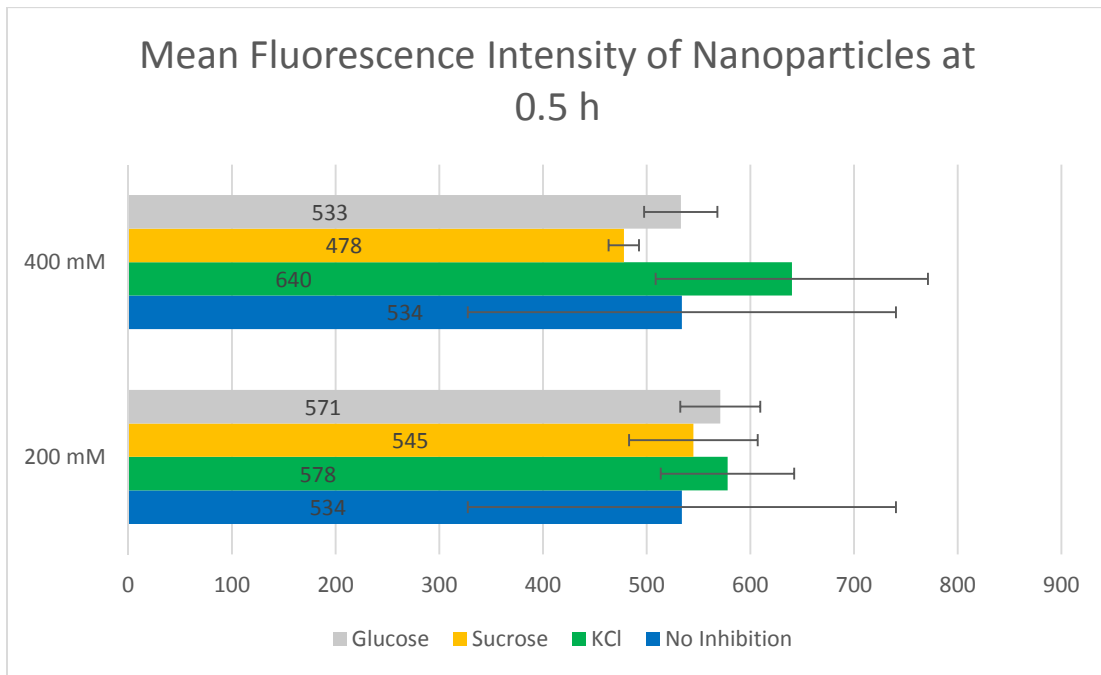


Figure 3.3. Mean Fluorescence Intensities of Nanoparticles after the Addition of Metabolic Inhibitors at 0.5 h. Values represent means \pm one std dev.; (n=3).

The results of this experiment suggested the ABC transporters in *V. gazogenes* either have minimal involvement in the transport of the nanoparticles into the cells or there was another transport mechanism capable of being exploited by the nanoparticles if the ABC transporters were not functioning properly.

Growth Curve

A bacterial growth curve is a visual representation of a bacterial species' generation time under optimal culture conditions. Growth curves outline the four stages of bacterial growth: lag phase, log phase, stationary phase, and cell death (Al-Qadiri et al 2008). During lag phase, bacteria are adjusting to the culture conditions after their inoculation into the culture medium (Vogel et al 2014). They begin to increase in size, activate their

metabolism, and synthesize proteins and nucleic acids in preparation for cell division (Vogel et al 2014). Since the bacteria have not yet replicated, the lag phase is marked by the lack of a substantial increase in cell concentration (Baranyi and Pin 1999). Following lag phase, the cells enter into log phase, and this is the period of maximal cellular growth. The bacteria are growing and dividing at an exponential rate, and all of the resources in the culture medium are being exploited to support their high rate of cell division (Hall et al 2014). The cells enter into stationary phase when they have exhausted the majority of their resources and are no longer able to sustain their rate of replication (Akerlund et al 1995). The culture medium becomes unfavorable due to an accumulation of metabolic waste products and a lack of nutrients, and this leads to a stabilization of the growth rate (Hall et al 2014). Death phase marks the stage where the rate of cell death is greater than the rate of cell replication due to the depletion of resources and increased toxicity of the culture medium (Al-Qadiri et al 2008).

In this experiment, a growth curve was produced to identify the phases of growth for *V. gazogenes* in Difco™ MB and to determine if there were any differences in the average OD₆₀₀ between the control and experimental samples after the addition of carboxylate nanoparticles to the culture medium over a twenty-four hour period. The OD₆₀₀ is a measure of the amount of visible light that is absorbed as it passes through a bacterial suspension at a set wavelength (i.e. 600 nm), and this provides information about the concentration of cells within the suspension (Matlock 2011). Greater light absorbance is indicative of a higher cell concentration. The only growth phases that could clearly be distinguished in the control and experimental samples during the twenty-four hour period were the lag phase and early to mid-log phase, which seemed to occur at the same time

points within both sample sets (Figure 3.4). In order to determine the stationary and death phases, the experiment would be required to run longer (~48 h) to give the cells more time to stabilize their growth and deplete the resources within the culture medium.

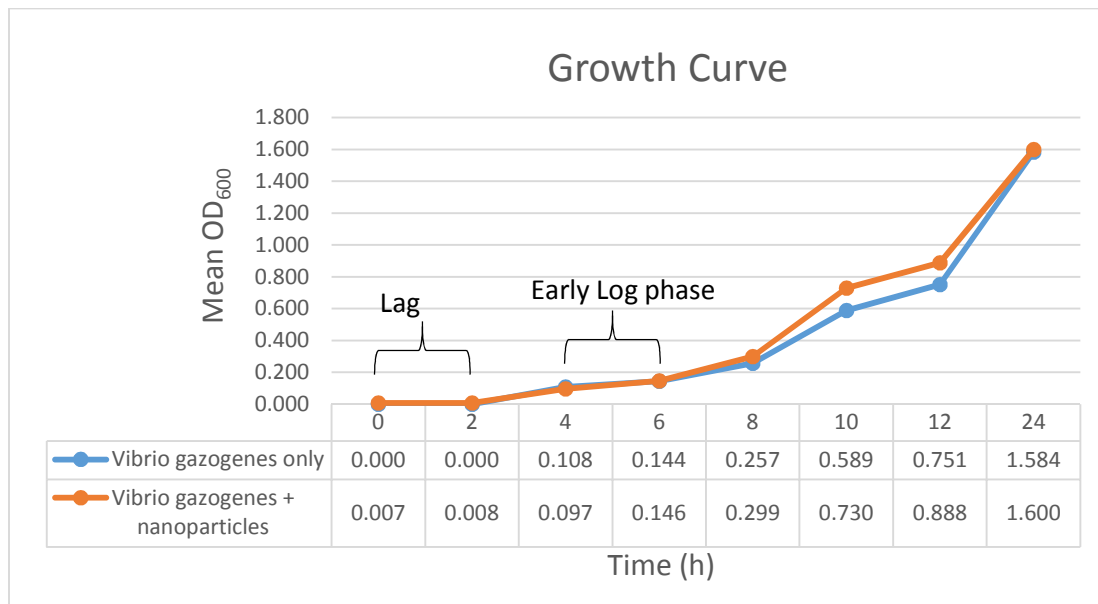


Figure 3.4. Comparison of the Growth Phases and Mean OD₆₀₀ of *Vibrio gazogenes* in the presence and absence of 20 nm (dia.) carboxylate polystyrene nanoparticles.

After the addition of the nanoparticles, there was a 16.34% increase in the average OD₆₀₀ of *V. gazogenes* at 8 h and a 23.94% increase at 10 h. At 12 h, the average OD₆₀₀ was increased by 18.24%, and at the 24 h mark, the average OD₆₀₀ was only 1% higher than the samples without nanoparticles. This was interesting because it suggested the carboxylate nanoparticles did not provide any major growth benefits for the cells unlike observations made in other studies using nanoparticles. To find a possible explanation for the absence of significant growth benefits for *V. gazogenes*, pH measurements of the Difco™ MB were taken over a twenty-four hour time period to determine if the carboxylate nanoparticles were capable of buffering the media (Table 3.1).

Table 3.1. pH Measurements of *Vibrio gazogenes* Culture Medium over 24 h

Treatment	Growth Time (h)								
	0	2	4	6	8	10	12	16	24
<i>Vibrio gazogenes</i>	7.00	7.06	7.04	7.05	7.32	7.45	7.46	7.75	7.73
<i>Vibrio gazogenes</i> + nanoparticles	7.00	7.00	7.04	7.03	7.42	7.46	7.56	7.57	7.61

The results in Table 3.1 show the average pH of the control and treatment groups increased over time, and there were very slight differences between the two groups at each time point, which suggested the carboxylate polystyrene nanoparticles did not have the same pH buffering effect on *V. gazogenes* as the magnetite nanoparticles were shown to have on *Bradyrhizobium japonicum* (Ghalamboran et al 2009). It is also possible that even if a pH buffering effect were present, it would not have a significant effect, either positively or negatively, on the growth rate because many of the *Vibrio* species are very tolerant to high pH values (Nishibuchi et al 1983; Harris et al 1996). *V. gazogenes*, specifically, has an optimal growth pH of 7.5 to 8.5 (Zhan et al 1997), and *Vibrio* species grow to the same maximal levels in the pH range of 6.0 to 9.0 (Nishibuchi et al 1983). The alternate theory of a nutrient-rich protein corona on the surface of the nanoparticles causing increased nutrients to be taken into the cell and accelerating their growth did not seem to occur in this experiment as well. Based on the results from this experiment, the 20 nm carboxylate polystyrene nanoparticles did not have a major effect on the growth of *V. gazogenes* after their introduction into the culture medium.

CHAPTER 4

CONCLUSION

V. gazogenes was capable of taking up 20 nm carboxylate-modified, fluorescent, polystyrene FluoSpheres® microspheres from the culture medium under normal culture conditions. While it was almost certain the process of uptake occurs through an active transport system, it was unclear which system is responsible. The results of the inhibition assay showed the ABC transporters of *V. gazogenes* did not play a major role in the uptake of nanoparticles, contrary to the hypothesis initially proposed. More research is necessary to begin elucidating the pathways involved in nanoparticle transport in this species.

The nanoparticles in this experiment did not seem to provide any significant growth benefits for *V. gazogenes*, which could be important when determining what type of nanoparticles to use in nanotherapies aimed at treating antibiotic-resistant Gram-negative bacteria. It is possible, however, that the nanoparticles may have a beneficial growth effect on other species, so it is necessary to experiment with different species to determine if the growth effects are unique to certain organisms.

Nanomedicine is a complex field with many unknowns that need to be examined further before deciding whether nanoparticle treatments are a viable option for the treatment of recalcitrant bacteria. While there is much progress being made in this field, future research should be geared toward determining the long-term effects of the treatments on the hosts and the specific mechanisms responsible for the observed effects of the nanoparticles on the bacterial cell.

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