# THE VIRUCIDAL PROPERTIES OF SILVER ION-EXCHANGE RESINS AND METAL-BASED NANOPARTICLES AND THEIR POTENTIAL USE IN WATER PURIFICATION

by

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# Abstract

Contamination of water with various disease causing agents such as bacteria and viruses leads to 4 billion cases of diarrheal disease and 1.8 million deaths per year worldwide. The World Health Organization estimates that 94% of these cases can be prevented by increasing the availability of clean and safe water to those at risk. They also claim that 1.1 billion individuals worldwide do not have access to clean water sources, and suggest the best way to improve this situation is to increase household water treatment and safe storage (HWTS). HWTS can dramatically improve water quality, leading to a significant reduction in diarrheal disease. Being able to produce a small and inexpensive device that can be used in a household to improve water quality will significantly aid in preventing diarrheal disease. Water purification systems have been manufactured in the past in order to turn potentially disease causing water into safe drinking water. The metal iodine has been used in some of these systems. However, iodine is known to leach off and become part of the drinking water, which can lead to non-infectious diseases such as hyperthyroidism. This project shows how the development of new water purification systems utilizing silver and other heavy metal nanoparticles may be used to help purify water and have the potential to prevent diarrheal disease. Various heavy metals, including silver nitrate, magnesium oxide, brominated magnesium oxide and titanium-silicon-manganese dioxide nanoparticles, are used in conjunction with ion-exchange resins to develop a biocidal column to sanitize water. A model virus from each of the following families is used: Reoviridae (rotavirus), Picornaviridae (enterovirus), and Caliciviridae (calicivirus). This research shows not only that some of these metals can be used to purify water by inactivating viruses, but also shows the mechanism of viral inactivation. This includes protein denaturation and destruction of viral RNA.

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# Dedication

This research is dedicated to Kent Mulkey and my family. They always believed in me and supported me. If it wasn't for you, I would have never been motivated enough to improve my life and career.

# CHAPTER 1 - Literature Review: Water quality, water-borne viruses, and heavy metals

## Introduction

Water is an essential part of life on earth. For mammals, the drinking of water is the main source of uptake of this important molecule. However, many microorganisms have co-adapted with mammals by living in water. When these microbes are ingested by mammals, they have the potential to cause disease. Humans have done many things throughout time in order to remove these microbes from the water. As early as 2000 BC, water purification practices were used, although these practices were mainly used to improve taste. The ancient Greeks of this time heated water, and also practiced sand and gravel filtration. By 1500 BC, the Egyptians mixed alum into their water in order to make particles to settle easier. In 500 BC, Hippocrates invented a sleeve, which was basically an early form of a bag filter (Baker 1948, EPA 2000, Outwater 1996). Once again, this was mainly used to trap large particles and make water clear looking. This did not protect against microbiological infections. However, when van Leeuwenhoek observed microorganisms in his microscope in the 1670's, this changed many things including water purification (Palm and Snelders 1982). For example, in 1804 the first public water treatment plant was built in Scotland by Robert Thom (EPA 2000). Water purification took a huge step forward when the modern founder of epidemiology, John Snow, showed that cholera was spread by ingestion of water from the Broad Street pump in London in 1854. Since the water coming out of the pump looked and smelled normal, this was somewhat of a paradigm shift for understanding what clean water meant. Snow treated the water with chlorine, and in addition to his other accomplishments, founded the practice of water disinfection (Hempel 2007). In 1903, the practice of softening water was invented. Softening water is done by the removal of ions from the water, and so this became useful in the desalination of water (EPA 2000). Safe and clean drinking water reached a new height in the eyes of the American public when, in 1974, the Safe Drinking Water Act (SDWA) was passed. This act said that people have the right to safe and clean drinking water (EPA 2000).

Since then, techniques such as aeration, flocculation, the use of activated carbon, and reverse osmosis technology have been used to treat and purify water. Aeration is mainly used in wastewater treatment. During the aeration treatment, wastewater is placed in pools for one to two weeks and air is pumped through the water. This aerates the water and reduces the bacterial load in the water by 80-90 percent during the holding time (Beychok 1971). Flocculation is a general chemistry process that can be used in many ways. In terms of water purification,

colloidal contaminants are forced to precipitate by the addition of flocculants. The precipitates, which can be chemical and biological, are then removed from the water resulting in cleaner water (Sridhar et al. 1988). Activated carbon is a form of refined charcoal that has a large amount of pores in it, making it very good at absorbing chemical and biological agents (American Water Works Association. 1989). Reverse osmosis is another useful technique where water is pumped against the normal flow of osmosis through a membrane or series of membranes. This process allows the removal of many different types of large atomic molecule, and can be used in the desalination of water (Popper et al. 1968). In many parts of the world, finding safe drinking water is still a major problem, though. In order to solve this problem, new and innovative methods may be needed. This may include the use of heavy metals and ion exchange resins to inactivate these microorganisms.

## Water Quality Across the Globe

While water quality is generally considered high in the more modernized parts of the world, this is not true for most of the 3rd world and some of the 2nd world. Even in the more advanced parts of the world, water can be considered somewhat in a fragile state during natural disasters such as flooding, hurricanes, and even earthquakes. Contamination of water with various disease causing microorganisms leads to 4 billion cases of diarrheal disease and 1.8 million deaths per year worldwide (Prüss-Üstün 2008, WHO 2007). They also claim that 1.1 billion individuals worldwide do not have access to improved water sources, and suggest the best way to improve this situation is to increase household water treatment and safe storage (HWTS). HWTS can dramatically improve microbial water quality, leading to a significant reduction in diarrheal disease (WHO 2007). The WHO claims that 4% of the total global burden of disease is due to diarrheal diseases. The WHO estimates that 88% of diarrheal disease is due to unsafe water supply, sanitation and hygiene. Finally, they also claim that improving the drinking water quality through household water treatment can reduce morbidity of diarrheal disease by 94% (WHO 2007).

### *Cholera in the Underdeveloped World*

There is perhaps no better example of how poor the water quality is in the under-developed world than the severe and seemingly constant cholera epidemics in Africa. Cholera is

a severe, acute diarrheal disease caused by the bacterium *Vibrio cholera.* In Zimbabwe from 2008-2009, there was a cholera epidemic where over 92,000 cases were and over 4,000 deaths reported. It was determined that the breakdown of the water supply, specifically in high density urban areas was one of the major determinants of the cause of the outbreak (Mason 2009).

### *Problems in the Developed World*

As mentioned previously, waterborne diseases are not restricted to the 3rd world. A recent example of this was the well studied and scrutinized aftermath of Hurricane Katrina. This was a devastating hurricane that struck the New Orleans area on August 29, 2005. The intense coastal flooding caused nearby Lake Pontchartrain to overflow and spill into New Orleans. This caused it to be one of the costliest and deadliest hurricanes in history (Knabb 2005). The intense flooding in the New Orleans area had immediate detrimental effects, but has had and still has lingering effects for the residents of that area. In addition to finding high levels of disease causing bacteria, high levels of pesticides and lead were found. The pesticides were concluded to come from the surrounding uprooted vegetation and the lead was concluded to come from industrial parts of the flooded city (Lubick 2006). The diseases that residents and evacuees of New Orleans became infected with were numerous. A group of evacuees that relocated to Texas became infected with norovirus, and enteric virus from the family Caliciviridae. Non-typhoidal *Salmonella* and non-toxigenic *V. cholerae* were shown to have caused gastrointestinal distress and diarrheal disease. In addition, there were numerous reports of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Vibrio parahaemolyticus* skin infections. These infections resulting in six deaths (Lubick 2006). This illustrates very well how even in developed countries clean, safe water is only one unexpected event away from becoming contaminated.

## Pathogenic Water-Borne Viruses

### *Rotavirus*

Numerous viruses are spread by the fecal-oral route. Many of these viruses cause diarrheal disease, but some can cause other complications such as meningitis. Of the gastrointestinal viruses, none are currently as widespread as rotavirus. Rotavirus (Family: Reoviridae) is a non-enveloped virus of about 75 nm with icosahedral symmetry. Its genome consists of 11 segments of double stranded RNA. Its two major proteins, the P and G proteins

are the spike proteins. They are the major determinants of serology of these viruses. Due to the fact that this is a segmented virus, it can undergo re-assortment and mutate by both genetic shift and genetic drift, just like the influenza viruses (Flint 2004). Additionally, rotaviruses were the first viruses to have shown to encode for a protein (NSP4) that acts as a toxin. NSP4, which is produced by viral RNA 10, disrupts intracellular calcium levels leading to leaky cells and cell death. On a larger scale, this is one of the major driving forces of diarrhea (Dong et al. 1997). By the age of five years old, 95% or more of children worldwide have been infected by at least one strain of rotavirus (Velazquez et al. 1996). The prevalence of this virus in drinking water leads to an estimated 450,000-700,000 deaths per year worldwide (310,000-590,000 of which being infants and young children), and an additional 2 million hospitalized due to rotavirus infection (Bresee et al. 2005, Parashar et al. 2006). Rotavirus is such a widespread problem in the whole of Asia that it should be considered endemic in that region of the world. Asia accounts for 55% of all rotavirus related deaths. In addition, of the top ten countries with rotavirus disease related deaths, six of the ten come from Asia (Bresee et al. 2005). This is a worldwide virus. It is also found in US drinking water at a rate as high as 5 particles per gallon (Rao et al. 1984). Additionally, only 10-100 particles are required for an infectious dose, and so infection in developed countries like the US is not only likely but probable (Ball et al. 2005). Bovine rotavirus was used in this study. Bovine rotavirus is a virus closely related to human rotavirus that causes diarrheal disease in young calves. These viruses are highly infectious and can lead to numerous deaths, which ultimately result in the potential for large economic losses (Holland 1990).

### *Enterovirus*

In addition to rotaviruses, enteroviruses are known to be transmitted fecal-orally. Enteroviruses (Family: Picornaviridae) are non-enveloped, single stranded positive sense RNA, and are about 30 nm in diameter (Flint 2004). The most famous enterovirus is the causative agent of poliomyelitis. Poliomyelitis, or polio, is a disease of numbers. About 90% of those infected show no signs of infection. The other 10% may show mild diarrheal symptoms or other gastrointestinal distress. However, in about 1% of those infected, the virus is able to evade the immune system and escape the gastrointestinal tract. It does this by infecting and destroying motor neurons. The virus can travel to and infect the spinal cord, which is the most common

form of the disease. When this occurs, patients generally have asymmetrical paralysis. If infection occurs in a growing youth, one may see stunted growth and as an adult one leg can be longer than the other. If the virus invades the cranial nerves, respiratory paralysis can occur, resulting in an inability to continue breathing, which can result in death (Atkinson W 2007). "Iron Lungs" were made famous as a machine that kept people breathing during the polio infection (Paul 1971). Although poliovirus was first recognized as a distinct disease in 1840, disease symptoms describing poliomyelitis have been recorded as far back as the ancient Egyptians. Around the turn of the 20$^{th}$ century, polio reached epidemic levels in the most developed countries in the world, including most of Europe and the United States. As a result, there was a strong push to create a vaccine against it and prevent its spread (Paul 1971). In 1988, the WHO, UNICEF, and The Rotary Foundation launched a campaign to fully eradicate polio from the face of the earth, much like what was done with smallpox. This has proven to be a much more difficult task than smallpox, as the virus still persists and is able to infect many individuals specifically from the Northern Africa and Southern Asiatic regions (Heymann and Aylward 2004). Since the beginning of the polio eradication efforts in 1988, a new enterovirus, enterovirus 71 (EV71), has emerged with the same neurotropic capabilities as polio (Blomberg et al. 1974). In addition, according to the Centers for Disease Control and Prevention, the most common causes of viral meningitis are enteroviruses. This clearly demonstrates how enteroviruses are of major significance and will continue to be in the future. In this study, an enterovirus called porcine teschovirus was used. Porcine teschovirus causes a disease similar to polio in pigs, where mild or almost non-existent infection occurs in the gastrointestinal tract of pigs. In a small percentage of the infected, the infection becomes viremic and can cause infection of the central nervous system. Once this happens, the disease usually manifests itself in the form of encephalomyelitis. These series of events are generally followed by death of the animal. Once a widespread disease of pigs, infection from this virus is considered a rarity. In most countries, it is reported to cause mild disease or is not reported at all (Merck 2008).

### *Calicivirus*

An emerging group of viruses that cause diarrheal diseases, and are spread fecal-orally are the noroviruses. Noroviruses (Family: Caliciviridae) are non-enveloped, have single stranded positive sense RNA, and are about 35 nm in size (Flint 2004). Norovirus is responsible

for the disease commonly called the stomach flu, but has also been called the cruise ship virus due to many outbreaks being linked to cruise ships (Khan et al. 1994). Norovirus is not as old of a virus as rotavirus or poliovirus. Instead it came on the scene in 1968 in Norwalk, Connecticut causing diarrheal disease in school children. In 1972, electron microscopy was used to show that stool samples from individuals with norovirus-like disease were loaded with virus particles. This essentially made the connection that a virus was the causative agent of norovirus-like disease (Thornhill et al. 1975). Norovirus is extremely infectious, causing 90% of all non-bacterial outbreaks of gastroenteritis. It causes around 21 million cases of gastroenteritis annually in the United States, and over half of all food-borne disease outbreaks are attributed to norovirus. On average one infected individual will infect 14 other people, and it is thought that 1 virus particle is enough for an infectious dose (Heijne et al. 2009). Norovirus related disease is usually self-limiting and tends to be less severe compared to rota- and enteroviral infections. It can become severe, however, in elderly and immunocompromised individuals by causing severe dehydration. These exceptions lead to at least 300 deaths per year in the United States (Goodgame 2006). This does not mean that this virus is limited to the United States. Indeed, this virus has been found all over the world. In a study of oysters from around the world, norovirus was found at nearly all locations tested. Since oysters are filter feeders, if the oyster filters norovirus contaminated water, then it retains and accumulutes the norovirus. This illustrates the hardiness of this virus to persist in different environments across the globe (Cheng et al. 2005). Feline calicivirus is the model virus used in this study. Feline calicivirus infects cats causing severe rhinosinusitis. Feline calicivirus often co-infects with other feline viral and bacterial pathogens to cause the disease complex known as Feline Respiratory Disease Complex. While feline calicivirus is not spread fecal-oral, and therefore does not cause diarrheal disease, it is a close relative to the human norovirus and can be successfully used as a model virus for this family (Merck 2008).

## A Brief History of Heavy Metals

### *Lead*

Heavy metals have been known to possess toxic properties since ancient times. The Greek poet Nicander of Colophon wrote in the second century B.C. that paralysis can occur after

lead ingestion (Needleman 2004). Lead might be considered a model for how heavy metals can cause toxicity because it has been studied so well, and it has been shown to be particularly neurotoxic. It is one of the several metals known to man that one should not be exposed to, as its exposure has a direct correlation with lowering IQ. Lead has been shown to interact specifically with proteins. This has many consequences. When lead binds an enzyme, the binding of lead can alter the ability of the enzyme to function properly by impacting on the ability of enzymes to bind their ligands. It may affect protein folding, the rates of protein synthesis and degradation, and possibly the intracellular localization of the affected protein (Goering 1993). Lead has specifically been shown to alter the function of neurons. First, it has been shown to alter the release of neurotransmitters from neurons, specifically glutamate. Additionally, it has been shown to cause apoptosis, or programmed cell death, in brain cells (Needleman 2004). Since lead has such a high affinity for staying in the organs, especially bone, of the animals that have ingested it, treatment is difficult. Often, chelation therapy is one of the most effective treatments. In this treatment, a chemical that has a higher affinity for lead than the tissues to which the lead is bound is used. One such chemical is ethylenediaminetetraacetic acid (EDTA). EDTA has four carboxylic acid arms on it and almost literally rips the lead from wherever it is bound. This works particularly well if there are still high levels of lead in the blood (Goldfrank and Flomenbaum 2006).

### *Copper*

Copper is another metal that has been shown to cause toxicity in humans and animals. Ingestion of excess free copper usually leads to self-limiting gastroenteritis. However, when excess copper salts (copper(II) sulfate) are ingested, or absorbed through the skin or mucous membranes, a more severe form of toxicity can develop in the form of hemolytic anemia. Additionally, excess chronic exposure to copper can lead to cirrhosis. Effective treatments for copper toxicity are similar to that of treatments for lead toxicity. Chelation is once again the most common treatment. However, instead of using EDTA, copper has a higher affinity for both penicillamine and dimercaprol (Merck 2008). Unlike lead, copper is required as a nutrient for the human body. If used correctly, copper can be a useful antimicrobial agent as well. As previously mentioned, copper(II) sulfate can be very toxic if ingested in large quantities. However, it can be used with little relative risk to humans as an herbicide, pesticide, fungicide

and even bactericide. Currently, research is being done that shows that when surfaces are coated with copper, this can significantly reduce the amount of bacteria that colonize on that surface (Gould et al. 2009).

### *Silver*

There is a long history of silver use to preserve and extend the life of food, milk, wine and other perishable commodities. Hippocrates, considered by many as the father of medicine, is well known for writing that silver had beneficial healing and anti-disease properties (Institute 2010). More recently, Carl Crede implemented the use of dilute silver nitrate dropped in the eye of infants to prevent gonorrheal opthalmia (Dunn 2000). This technique was first practiced in 1881 and has been used widely ever since. In 1893, Carl von Naegeli noticed that algae would disappear after exposure to silver (Tilton and Rosenberg 1978). von Naegeli was also the first to propose a mechanism for the activity of silver. He said that activity was mainly due to the silver ion and coined the term 'oligodynamic' to describe how the silver ion works. While several biological and non-biological hypotheses exist to describe why silver has antimicrobial properties, no theory for the reactivity of silver is more widely accepted than the oligodynamic effect hypothesis (from Greek *oligos* = few, and *dynamis* = power) (Addicks et al. 1940). The general explanation for the hypothesis is that metal ions have a toxic effect on microorganisms of all types. In the case of silver, the ionic form of silver is responsible for the killing of microorganisms. Heavy metals are specifically known to show this effect. For example, the monoatomic form of silver loses an electron and exists as a positively charged ion.

However, there is no widely accepted agreement on how the silver ion leads to bacterial cell death or viral inactivation. Some groups have suggested that the reactivity of the silver ion leads to the mass formation of reactive oxygen species (ROS) (Park et al. 2009). ROS leads to oxidative damage within the cell, which lead to a number of outcomes. Long term oxidative damages tend to lead to DNA damage and results in apoptosis of the cell (Evans and Cooke 2004, Lee and Shacter 1999). However, if the oxidative damage is high and acute, a different set of events can occur. High oxidative damage can lead to ATP depletion within the cell. Since apoptosis is an ATP dependent event, the cell is unable to undergo apoptosis and instead becomes necrotic (Lelli et al. 1998, Lennon et al. 1991). Others have suggested that silver interacts with disulfide bridges of proteins, causing protein denaturation and loss of function

(Elechiguerra et al. 2005). This theory is similar as to what has been described with lead toxicity. In this study, silver was used to inactivate the Human Immunodeficiency Virus (HIV). When the virus particles were viewed under an electron microscope, silver was shown to be bound to the spike proteins of the virus. This study not only illustrates one way that silver may be toxic, but also shows how silver can be used in a positive way in order to sequester and possibly eliminate disease. Numerous others have described different mechanisms, but ultimately the mechanism of silver ion killing is not clear. Just like lead toxicity, silver toxicity most likely has multiple mechanisms.

As previously mentioned, silver has been used in the past to prevent food spoilage and infection. This is especially true before the advent of modern refrigeration and antibiotics. For example, in World War I, leaves of silver and silver nitrate solutions were successfully used to treat infections (Institute 2010). The discovery of antibiotics, such as penicillin, led to the end of widespread use of silver to treat infections. However, the widespread use of modern antibiotics has led to bacterial resistance. The emergence of drug resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and the XDR strain of *Mycobacterium tuberculosis* (XDR-TB) have inspired many to search for novel antimicrobial compounds. In the late 1960's silver sulfadiazine was shown to possess anti-bacterial, anti-viral, anti-fungal, and anti-treponemal activity (Chang and Weinstein 1975). It is thought that the silver atom is loosely attached to the sulfadiazine molecule via ionic interactions. The silver molecule is highly reactive and is easily stripped from the sulfadiazine molecule, and this is thought to be its mode of action. Since the discovery of silver sulfadiazine, it has become a mainstream product under the brand names Silvadine and Flamazine, and is mainly used on burn victims. In addition to silver sulfadiazine, researchers have started to use silver and other heavy metal nanoparticles to kill and inactivate various microbes. In these studies, heavy amounts of silver are found associated with the bacteria that had been exposed to the silver (Smetana et al. 2008). This supports the previously mentioned report, where silver binds heavily to the surface proteins of the HIV virion.

### *Heavy Metal Nanoparticles*

Nanoparticles are defined as any particle between 1-100 nm in size. They can come in many different shapes – spheres, rods, helices, or even zigzags. Since size is the only real

determinant upon defining what is and what is not a nanoparticle, they come in many different formats. For example, much of what makes up dust is in the nanoparticle range. Also, when haze from a nearby forest fire or possibly even when a volcanic eruption hangs over a nearby town, much of that is nano-scaled. Even the exhaust from a gasoline engine are made of nanoparticles, and so is smog. Nanoparticles are not always associated with dust or dirt. They can be biological. Indeed, most viruses are in the nano-range and may be more appropriately titled nano-viruses.

Artificial nanoparticles have some of the unique shapes, like the zigzag or spiral shapes (Buzea C 2007). The creation of metal based nanoparticles has resulted in compounds with interesting properties. Often, but not always, these nanoparticles will have properties and characteristics that differ from their bulk counterparts. For example, materials containing iron nanoparticles have the ability to change their magnetic polarity depending on the temperature of their surroundings. In addition, nanoparticles may lose malleability. For example, when a certain copper nanoparticle was made, it was found to be many time harder than its bulk counterpart. The authors theorized that the bonds between atoms were not as abundant and so bending was not as easily done (Buzea C 2007). Not only that but some metal nanoparticles have been shown to be soluble in the proper solvent. In doing so, a nanoparticle like gold changes its appearance to deep red or even blackish in solution (Turkevich et al. 1951).

Recently, magnesium oxide molecules of nanomaterial size have been made. These nanoparticles form a complex structure, four stacked hexagons, each hexagon composing of a three magnesium oxides. These nanoparticles have a unique ability to bind to aldehydes and ketone bodies, specifically formaldehyde, acetaldehyde, and acetone (Kakkar et al. 2006). Additionally, when titanium dioxide nanomaterials were made in the presence of silver, this nanoparticle compound showed the ability to kill both *Escherichia coli* and *Bacillus subtilis* spores (Hamal et al. 2010).

The titanium and magnesium based nanoparticles previously mentioned have unique properties and, if applied appropriately, have the ability to be used in things like water purification. Another researcher has incorporated nanotechnology into immunobiology. In this instance, gold nanoparticles were conjugated to secondary antibodies. The reasoning behind this was to help treat bone infections by *Staphylococcus aureus.* This is a type of nosocomial infection that is very difficult to treat and often results in amputated limbs and sometimes death.

The researcher proposed that a primary monoclonal antibody, specific to *S. aureus* be used to first bind the bacterium. Then, the gold nanoparticle conjugated antibody was used to bind to the primary antibody. A high intensity laser was shot at the sight of the infection. Through the skin of the individual, the laser interacts with the gold nanoparticles to raise the temperature of that microenvironment from several hundred to several thousand degrees higher than normal. This essentially raises the temperature on the surface of the bacteria to a high enough point that the bacteria are lysed. This therapeutic technique, if applied properly, has the ability to clear up an otherwise untreatable infection (Zharov et al. 2006). Needless to say, nanotechnology is a new and developing field, and for now the ideas and applications are only limited to the minds of researchers.

**Table 1.1** Ion-Exchange Functional Group Affinities

| Resin Type | Example of Active Group | Order of Selectivity |
|---|---|---|
| **Strongly basic** | $R\text{-}CH_2N^+ (CH_3)_3$ | $I>NO_3>Br>CN>Cl>HCO_3>OH>F$ |
| **Strongly acidic** | $R\text{-}SO_3^-$ | Ag>Rb>Cs>K>Na>H>Li |
| **Weakly basic** | $R\text{-}NH_3^+$ | $H_3PO_4>HNO_3>HI>HBr>HCl>HF>HCOO$ |
| **Weakly acidic** | $R\text{-}COO^-$ | H>>Ag>K>Na>Li |

### *Ion-Exchange Resins*

Ion-exchange resins are beads of about 1-2 mm in diameter that have different chemical functional groups on their surface. These beads are usually made from cross-linked polystyrene (BioRad). On the surface of these beads contain the functional groups. The functionality of the bead is generally dependent upon the function group it contains. The functional groups can be a weak base, like ammonia or other amino based groups. They can also be strongly basic, consisting of tertiary or quaternary amino groups. Trimethylammonium is a common strongly basic functional group. Similarly, weak and strong acids can be applied to these resins. Ions with carboxylic acid residues are considered weak acid resins. The sulfonic and phosphonic acid groups are generally indicative of strong acid resins. Acidic resins are good at interacting with cations such as sodium or potassium. Basic resins are good at interacting with anions such as

chlorine or other halogens. Additionally, there are also resins that chelate. Resins with iminodiacetic acid or even a full EDTA molecule are common examples of chelating resins. These chelating resins can have multiple uses. For example, since EDTA can chelate lead, a resin with EDTA functional groups could be used to remove lead from water (Helfferich 1962). These resins work by replacing one ion with another. For example, what might be loosely attached to a carboxylic acid functional group would be a sodium ion. What then happens is that another cation may come along with a higher affinity for carboxylic acid and displace the sodium. The new cation is then loosely attached to the resin and the sodium leaves the resin (BioRad , Helfferich 1962). Depending upon the ion and the resin used, the affinities can differ dramatically (Table 1.1). Due to the effectiveness of these resins to exchange ions, the history of ion-exchange resins is very closely related to the recent history of water purification and water softening. Some researchers have taken the use of these resins one step further by adding additional atoms to the functional groups already present. This can dramatically change the properties of the functional group. For example, in a recent study iodide was attached to a resin and this resin then showed its ability to kill bacteriophages. A strong base quaternary ammonia functional group had pentaiodide added to it. Water containing various bacteriophages was passed through a column containing these pentaiodide beads and the viral titer was decreased (Brion et al. 2004). This clearly illustrates how these ion exchange beads can be used in conjunction with ions and possibly heavy metals in order to disinfect water.

## Scope of Thesis

It has been said that the wars of the 20$^{th}$ century were fought over oil, and the wars of the 21$^{st}$ century will be fought over water. Not only is water quality poor in some undeveloped parts of the world, but it will become increasingly more valuable of a commodity as the world population increases. Currently there are few alternative methods for the individual on the household level to purify water. Companies such as Katadyn and LifeStraw have commercialized products for individual use that are composed of iodinated resins. However, due to the tendency for iodine to leech off the column and end up in the drinking water, their use is being reconsidered. In 2009, the European Union essentially banned and phased out the use of iodine for any use in water purification (EU 2009). Because of these facts, new ideas and research are needed in order to come up with ways to treat and purify water. This research

attempts to use some new ideas and technologies to do just that. In chapter two, the uses of silver impregnated ion-exchange resins are evaluated. Different resins with differing functional groups were evaluated for their ability to inactivate viruses. The differing functional groups include iminodiacetic acid and phosphonic acid, in macroporous and microporous form. Three different model viruses are used to evaluate the ability of these resins. These viruses include a strain of bovine rotavirus, porcine teschovirus, and feline calicivirus. Additionally, nanoparticles were also evaluated for their ability to inactivate viruses. Specifically, three nanoparticle powders were used. These include magnesium oxide (MgO), brominated magnesium oxide (MgOBr), and a titanium dioxide – silicon dioxide – manganese ($TiO_2SiO_2Mn$) nanoparticle. In chapter three, an investigation on mechanisms of viral inactivation is described. This was first done by visualizing the viruses using transmission electron microscopy (TEM). This gave an insight as to what was happening and led to a major hypothesis. It was hypothesized that viruses were being inactivated by changes in the proteins found on the surface of the viruses, or the coat proteins. It was also hypothesized that alteration of the viral RNA was occurring. Depending on the format of the disinfectant, one or both of these events was happening to the virus. In order to evaluate this hypothesis, native gel Western blots and reverse transcriptase polymerase chain reaction (RT-PCR) was performed. Finally, chapter four summarizes the work and provides a possible conclusion and application of this work for the future.

# CHAPTER 2 - The virucidal activity of various nanoparticles and silver coated resins

## Abstract

Joseph Lister is widely considered the first to use a disinfectant when he used phenol in order to sterilize his tools he used in surgery. Since then, many compounds have been shown to have unique disinfectant properties. Previous studies have shown how iodine is a powerful disinfectant. It can be applied in a tablet form or can be applied to an ion-exchange resin in order to achieve its anti-microbiocide goals. More recently, researchers have shown how silver has the capabilities to be used as a disinfectant. The inactivation of bovine rotavirus, feline calicivirus and porcine teschovirus were evaluated against different types of disinfectants. Using a viral titration method, it is demonstrated that silver impregnated ion-exchange resins all have at least some ability to inactivate viruses. It is demonstrated that this is due to the addition of silver to the resins and that the presence of salts can interfere with the inactivation process. Additionally, nanoparticles are evaluated for their ability to inactivate viruses. It is demonstrated that while magnesium oxide nanoparticles contain no innate virucidal properties, its brominated counterpart is a potent viral inactivator. Within minutes of exposure to this nanoparticle, all the viruses tested were shown to no longer be infectious. When the titanium-manganese based nanoparticle was evaluated, it was also shown to inactivate viruses, although the process was much slower.

## Introduction

Rotaviruses, enterovirus, and caliciviruses are all common viruses found in fecal contaminated water that have potential to cause serious human health complications. Both rotaviruses and caliciviruses are closely linked severe diarrheal disease (Bresee et al. 2005, Goodgame 2006). Enteroviruses, while not associated with diarrhea, still cause serious disease by their affinity to become neurotropic. This can lead to encephalitis and limb disfigurement if infection occurs early in life (Paul 1971). These viruses continue to be a problem in both the developed and underdeveloped world due largely in part to sub-standard drinking water quality and poor sanitation. It is important that new alternative methods for disinfecting water, and thus improving water quality, can be found because this will directly correlate to a decrease in the amount of water-borne disease. In the past, iodine has been the main atomic molecule at the forefront of water disinfection. Campers and other outdoors people have used iodine tablets in their water to help kill bacteria, viruses, as well as *Cryptosporidium* and *Giardia*. This is a short term solution, as it leads to an over abundance of iodine consumed by the drinker. This can lead to thyroid disease, (Trumbo et al. 2001). To circumnavigate this issue, researchers have added triiodide and pentaiodide to ion-exchange resins in order to avoid this large intake of iodine into the body of the drinker. The resins proved to be very effective at disinfecting water, but it was shown that iodine leeched off the resins and still led to an undesirable amount of iodine ingested (Brion et al. 2004, EU 2009).

In this research, a different metal is explored for its ability to inactivate viruses on an ion-exchange resin. Silver nitrate solutions are added to resins containing either iminodiacetic acid or phosphonic acid functional groups. The resulting resin holds the silver loosely to the negatively charged oxygen and allows the silver ion to inactivate viruses. Additionally, magnesium oxide, brominated magnesium oxide, and titanium-manganese nanoparticles are evaluated for their virucidal activities. The data present shows clearly that iminodiacetic acid resins impregnated with silver ions are very efficient at inactivating all the viruses tested, while phosphonic acid resins are not as efficient. Additionally, it is shown that brominated magnesium oxide is efficient at inactivating viruses. The titanium-manganese nanoparticles also show some ability to inactivate viruses, although they are far less effective compared to that of the MgOBr nanoparticles. Bovine rotavirus, porcine teschovirus, and feline calicivirus are all used in this

study as model viruses with close relatedness to human rotavirus, poliovirus, and norovirus, respectively.

## Materials and Methods

### *Viral Strains and Growth Conditions*

Each virus used required its own set of unique growth conditions and cells required to infect. For bovine rotavirus, strain C486 was grown on MARC-145 cells (see Figure 2.1). MARC-145 cells are of African green monkey kidney origin and rotavirus strain C486 is of bovine origin. Confluent monolayers of MARC-145 cells were grown in T-75 flasks (Corning, Corning, NY). Growth media consisted of 1x Eagle's Minimum Essential Medium (EMEM) (BioWhittaker, Walkersville, PA). To the EMEM, the following supplements were added: 70 ml/L (7% final) defined fetal bovine serum (FBS) (HyClone, Logan, UT), 20ml/L (4mM final) 100x GlutaMAX-1 (Gibco, Grand Island, NY), 5ml/L 250ug/ml Fungizone/Amphotericin B (Gibco, Grand Island, NY), and 5ml/L 100x PSN antibiotic mixture (5mg/ml penicillin, 5mg/ml streptomycin, 10mg/ml neomycin) (Gibco, Grand Island, NY). The supplemented EMEM will be referred to as complete growth media.

10ml of complete growth media were added to each flask of MARC-145 cells. The cells were allowed to grow for 3-7 days, and when they reached full confluency, were passed by splitting the cells 1:4. This means that one quarter of the cells in the old flask were used to seed a new flask. Thus, up to 4 new flasks could be planted from the old flask. This was accomplished by first dumping off the old growth media and then washing the cells with 10ml of 1x phosphate buffered saline (PBS) (7.65g/L sodium chloride, 1.27g/L disodium phosphate, 0.1 g/L monosodium phosphate, and 0.21 g/L monopotassium phosphate). The PBS was then discarded and 3ml of 0.25% trypsin (Gibco, Grand Island, NY) was added and the cells were incubated at 37$^{o}$C for 5-15 minutes, depending on the density of the cells.

The protease trypsin destroyed the extracellular matrix the cells use to adhere to the plastic of the flask, allowing the cells to then be divided evenly in solution. New flasks are planted 1:4 and 10ml of complete growth media is added. The freshly planted flasks are placed in a 37$^{o}$C, 5% $CO_2$ incubator until confluency is reached once again. If it is desired to grow rotavirus, the MARC-145 cells are allowed to grow for a full seven days so that a high density of

cells can be achieved. At this point the old growth media is discarded and the cells are washed with 10ml 1x PBS.

It has been well established that rotavirus requires trypsin or some other related protease in order to become infectious (Babiuk et al. 1977). It is thought that the surface proteins of rotavirus are essentially produced as precursors to the active form of the protein, and that trypsin is required full activation of the protein and thus infectivity of the virus (Lopez et al. 1985). Since FBS contains many anti-trypsinases and other related enzymes, and trypsin is required for infectivity of rotaviruses, FBS must be removed from the growth media and a small amount of trypsin must be added. To 1g of lyophilized trypsin (Sigma, St. Louis, MO), 1ml of de-ionized water was added. From this stock solution of trypsin, 5ml/100ml viral growth media can be added. The EMEM lacking FBS and with the trypsin supplement added is referred to as the viral growth media. Once the cells have been washed with PBS, 10ml of the viral growth media is added. To this, 10ul of rotavirus stock (~$10^7$ to $10^8$ infectious virions per ml) is added to the cells. This led to an approximate MOI of 0.1. The cells are placed back in the 37$^o$C, 5% $CO_2$ incubator for an additional 4-6 days. During this time, virus replication occurs and can be seen and evaluated by the number of plaques present on the cell monolayer. The infection was considered complete when 95+% of the cells had come off the plastic and the presence of a cellular slime layer was present in the flask. Each flask would yield 10ml of viral fluids at a concentration of 7.5 to 8.5 $\log_{10}$ of infectious virus particles per ml. From here, the virus was frozen and stored at -20$^o$C (for short term storage) or in liquid nitrogen (for long term storage).

Crandell feline kidney (CrFK) cells are required for the growth of feline calicivirus. These cells were specifically developed for the intent of growing feline viruses (Crandell et al. 1973). These cells were grown in the same way that MARC-145 cells were grown. 10ml of complete growth media was used in T-75 flasks. The cells grew for 3-7 days, just like the MARC-145 cells. However, when attempting to propagate feline calicivirus, the method is slightly different. Once again, the cells are grown to confluency, or about 7 full days of growth. When the cells are ready, the old media is discarded and the cells are washed with 10ml of 1x PBS. The PBS is then dumped off and replaced by complete growth media. Trypsin is not required for the propagation of this virus, and so it can be grown in the presence of FBS. Lastly, only 1ul of virus (from a stock of $10^6$ to $10^7$ infectious virions per ml) is needed for full infection. This was an approximate MOI of 0.0005. After 1-3 days of viral replication, the cells will have

come completely off the plastic and the course of infection is considered complete. Each flask would yield 10ml of viral fluids at a concentration of 6.5 to 7.5 $\log_{10}$ of infectious virus particles per ml

Porcine teschovirus is grown on a different cell line, although the infection protocol is very similar to feline calicivirus growth. Swine testicular (ST) cells are used for the growth of porcine teschovirus. These cells, once again can be grown in the same media and manner as MARC-145 and CrFK cells. When the cells are 7 days old, they are ready for viral inoculation. For this, the old media is dumped off and the cells are washed with 10ml 1x PBS. Then the PBS is dumped off and 10ml complete growth media is added. For proper growth of this virus, add 100ul (of a $10^5$ to $10^6$ infectious virions per ml) to the aged cells. This was an approximate MOI of 0.005. The infection time for this virus was much slower, taking about 7 days to come to completion. Infection was considered complete when the cells had come off the plastic, or when there was so much cellular debris that the flask could no longer be observed through the microscope. Each flask would yield 10ml of viral fluids at a concentration of 6 to 7 $\log_{10}$ of infectious virus particles per ml

### *End Point Dilution Assay*

In order to determine the amount of virus present in a sample, end point dilution assays were performed. In this assay, 96-well plates (BD Falcon, Franklin Lakes, NJ) were planted with the cell type of choice at a rate of 200ul per well. Once planted, the cells were allowed to reach confluency, usually lasting 3-4 days. Once confluent, virus samples can be tested. To do so, the cells are washed by dumping off the growth media and replacing it with 200ul per well of 1x PBS. In the case of rotavirus, the PBS is replaced by the viral growth media (lacking FBS and containing trypsin). In the case of teschovirus and calicivirus, complete growth media can be used also at a rate of 200ul per well. Once the cells were prepared, the virus was added. To do this, the virus underwent a series of ten-fold dilutions. Each ten-fold dilution was repeated 5 times at a minimum. The ten-fold dilutions were performed by on the same plate as the cells. First, 22ul of sample virus was added to the first row of wells and mixed 3-5 times to ensure proper distribution of the virus within the well. This created a 1:10 dilution in this row of wells. Then, using a multichannel pipette and a new set of pipette tips, 22ul of virus was taken from the first row of wells and transferred to the second row of wells. Once again, the sample was mixed

well and the process was repeated again. Special attention was paid to changing tips between each ten-fold dilution so that carry over of virus was avoided. Once all the dilutions were made, all the way out to $10^{-8}$, the plate was then incubated at 37°C for the designated time as described previously for each particular virus. Once the virus replication cycle was complete, the plates were read for the presence of virus. Each well was scored either a positive or a negative. A positive result was marked as a well showing any sign of infection. For example, a well that was completely destroyed by viral replication was marked a positive, and a well that had one foci of infection was also marked positive. A negative well meant that no visible signs of infection were present, and looked the same as the negative controls that lacked virus all together. Once all the wells were marked either positive or negative, tissue culture infectious dose ($TCID_{50}$) was then calculated by using the Spearman-Kärber method. The Spearman-Kärber formula is as follows:

$$\mathbf{TCID_{50} = (X_o - (d/2) + d(\Sigma\, r_i/n_i))}$$

Where $X_o = \log_{10}$ of the reciprocal of the lowest dilution at which all wells are positive

$d = \log_{10}$ of the dilution factor (the difference between the log dilution intervals)

$n_i$ = number of wells used at each dilution

$r_i$ = number of positive wells (out of $n_i$)

$\Sigma\, r_i/n_i$ = sum of the proportion of positive wells (beginning at the lowest dilution showing 100% positive result)

All $TCID_{50}$'s are reported in $\log_{10}$ and represent the number of infectious virus particles per milliliter.

### *Preparation of Silver Impregnated Resins*

Most, but not all, commercially bought resins do not come pre-loaded with silver ions. So, once the resins are received from the manufacturer, silver must be loaded on the ion-exchange beads. Depending upon the chemical structure of the functional group (iminodiacetic acid or phosphonic acid; see Figure 2.1), varying amounts of silver was added to the resin. According to BioRad, Chelex-100 (iminodiacetic acid) has a total binding capacity of 2 meq/dry gram of resin. Therefore, if 30 grams are to be loaded with silver at 50% capacity, 30 meq (5.1g) of silver nitrate are required. Phosphonic acid resins were loaded in a similar way, based on the manufacturers recommended total binding capacity.

**Figure 2.1 Silver Loaded Functional Groups**

### *Preparation of the Water Purification Column*

Two sizes of columns were prepared in the testing of the resins. First, there are the full sized columns. These are 20mm x 2mm cylindrical tubes. These were primarily used for large batches of water with a resin that is known to work. For resins that are being tested for the first time or a quick sample condition needs to be tested, a mini-column was used. This was a 10ml needless syringe (BD, Franklin Lakes, NJ). To the ends of these columns, a layer of fiberglass cotton was used in order to keep the beads inside the column while still allowing the water to pass freely. Then the silver loaded resin was added to the column (see Table 2.1 for a complete list of resins used). Depending upon the test conditions, the amount was variable. Once again, depending upon the test conditions, other components were added. For example, granular activated carbon (GAC) was added to many of the columns. Due to the high surface area of GAC (from the vast amount of pores present), GAC can successfully be used as a media to remove contaminants from the water like pesticides and even some ions. Additionally, some of the columns contained another resin called MB400. MB400 is known as a mixed bed resin. This means that it has two different functional groups on the surface of each bead. In the case of MB400, the two functional groups are sulfonic acid (strong acid) and type 1 quaternary ammonium (strong base). The sulfonic acid will exchange the hydrogen bound to it by another positively charged ion, like sodium. The ammonium will exchange the hydroxyl group bound to it by a negatively charged ion, like chlorine. In doing so this resin has the ability to de-ionize water, and was used experimentally for that very purpose. The silver impregnated resin, GAC, and MB400 were the only components of the columns. Once these components were added, the column was sealed off and was ready for use.

**Table 2.1 Silver Impregnated Resins and Their Functional Group**

| Resin Name | Manufacturer | Functional Group |
|---|---|---|
| **Chelex-100** | Bio-Rad | Iminodiacetic Acid |
| **Dowex IDA-1** | Dow | Iminodiacetic Acid |
| **D5134** | Purolite | Iminodiacetic Acid |
| **D5194** | Purolite | Iminodiacetic Acid |
| **S393** | Purolite | Phosphonic Acid |
| **S950** | Purolite | Phosphonic Acid |

### *Preparation of Nanoparticle Powders*

The three nanoparticle powders tested (magnesium oxide, brominated magnesium oxide, and titanium dioxide with manganese) were all obtained from Dr. Kenneth Klabunde and his group in the chemistry department at Kansas State University. All samples were in powder form and were tested for their biocidal capabilities by dissolving them in aqueous solution containing viruses.

## Results

### *Evaluation of Resins Using the End Point Dilution Assay*

Virus samples were prepared and exposed to different resins by passing the viral solutions through mini-columns. All virus samples were diluted in de-ionized water. A positive control was used, which was a viral sample that was not exposed to any viral inactivator. Additionally, other controls were tested. A GAC only column and an MB-400 only column were also used to show that extra components of the column are not responsible for any viral inactivation. Finally, the virus was ran through the mini-column containing GAC, MB-400, and the resin in question (Table 2.1) at the normal flow of gravity. The lowest detectable limit of viral detection is 1.2 $\log_{10}$ $TCID_{50}$/ml of virus. This implies that anything lower than that value is considered a negative result. Anything above that value implies that virus was present in the sample. It is clear that GAC and MB-400 have no inherent ability to inactivate viruses. It is also clear that rotavirus is the hardiest of the three viruses because it resists inactivation, whereas the other two viruses tend to be more easily inactivated. The data shows that Chelex-100, Dowex

IDA-1, and Purolite's D5194 resins are all capable of complete inactivation of all viruses. The other resins tested, including two phosphonic acid resins, show partial but incomplete ability to inactivate viruses (Figures 2.2).

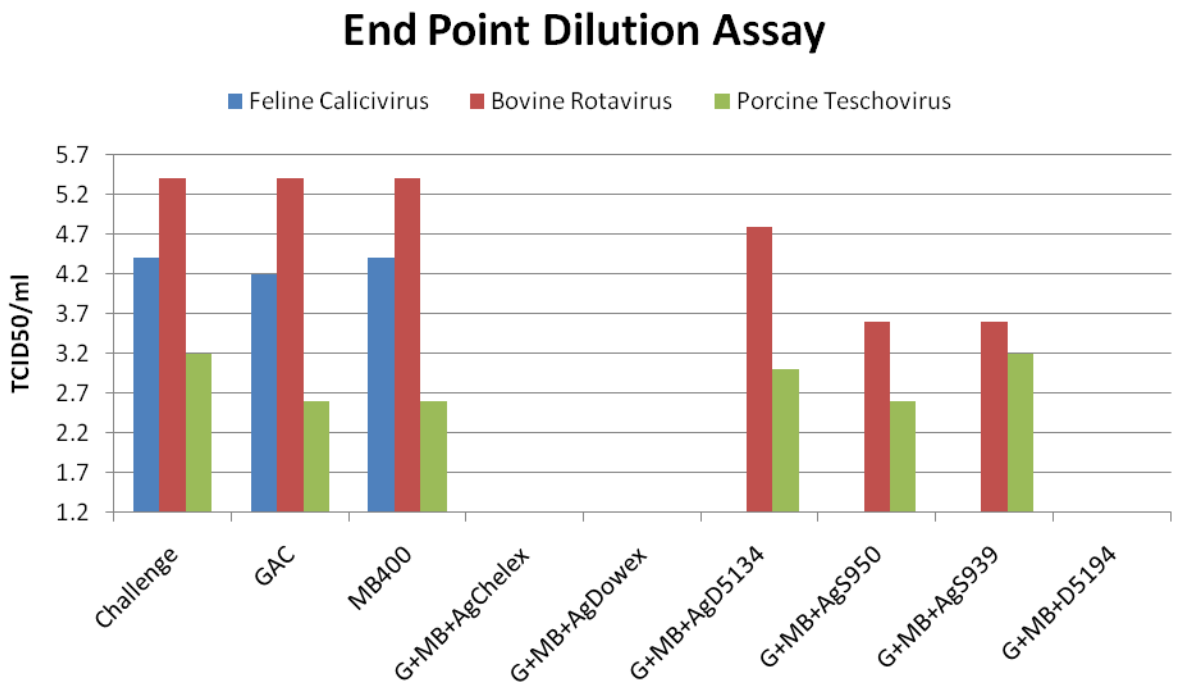


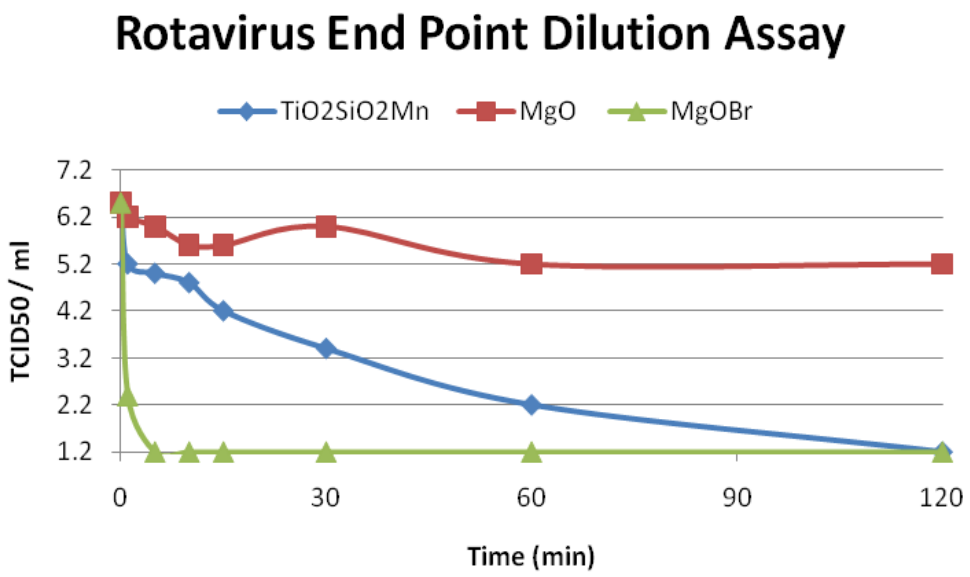

**Figure 2.2 Silver Loaded Functional Groups**

GAC and MB400 represent mini-columns only filled with each of the components, respectively. These columns showed the same virus titer as the positive control (challenge). Chelex-100, Dowex IDA-1 and D5194 columns showed complete inactivation of all the viruses challenged. D5134, S950, and S939 showed complete inactivation of calicivirus, but lacked the ability to inactivate rotavirus and teschovirus. Y-axis values are represented as infectious virus particles ($\log_{10}$) per ml.

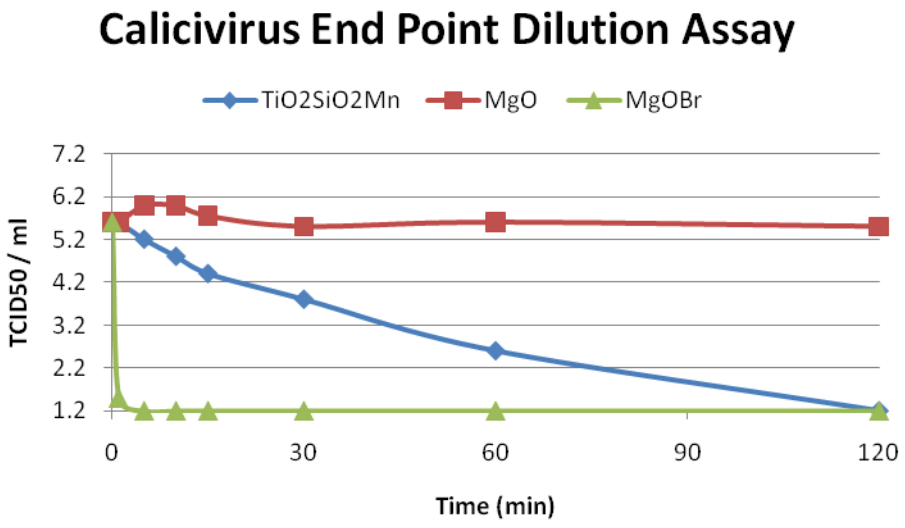
### *Evaluation of Nanoparticles Using the End Point Dilution Assay*

Virus samples were prepared and exposed to nanoparticles. This was done by taking the undiluted sample of virus and dissolving nanoparticles to 2ug/ml. The samples were then placed on a shaker and allowed to interact for differing amounts of time. Each sample was tested at room temperature (22$^{o}$C), at physiological pH (pH 7), and under natural indoor lighting. At each time point, the sample was then removed from the shaker and briefly centrifuged to remove

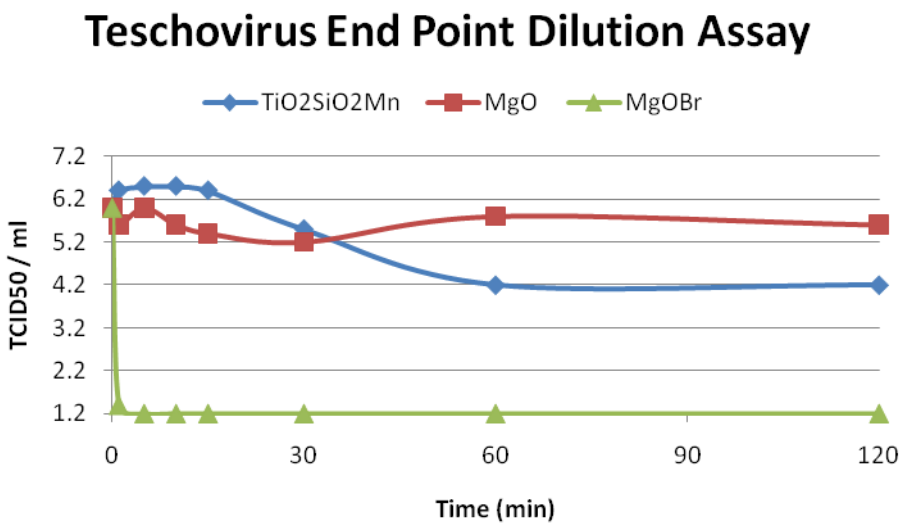
excess nanoparticle powder. The supernatant was then tested for presence of infectious virus using the end point dilution assay. For all three viruses tested, there was no detectable virus present in the supernatant after 5 minutes of exposure to the brominated magnesium oxide nanoparticles. For calicivirus and rotavirus, the titer slowly dropped when these viruses were exposed to titanium dioxide manganese nanoparticles. After two hours of exposure, these viruses were at non-detectable levels. Curiously, porcine teschovirus did not show the same sensitivity to the titanium dioxide manganese nanoparticles. For all three viruses tested, there was no virucidal effect noticed after exposure to the unaltered manganese oxide nanoparticles (Figure 2.3).




(a)




(b)




(c)

**Figure 2.3 Viral interactions with nanoparticles**

Viral inactivation curves showing how much infectious virus was present in a sample after a given amount of time exposed to the nanoparticle of choice. In all viruses tested, complete inactivation was achieved after only 5 minutes of exposure to brominated magnesium oxide (MgOBr, green). Titanium dioxide manganese ($TiO_2SiO_2Mn$, blue) showed slight virucidal capabilities against rotavirus and calicivirus, but no ability to inactivate teschovirus. In all viruses tested, magnesium oxide (MgO, red) showed no ability to inactive any of the three viruses. Y-axis values are represented as infectious virus particles ($log_{10}$) per ml.

## Discussion

It has been well documented how iodine can be used in the sterilization of water (Brion et al. 2004). It has also been shown how nanoparticles can be used in the inactivation of viruses and the killing of bacteria (Elechiguerra et al. 2005, Smetana et al. 2008). Building on those concepts, here a different set of nanoparticles and another metal ion is used in order to show how they could potentially used in the sterilization of water. It is demonstrated very clearly that resins with iminodiacetic acid functional groups that are loaded with silver ions are generally very efficient at inactivating viruses in aqueous solution. It is demonstrated that the silver ion, not the resins or the components of the resins, are responsible for the inactivation of these water-borne viruses. Additionally, it is shown that alternative resins that are capable of binding silver, such as phosphonic acid, are capable of inactivating viruses, although to a much lesser extent.

It is also shown that brominated magnesium oxide nanoparticles have an ability to inactivate viruses. The ability to use this nanoparticle in a water purification scheme is more fully addressed in Chapter 3 and 4, where the mechanism of inactivation is addressed and the possibility of usage is determined. It is well demonstrated here that common water-borne viruses are not inactivated by magnesium oxide nanoparticles. This, along with the fact that brominated magnesium oxide is so effective, implies that bromine is somehow causing the inactivation. This would make sense, considering other halogens such as chlorine and iodine are known to be very efficient water purifiers. While titanium dioxide manganese nanoparticles do have virucidal capabilities, the time in which it takes puts the application of these nanoparticles in question.

In summary, the results presented in these viral inactivation studies show that water-borne viruses continue to be inactivated by halogen based disinfectants. Additionally, silver, which was long thought to be thought of as anti-bacterial, is shown to be anti-viral as well. Future studies will be likely to include the use of dirty water with these nanoparticles and resins. It is unclear how the presence of other ions and contaminants of water would interfere or enhance the viral inactivation. Additionally, any future studies would have to include application of the brominated magnesium oxide nanoparticles into a possible water filter that could be used on the personal or household level.

# CHAPTER 3 - Mechanisms of Viral Inactivation

## Abstract

Joseph Lister is considered the modern founder of disinfection. He was the first to use a chemical in order to sterilize when he used phenol to sterilize his medical equipment to cut down on infections (Bankston 2005). While Lister was not exactly aware of how the phenol sterilized, it is now known that phenol can cause oxidative stress and damage to the cells that come in contact with it (Murray et al. 2007). Chlorine and chlorine compounds are commonly used in water purification today. A common chlorine based compound that is used at the individual and household level is sodium hypochlorite, commonly referred to as bleach. Bleach has long been known to be a good oxidizer at high pH (Gehrmann and Remmer 1952). It is not exactly known how newer compounds used for disinfection, such as silver based products, are able to disinfect. In my research, insight is given to how silver loaded ion-exchange resins, brominated magnesium oxide, and titanium dioxide manganese nanoparticles are able to disinfect virus from water by using electron microscopy, immunoblotting, and polymerase chain reaction techniques. The data presented provides evidence that titanium dioxide manganese nanoparticles are capable of inactivating virus from water by binding to the viruses and preventing them from infecting cells. Additionally, it is shown that silver impregnated resins are capable of inactivating viruses by binding to the surface proteins and putting the proteins in a denatured state. Finally, it is demonstrated that brominated magnesium oxide does not alter protein structure, but rather alters the genome of the microorganism to a non-functional state.

## Introduction

Rotaviruses, enteroviruses, and caliciviruses are among the leading viral causes of water-borne infectious disease. In fact, 95% or more of all humans are infected by rotavirus by just a few years of age (Parashar et al. 2006). These viruses cause disease ranging from severe and acute diarrhea to meningitis and encephalitis. These viruses show an inherent ability to persist in the environment. For example, rotavirus has shown to only undergo a 3-$\log_{10}$ reduction of viral titer in a two week period in both fresh- and estuarine-water (Hurst and Gerba 1980). Additionally, there is evidence to show that rotavirus is even more stable at lower temperatures (Moe and Shirley 1982). These results imply that lower environmental temperatures would cause a lesser reduction of titer over time. Compound these facts with data that shows that only 10-100 rotavirus particles are required for a potential infection, and it is clear as to why these water-borne viruses are so easily transmitted and infection is so common (Ball et al. 2005).

Rotaviruses, and all other reoviruses, have been shown to have very complex protein core structure. Rotavirus has icosahedral symmetry that forms a T=13 outer structure (Chen et al. 2009). Like all non-enveloped viruses, the outer protein layer contain the 'anti-receptors'. These are the proteins that interact with the cell in order to enter the cell and cause infection of the cell. Rotavirus capsid structure is somewhat unique in that it is formed of multiple layers of protein. The total particle consists of three total layers of proteins, the total virus is considered to be triple layered. What is referred to as the core particle is double layered. Finally, what is called the infectious sub-viral particle (ISVP) is mono-layered (Coombs 1998). During infection of a cell, the proteins on the outside layer of the virus interact with the surface of the host cell and this causes the virus to enter the cell. Once in the cell, the outer and middle layers are removed, leaving only the ISVP. During the course of infection, the ISVP is not removed. Instead, pores in the ISVP allow molecules required for replication of viral RNA and viral protein synthesis to move freely in and out of the ISVP (Flint 2004). Although the ISVP does not contain the proteins required to interact with the cell surface and enter the cell, it is capable of producing infectious viral particles (that can complete the infection cycle) if it is allowed to enter the cell. The complex structure of this virus adds to the stability and hardiness of the virus and is no doubt why it is so persistent in the environment.

These viruses are capable of being inactivated very easily with the use of chemical disinfectants. Bleach, for example, is a common disinfectant that is very efficient at inactivating

rotaviruses (Gehrmann and Remmer 1952). Chemically, bleach is known as sodium hypochlorite. This compound has a high pH around 11, and rotaviruses have been shown to be easily inactivated at pH's above 10 (Estes et al. 1979). Additionally, it is well known that bleach is a strong oxidizer. This two-fold attack on rotavirus explains why this is such a powerful tool in viral inactivation. However, this is not without consequences. It can also be harmful to the humans that use it, and humans would not want to ingest bleach laced water. Bleach is a chlorinated compound, in which the chlorine is very active and is capable of causing irritation of the gastrointestinal tract if ingested and irritation of the respiratory tract if bleach fumes are inhaled over a long period of time (Hostynek et al. 1990).

While chlorinated compounds are readily used in water purification, they are rarely at a high enough concentration to kill all of the potential biological contaminants. The need for alternative disinfectants is real. There has been a recent use of silver as a disinfectant. Silver was long known for its antimicrobial properties. Even today, silver nitrate is widely used by the medical community as a treatment for infant ocular gonorrhea and is even being used as a treatment for fungal infections of the eye (Xu et al. 2009). Recently, silver nanoparticles have been used in order to inactivate viruses. In two separate studies using silver nanoparticles, both *E. coli* and Human Immunodeficiency Virus-1 (HIV-1) have been shown to be inactivated by action of the nanoparticles (Elechiguerra et al. 2005, Smetana et al. 2008). In the case of HIV-1 inactivation, the nanoparticles were shown to interact with the surface proteins of the virus. The researchers concluded that the interaction with these proteins inhibited the ability of these proteins to interact with the host cell, thus preventing infection (Smetana et al. 2008). Intercalating agents such as ethidium bromide (EtBr) or binary ethyleneimine (BEI) can be used to inactivate viruses by altering the genome of the infectious particles. This provides another way that viruses can be inactivated. Our results show that silver loaded ion-exchange resins can inactivate rotaviruses by altering the surface proteins, and thus altering the ability of the virus to infect a cell. Additionally, it is shown that brominated magnesium oxide nanoparticles are capable of rotavirus inactivation by disrupting the double stranded RNA of the virus.

## Materials and Methods

### *Determination of Bromide in Solution*

It is unclear if the brominated magnesium oxide nanoparticles retain or lose the bromine in aqueous solution. In order to determine if bromine is present in aqueous solution, potassium iodide is added to the solution. If free bromine is present in solution, then it may have the ability to convert iodide, from the potassium iodide, into iodine. Iodine appears dark brown in concentrated solutions and a lighter yellow in more dilute solutions. A positive result would show a color change from clear to yellow or brown. In addition, iodine test strips (ITS, Rock Hill, SC) were used to detect the presence of iodine. Any level of iodine would confirm that iodide is being converted to iodine, once again showing that bromine is present in the solution.

### *Transmission Electron Microscopy Negative Stain*

Samples of virus were prepared in order to visualize them under TEM. To do this, a copper, formvar/carbon, 200 mesh grid (Ted Pella, CA) was exposed to the sample for one minute. Then the disc was exposed to 2% uranyl acetate for 1 minute. Finally the disc is briefly washed in water. The disc was then loaded into the TEM and virus was observed.

### *Native PAGE Western Blotting*

Multiple monospecific antibodies against rotavirus are available on the commercial market, mainly due to the diagnostic need for such a reagent. Additionally, there is much cross-reactivity between antibodies specific for human, bovine and porcine rotavirus antibodies. As a result, multiple monospecific antibodies were tested for their ability to detect rotaviral proteins in a Western blot setting. Ultimately, a monospecific polyclonal antibody against the native form of the coat protein of bovine rotavirus was used in this work (Millipore, USA). Since the antibody was specific against native protein and not denatured protein, no heating or SDS was used in preparation of the sample or running of the gel. First, 40ul of sample was mixed with 10ul of loading buffer (72g/L glycine, 15g/L tris base, 0.006g bromophenol blue in 10 ml of $dH_2O$). Once the sample is prepared, 40ul of sample is loaded onto a 10-well 12% PAGE gel (Lonza, Basel, Switzerland). The gel is placed in a box with around 800ml of 1x running buffer (14.4g/L glycine, 3.03g/L tris base). Additionally, one well is reserved for the protein ladder (FisherSci, USA).

Once the gel was loaded, the power was turned on and the gel ran at 125V (constant voltage) for 80 minutes. After the gel was done running, the Western blot sandwich was prepared in this order: back side of the cassette, one piece of transfer pad pre-soaked in transfer buffer, two pieces of Whatman paper pre-soaked in transfer buffer, the gel, one piece of PVDF membrane (Millipore, USA), two pieces of Whatman paper pre-soaked in transfer buffer, one piece of transfer pad pre-soaked in transfer buffer, the front side of the cassette. The cassette is closed and placed in to a gel box. Then, 1000ml of ice cold transfer buffer (14.4g/L glycine, 3.03g/L tris base, 200ml/L methanol) is poured in the gel box. The gel box is then placed into another open box and the outer box is filled with ice. A small magnetic bar is placed in the gel box and both of the boxes are placed on a stir plate. The stir plate was turned on and the gel was run at 100V for 60 minutes. If the ice melted too much, then more ice was added to the outer box in order to keep the transfer buffer as cool as possible.

Once the transfer was done, the PVDF membrane was placed in 5% powdered milk in TTBS (20mM tris base, 500mM NaCl, and 0.05% Tween-20) for 1 hour. Once done, then 10ul of goat anti-rotavirus antibody (Millipore, CA) was placed in 20ml of 1% milk in TTBS overnight at $4^{o}$C. The next morning, the membrane is washed three times in 10ml TTBS. Then, rabbit anti-goat IgG HRP antibody (Millipore, CA) was added by adding 10ul of the antibody into 20ml of 1% milk in TTBS. This is added to the membrane and is incubated on a stir plate at room temperature for at least one hour. When done, the gel was washed again three times with 10ml TTBS. The membrane was then blotted dry on Whatman paper. Finally, SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, IL) is added to the membrane for five minutes. Again the membrane is blotted dry and then the gel was exposed to film in a dark room. The film was then developed.

### *Reverse Transcriptase Polymerase Chain Reaction*

Since rotavirus is a double stranded RNA virus, reverse transcriptase polymerase chain reaction (RTPCR) is required in order to determine if alterations have been made to the viral genome. Additionally, since rotaviruses have segmented genomes (11 segments of double stranded RNA), it is capable to amplify an entire gene of the virus performing RTPCR. A known procedure (Falcone et al. 1999) was modified in the testing of viral RNA. First, using tissue culture fluids, viral RNA is isolated using the procedure recommended by the

**Table 3.1 Primers used for full length amplification of rotavirus gene VP7**

| Primer | Sequence | Location |
|---|---|---|
| Forward | 5'-GGCTTTAAAAGAGAGAATTTCCGTTTGG-3' | 1-28 |
| Reverse | 5'-GGTCACATCATACAACTCTAATCT-3' | 1039-1062 |

viral RNA isolation kit manufacturer (Qiagen, USA). Then, since the isolated RNA is double stranded, a pre-RTPCR step is required. 23ul of isolated RNA is mixed with 1ul of each the forward and backward primers, as described by Falcone, et al, and is warmed up to 97$^{o}$C for 5 minutes. The RNA is immediately placed on ice when the warming step is done. This step is done in order to denature the RNA and allow the primers to attach to the single stranded RNA. Then, 24ul of 2x master mix (magnesium, Taq polymerase, and dNTP's) and 1ul of reverse transcriptase (Promega, USA) is added to the isolated RNA mix. This brings the reaction mix to 50ul total. The reaction was over-layed with 80ul of mineral oil. The reaction then runs the temperatures and times described by Falcone, et al, except the reaction runs for 20 cycles instead of 39 cycles.

After the reaction was done, the samples were then run on an agarose gel. The agarose gel was prepared by dissolving 1.5g of agarose (FisherSci, USA) in TAE buffer (4.84g/L Tris base, 1142ul/L glacial acetic acid, 0.292g/L EDTA). This was best accomplished by microwaving the mixture for one minute. Once the agar is dissolved in the TAE, 10ul/100ml of ethidium bromide was added to the agar. The gel was then poured. The samples were prepared for loading onto the gel by mixing 2ul of loading dye (Promega, WI) with 10ul of sample from the RTPCR reaction. Additionally, 10ul of DNA ladder (Invitrogen, USA) was added to the gel. The gel was then loaded and ran at 100V for 40 minutes. The samples were then exposed to ultraviolet light and pictures were taken.

## Results

### *Detection of Bromide*

To test if bromine is present in a solution, 0.1M potassium iodide is added to the solution. If a color change from clear to yellow happens, then bromine is most likely present. If there is

no color change, then there is no bromine. When KI was added to MgO nanoparticles, no color change occurred. This is expected as there is no bromine present. However, when MgOBr nanoparticles are dissolved in water, and subsequently exposed to KI, the solution quickly turned yellow. This would indicate that bromine is present in the solution, and is converting iodide into iodine. These results indicate that when MgOBr nanoparticles are dissolved in water, that the bromide freely dissociates from the MgO nanoparticle. These results are further confirmed by the fact that high levels of iodine were present in the solution when tested using basic iodine test strips (ITS, Rockhill, SC).

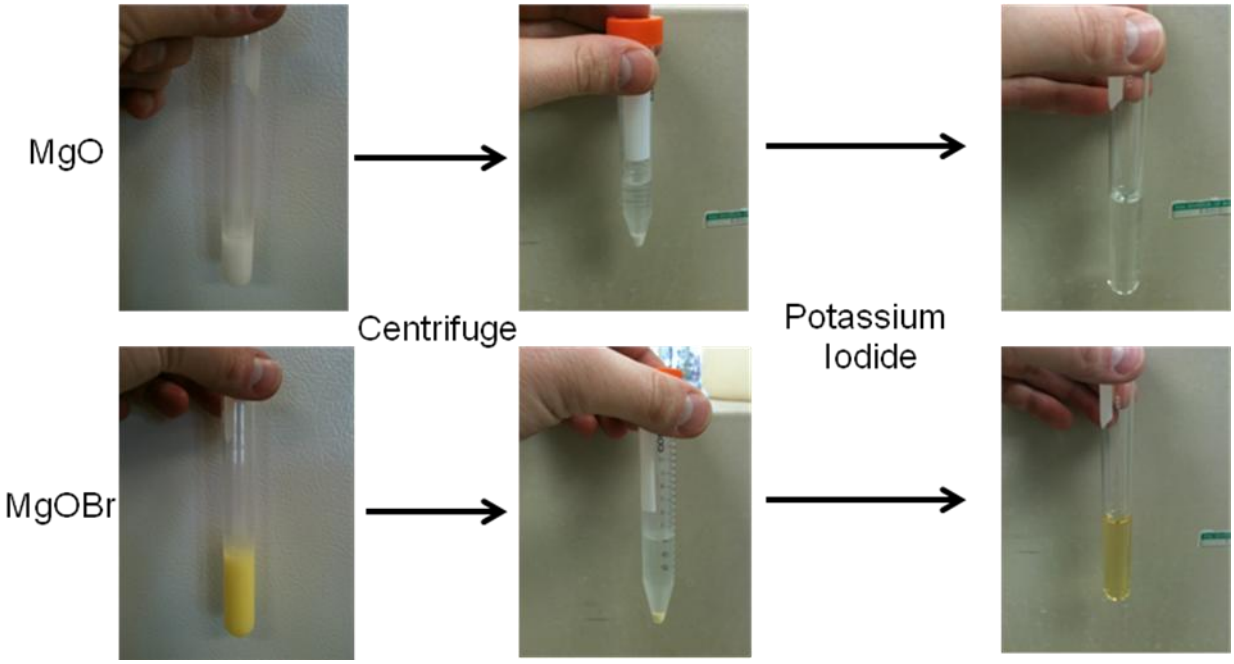


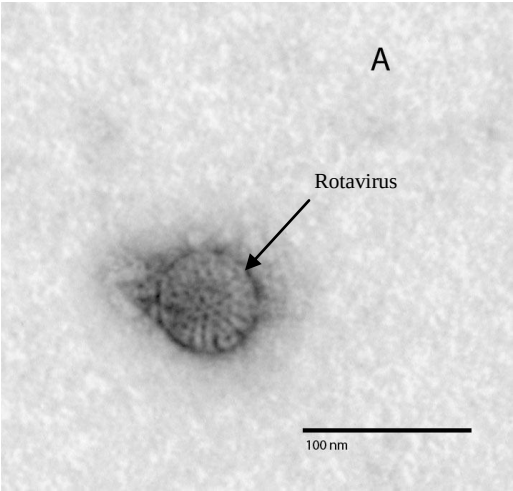

**Figure 3.1 Bromine dissociates from MgOBr nanoparticles in solution**

After MgOBr, or MgO nanoparticles have been placed in distilled water, and the centrifuged, the supernatant had equal parts of 0.1M KI added. There was no color change in the MgO supernatant, but there was a color change from clear to yellow in the MgOBr supernatant. Additional testing (data not shown) using iodine test strips show that iodine levels were high in MgOBr/KI solution, suggesting that iodide is being converted to iodine by the presence of free bromine in solution.

### *Electron Microscopy of Rotavirus*

Samples of rotavirus were exposed to the different nanoparticles and run through the silver loaded D5194 resin. The resulting fluids were then observed under transmission electron

microscopy (TEM). We were unaware of what the viral particles were to look like, but we were interested in if any physical changes were present after the different treatments. Untreated samples showed rotaviral particles around 65-75 nm in size (Figure 3.2a). Treatment with magnesium oxide nanoparticles showed unaltered rotavirus particles, as did treatment with brominated magnesium oxide (Figure 3.2b, e). Rotaviruses exposed to MgOBr did contain a large proportion (possible 10:1) of viruses that appeared hollow, possibly indicating the lack of viral RNA. There was a distinct lack of viruses in samples exposed to the silver loaded ion exchange resin. Instead, there were numerous (3-5) cases where small particulate the size of protein was found. It was thought that this was proteinaceous in nature mainly because of its size, but also because of its amorphous shape. There even was one account where it appears the viral particle was being ripped apart (Figure 3.2c). Finally, exposing the virus to titanium dioxide manganese nanoparticles showed viral particles, however they were most closely associated with the nanoparticle complexes. These nanoparticles had a tendency to clump, and in doing so clumped with the viral particles (Figure 3.2d).



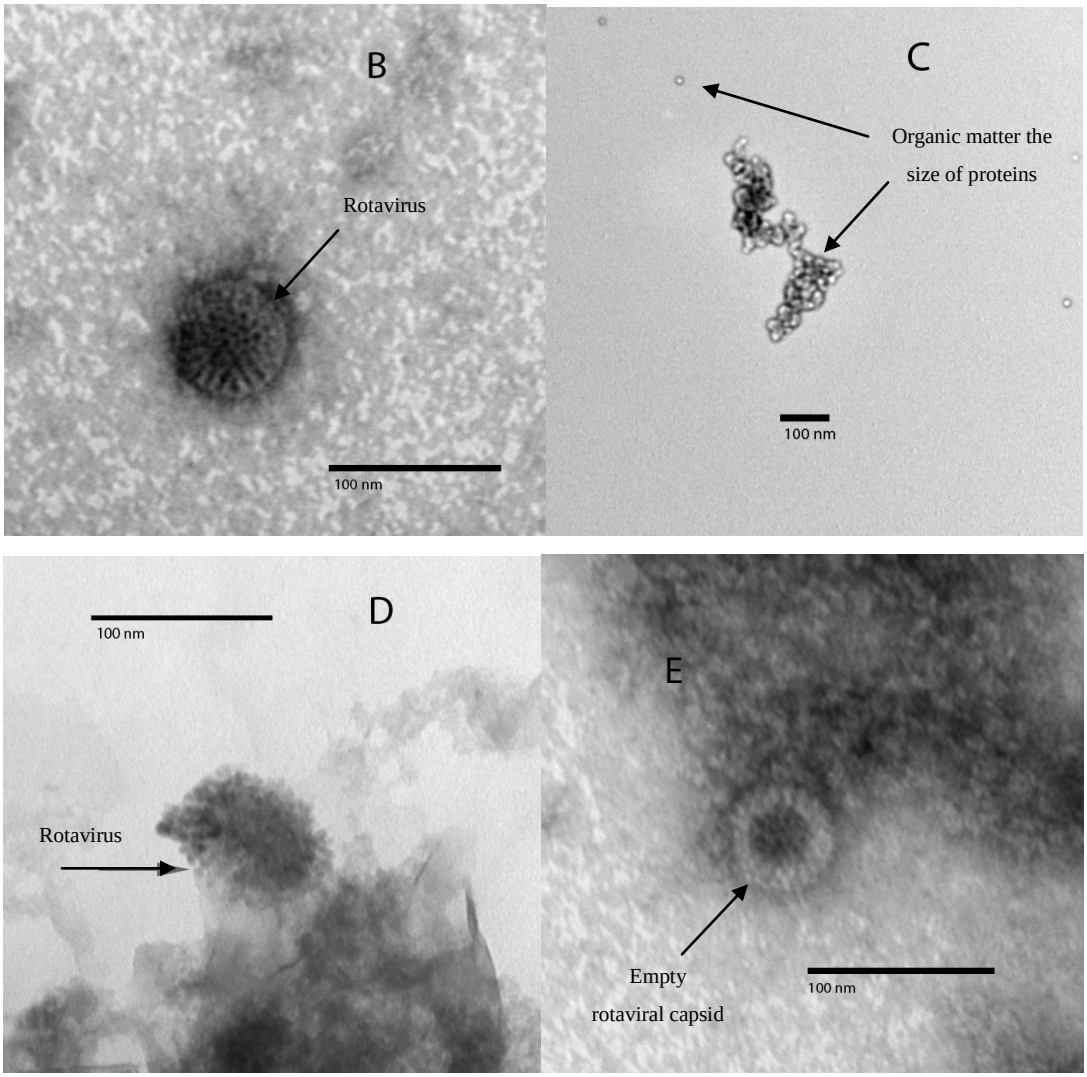



**Figure 3.2 TEM images after treatments**

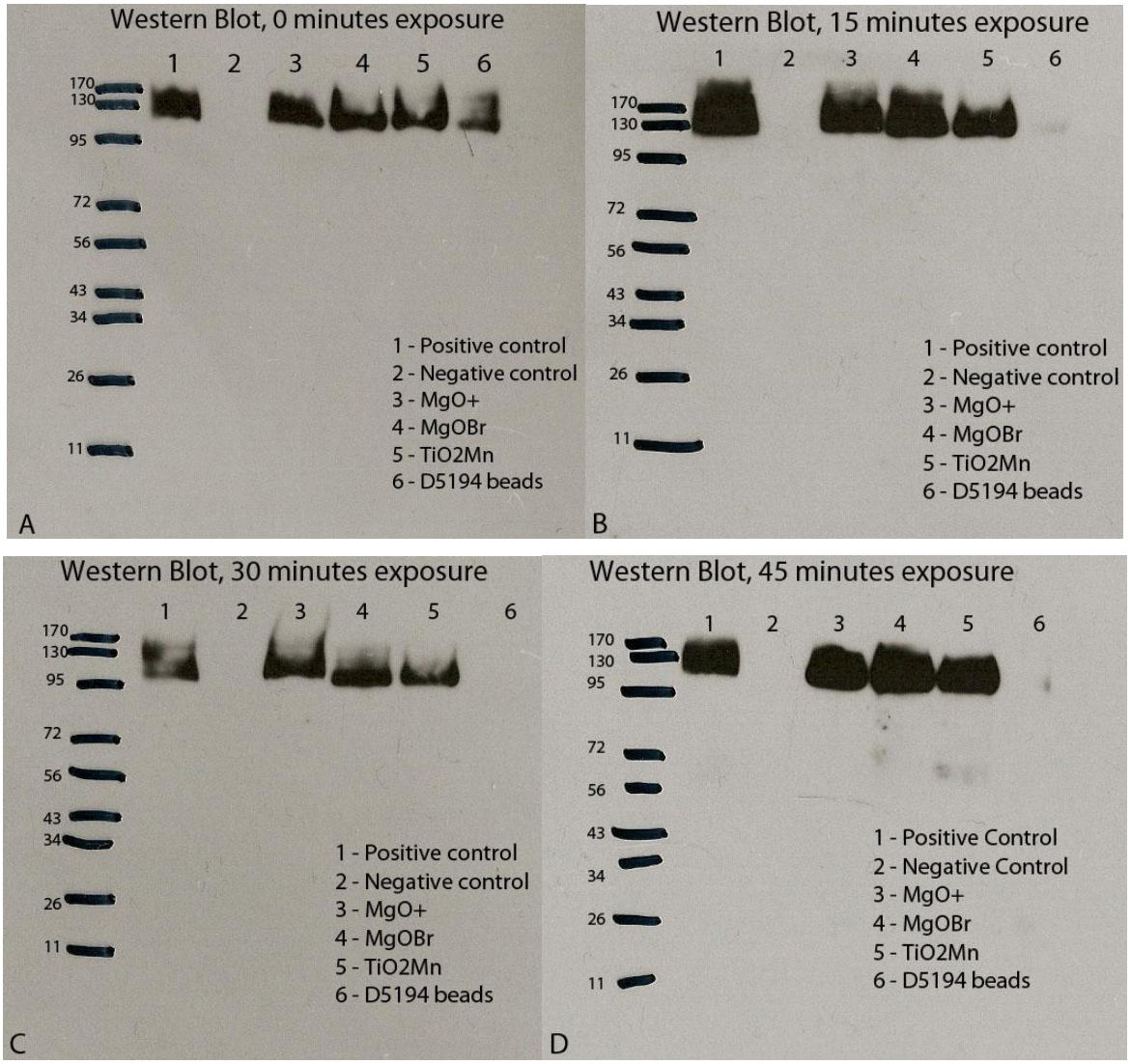
TEM images of rotavirus. Images A, B, D and E are at 130,000x and image C is 64,000x. Image A is an untreated sample of rotavirus. B are rotavirus particles treated with MgO nanoparticles. Image C are virus particles run through a column with the silver impregnated D5194 resin. While image C shows no complete rotavirus particles, the particulate matter is the size of, and looks like a viral protein capsid which has been 'ripped' open. D are rotavirus particles treated with Ti-Mn nanoparticles. In this image, the virus is closely attached and possible bound to the nanoparticles. E are rotavirus particles treated with MgOBr nanoparticles. Here is a complete viral particle, although it appears hollow (no viral RNA).

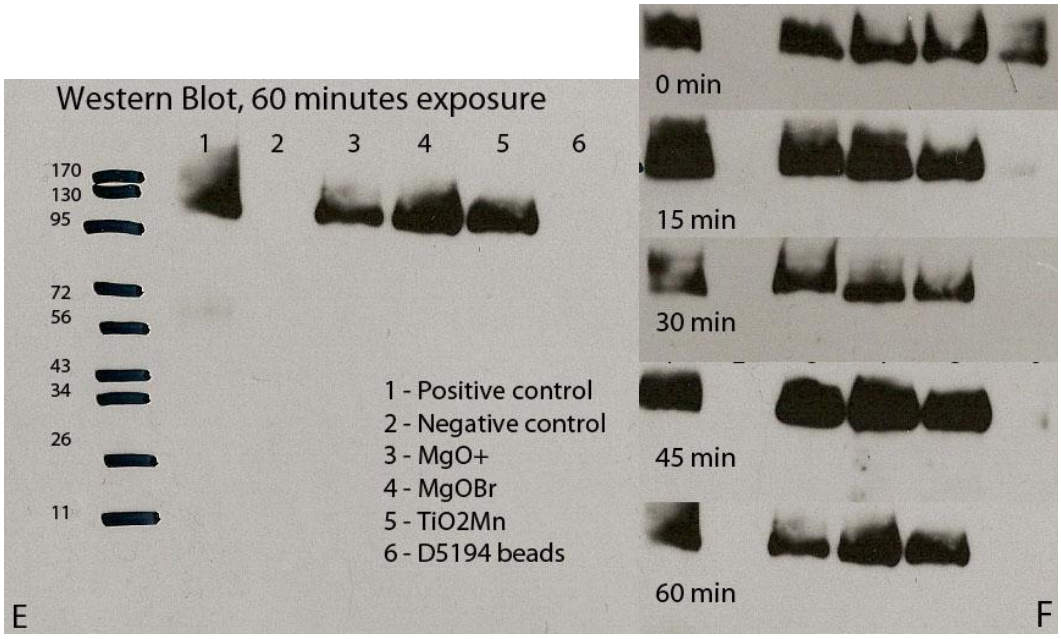
### *Western Blots of Treated Samples of Rotavirus*

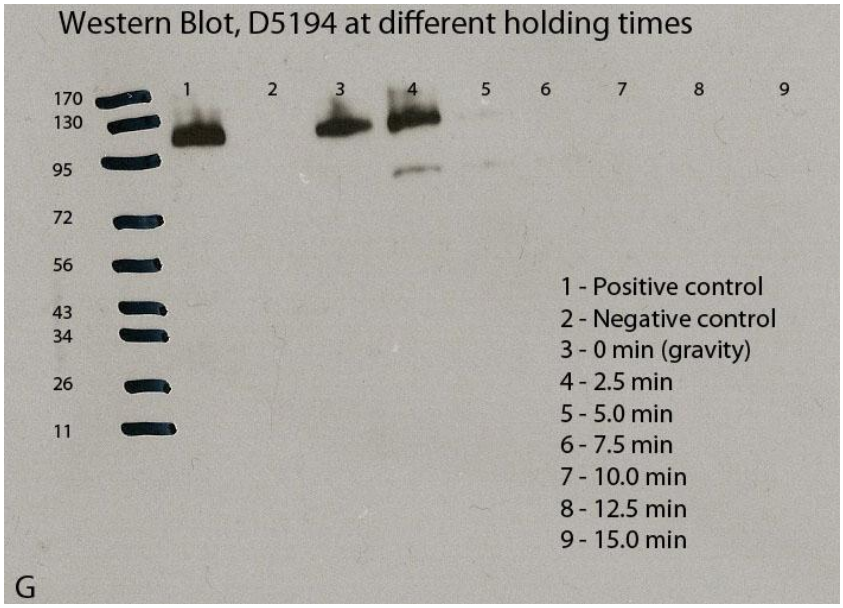
Previous studies have shown that silver interacts with proteins. In order to determine which conditions lead to alteration of proteins, the native gel Western blot was used. Samples of rotavirus were exposed to nanoparticles and silver loaded D5194 resin. For the nanoparticles, the virus interacted with 2ug/ml of the given nanoparticle for 0, 15, 30, 45, and 60 minutes. For the D5194, the virus was held in a column containing only D5194 for 0, 15, 30, 45, and 60 minutes. At 0 minutes, bands were present on the blot for all types of conditions tested. By 15 minutes and for every other time point beyond that, bands were present at all conditions tested except for samples that interacted with the ion exchange resin D5194. In order to further pin point the time in which viral protein is no longer detectable by immunoblotting, additional columns are prepared with 0, 2.5, 5, 7.5, 10, 12.5, and 15 minute holding times. By 5 minutes of expose to the silver ions of the resin, viral protein is no longer detectable. There is a slight qualitative difference between bands found on one membrane to the next. This is accounted for by the fact that both the membrane was exposed to the substrate, and the film was exposed to the membrane for slight variations in time. The membrane was exposed to the substrate for 5 +/- 1 minutes. The film was exposed to the membrane for 3 +/- 1 minutes.

**Figure 3.3 Western blots, D5194 destroys viral proteins**

Native Western blots at 0, 15, 30, 45 and 60 minutes (A-E). The summary (F) shows that by 15 minutes of exposure to the silver loaded D5194 resin, no detectable native rotavirus capsid protein is present. All other conditions tested showed the presence of native rotavirus capsid protein at all time points. Furthermore, when smaller time points are used, it is shown (G) that by as little as 5 minutes of exposure to the D5194 resin, that the native viral protein is no longer detectable. The positive control represents cell culture fluids that have been infected with rotavirus. The negative control represents cell culture fluids that have not been infected with rotavirus.


Western Blot, 0 minutes exposure
1 2 3 4 5 6
170
130
95
72
56
43
34
26
11
1 - Positive control
2 - Negative control
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194 beads
A
Western Blot, 15 minutes exposure
1 2 3 4 5 6
170
130
95
72
56
43
34
26
11
1 - Positive control
2 - Negative control
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194 beads
B
Western Blot, 30 minutes exposure
1 2 3 4 5 6
170
130
95
72
56
43
34
26
11
1 - Positive control
2 - Negative control
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194 beads
C
Western Blot, 45 minutes exposure
1 2 3 4 5 6
170
130
95
72
56
43
34
26
11
1 - Positive Control
2 - Negative Control
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194 beads
D



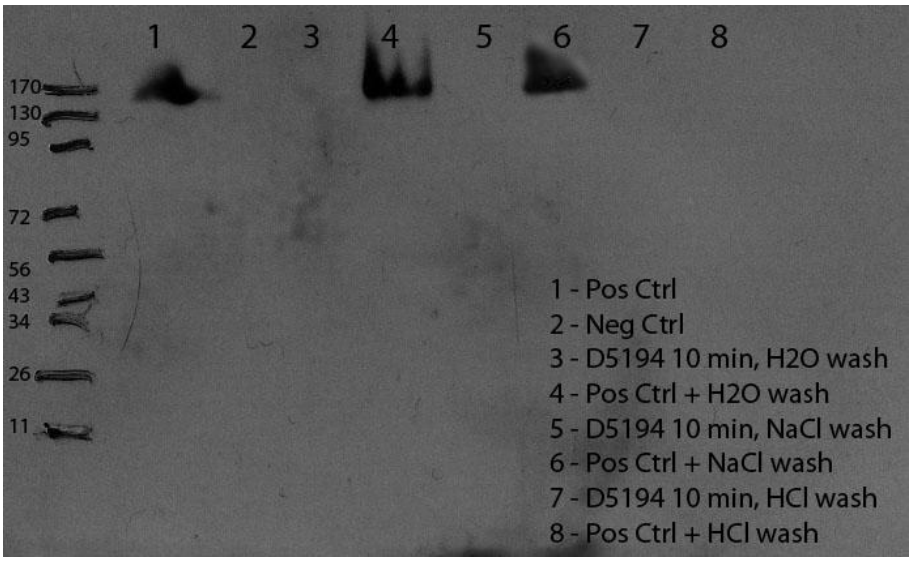

### *Detecting any Rotavirus Bound to the D5194 Resin*

Since the Western Blots previously shown do not directly address whether viral protein is being denatured or if virus is simply being bound by the resin, much like a mechanical filter, additional tests were required. In order to determine this, first an RT-PCR reaction was set up (results not shown) to see if the VP7 gene could be amplified without purifying the viral RNA. The idea would be that if it could not be amplified without purification, and then it became amplified after long term exposure to the column, that would indicate that the viral capsid is

being ripped open and viral RNA molecules are passing through the column. However, no amplification could be performed under any circumstances, indicating that the purification step is required. The next logical step was to then look at viral proteins using the Western blot technique. Six columns containing only silver loaded D5194 were prepared. To three of the columns, rotavirus was added for 10 minutes. Then, the virus was washed off with either de-ionized water, 35% hydrochloric acid, or 5M NaCl. The other 3 columns were simply washed off without prior exposure to the virus with water, HCl or NaCl. The reason to use HCl is to bring the pH inside the column so low that the silver on the iminodiacetic acid is replaced by protons. In doing so anything bound to the silver should flush off of the column as well. This flushing of the column can be visually confirmed by the formation of silver chloride, a white precipitate. In addition, high molar NaCl was used to elute the silver off. The high amount of chloride ions interact with the column and form silver chloride, and sodium replaces silver on the column. Again, anything bound to the silver will precipitate off the column. To these last three columns, positive control virus was added. These columns are required as controls to show that the effluent does not hinder the ability of the viral protein to be detected by the Western blot procedure. The date clearly shows a band for the positive control and no band for the negative control, as expected. When water effluent was added to the positive control, there was once again a band. There was also a band when the 5M NaCl effluent was added to the positive controAt ten minutes time of exposure, flushing the column with water, sodium chloride, and HCl did not show any bands.

**Figure 3.4 Viral protein cannot be detected after washing column**

Lane 1 is cell culture fluids infected with rotavirus and lane 2 are cell culture fluids not infected. Lane 3 had the positive control added to the D5194 column for 10 minutes and was then washed off with an equal amount of $dH_2O$. Lane 4 had the positive control unexposed to the column mixed with the effluent of flushing the column with $dH_2O$ to ensure that the flushing of the column doesn't inhibit detection of protein, especially for the NaCl and HCl treatments. Lane 5 is positive control in the column for 10 minutes and then washed with 5M NaCl. Lane 6 has the positive control not exposed to the column mixed with an equal amount of 5M NaCl after it has passed through the column. The same experiment was done in lane 7 and 8, respectively, except 35% HCl was used instead of $dH_2O$ or 5M NaCl.



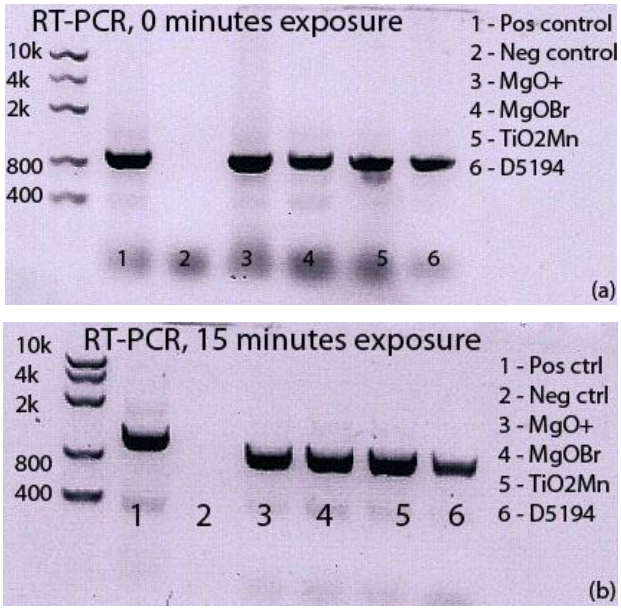

### *Reverse Transcriptase Polymerase Chain Reaction of Rotavirus*

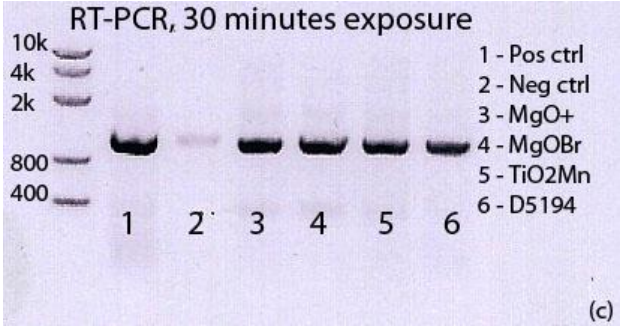
Rotaviruses have a genomic make up that is 11 unique segments of double stranded RNA. In order to amplify this RNA in a PCR style reaction, the RNA must first be amplified. Then, the double stranded RNA must be denatured to allow the gene specific oligonucleotides to bind the viral gene. Finally, all of the RNA must be converted to a cDNA intermediate. This intermediate is what is amplified in the RTPCR reaction. Magnesium oxide, brominated magnesium oxide, titanium dioxide/manganese, and the silver loaded D5194 resin were tested at 0, 15, 30, 45, and 60 minutes of exposure time. For each condition and each time point, the viral RNA was isolated and RTCR was performed. A band of about 1000 base pairs on the PCR gel would indicate a positive result, meaning that viral RNA is present in the sample. Lack of a band

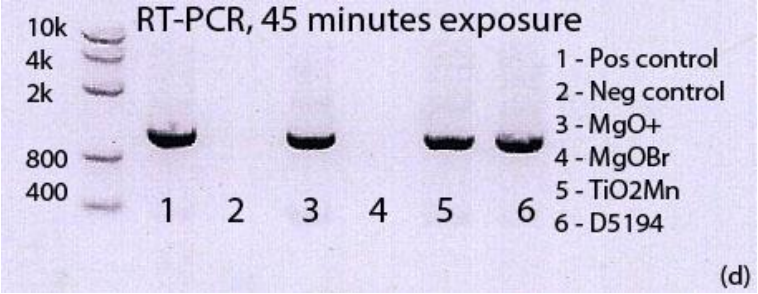
would indicate a negative result, meaning that there is no viral RNA detectable in the sample. The positive control is a sample of virus that has not been exposed to any conditions. This control shows a band, and so if the band disappears that would indicate that the nanoparticle or resins is causing the viral RNA to become not detectable by the oligonucleotides and thus not amplified. If the RNA cannot be amplified, it is likely that it cannot be replicated or translated either. The data shows that MgO and D5194 do not alter viral RNA. By 45 minutes, viral RNA is no longer detectable after exposure to MgOBr nanoparticles. Similarly, bands are lacking at 60 minutes of exposure to MgOBr. After 60 minutes of exposure to $TiO_2Mn$ nanoparticles, viral RNA is no longer detectable.

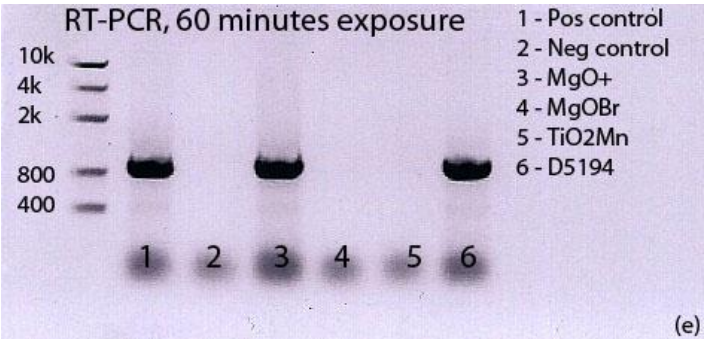
**Figure 3.5 RT-PCR, MgOBr and $TiO_2Mn$ alter viral RNA**

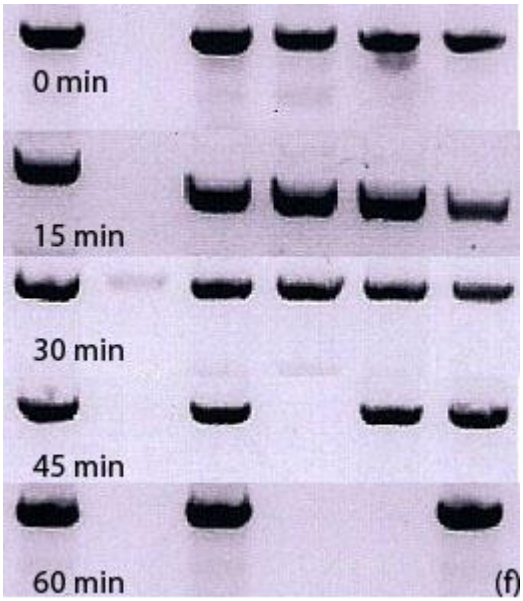
On all gels, the positive control is cell culture fluids infected with rotavirus. Alternatively, the negative control is cell culture fluids not infected with rotavirus. All other conditions are cell culture fluids that have been infected with rotavirus, and then treated appropriately. Gels (a-c) show detectable viral RNA at 0, 15 and 30 minutes. By 45 minutes (d), there is no longer detectable virus in fluids that have interacted with brominated magnesium oxide. At 60 minutes (e), not only was virus not detectable for MgOBr, but viral RNA was not detectable for the sample that has interacted with $TiO_2Mn$. Additionally, a summary of all five gels is shown (f). Again, the positive control represents cell culture fluids that have been infected with rotavirus. The negative control represents cell culture fluids that have not been infected.




RT-PCR, 30 minutes exposure
10k
4k
2k
800
400
1 2 3 4 5 6
1 - Pos ctrl
2 - Neg ctrl
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194
(c)


RT-PCR, 45 minutes exposure
10k
4k
2k
800
400
1 2 3 4 5 6
1 - Pos control
2 - Neg control
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194
(d)


RT-PCR, 60 minutes exposure
10k
4k
2k
800
400
1 2 3 4 5 6
1 - Pos control
2 - Neg control
3 - MgO+
4 - MgOBr
5 - TiO2Mn
6 - D5194
(e)


0 min
15 min
30 min
45 min
60 min
(f)

## Discussion

Due to its toxicity in humans, lead is one of the most well studied metals. For lead, it is shown that it interferes with many different proteins. First, it interferes with blood proteins such as ferrochelatase which inhibits heme synthesis and results in anemia. However, lead also acts as an analog to calcium. This causes a dysfunction in calcium pumps and is a leading reason why lead disrupts the nervous system (Cohen et al. 1981). Very few other proteins are as well studied as lead. However, silver is starting to get attention due to its inherent toxic properties. For unknown reasons, silver has been shown to have an affinity to kill and inactivate microbes ranging from bacteria to fungi to viruses, while causing little to no negative side effects on higher organisms like humans other than argyria, a condition where the skin turns blue. Recently, silver nanoparticles have been shown to kill *E. coli*. In their study, silver was shown to accumulate on the surface of the bacterium (Smetana et al. 2008).

In another recent study, silver nanoparticles were shown to inactivate Human Immunodeficiency Virus (HIV). In thier study, it was shown once again that silver tended to accumulate on the surface of the microorganism. In their study, the data suggests that silver is likely to interact with and cause dysfunction in viral surface proteins. The authors take it one step further by showing that nanoparticles greater than 10nm in size did not interact well and thus did not inactivate HIV. They showed a size dependent correlation, explaining that small nanoparticles are required in order to disrupt the viral protein (Elechiguerra et al. 2005).

The data observed in my study continues to suggest that silver plays a role in viral inactivation by causing disruption of viral surface proteins. By performing Western blots using an antibody specific for one of the viral capsid proteins, it was shown that ionic silver that has been loaded onto ion-exchange resins inactivates rotavirus by disrupting the capsid. In this portion of the study, the antibody used was specific for the native form of the capsid protein. Thus, if the protein was detected, the protein was in the native form. However, if the conformation of the protein is not completely in the native form, the protein can no longer be detected. It is not known if the protein is completely denatured or if the silver disrupts the detection of the protein by the antibody. The data does show that by 5 minutes of interaction with the silver loaded resin the antibody is no longer able to detect the viral capsid. This data correlates with what is seen in the TEM images. Here, there was a distinct lack of complete viral particles. Instead, there was a lot of particulate matter on the size order of protein. It is likely, as

some of this forms a higher ordered structure, that this is viral protein that has been denatured by the silver ions.

The data also showed that rotavirus can be inactivated by alteration of the viral genome. Due to the limited genome size of many viruses, genomic conservation is observed. This can manifest itself in various forms. Some viruses encode for proteins that are only translated as a result of the read-through of a stop codon. Others might conserve the size of their genome by having nested genes (Flint 2004). Space for essential genes is at a premium within the genome of most viruses. With this in mind, it is clear that very few viruses are capable of disposing of genes and still being able to replicate. In this study, VP7, or the gene that encodes for the G (spike) protein, is probed for using gene specific oligonucleotides. Using RT-PCR, it is shown that brominated magnesium oxide and titanium dioxide manganese nanoparticles are capable of disrupting the VP7 gene. This data once again agrees with the images produced by TEM. Here, empty viral particles are seen in a large abundance when exposed to MgOBr. Empty virions are generally considered to be lacking the viral genome. Additionally, when the MgOBr was tested for aqueous bromide ions, they were found in abundance. It is also shown that in all cases the magnesium oxide nanoparticle is rather inert, never being shown to inactivate virus. This suggests rather clearly that the mechanism of action for MgOBr in aqueous solution is that bromide ions form and are solely responsible for the inactivation of viruses. The inactivation is done by interfering and possibly destroying the viral RNA.

It is very clear that some of the tested compounds are capable of inactivating water-borne viruses. What is not clear is how these compounds will be best applied in a usable commercial product. For the ion-exchange resin, it is clear that this can simply be applied in a column and sold as such. Details that must be looked at include how salt water might affect the efficacy of the column and how long the column would last. For the nanoparticles, it is a little more complicated. If bromide is formed in solution from MgOBr, then it would be inevitable for the bromide to be consumed with the water. Instead of using these nanoparticles, the best usage for bromide might be to simply load it onto an ion exchange column. This data provides a basis of understanding for the mechanism of action for the tested compounds, and it is clear that further studies are needed in order to make these compounds functional in a commercial setting.

# CHAPTER 4 - Summary and Discussion

## General Summary

Water has long been a potential source of disease. Some of the earliest records of modern history include accounts of people trying to purify water to make it safe for drinking. At first, the grade of water was determined solely on its clarity and smell. It wasn't until the founding of microbiology, and then later the discovery of viruses, that man was able to understand that the source of the water-borne disease was microbiological in origin. These discoveries drastically changed the direction of how to go about purifying water. These discoveries proved that clear water that did not smell could still potentially cause disease. Microbiological grade filters were soon invented, which allowed bacteria to be mechanically removed from the water by not being able to pass through the size exclusion limit of the given filter. However, these filters were not capable of removing viruses, which are somewhere in the range of $1/100^{th}$ or $1/200^{th}$ the size of an average bacterium. Since these small infectious agents are not capable of being filtered, alternative methods were required. Early in chemical disinfection, sodium hypochlorite (bleach) was shown to be a potent disinfectant. However, at concentrations high enough to kill bacteria and viruses, the bleach can also be harmful to the individual ingesting the bleach laced water (Baker 1948). Instead, chlorines and chloramines were added to the water. This allowed a large portion of the disease causing microbes in the water to be killed without causing any harm to the consumer of the water. Additionally, chlorine was added to bathing pools in high enough quantities to kill poliovirus. Poliovirus is a water-borne virus that was commonly spread in public bathing pools. The addition of chlorine effectively killed the virus in the water, and was one of the major ways that polio infections have been significantly reduced around the world (Paul 1971).

Generally, water-borne viruses are very infectious, and only a few infectious virus particles are required for an infection. Not only that, but they are extremely resilient in normal environmental conditions. The viruses that are capable of surviving these conditions are still capable of causing disease. Couple that with the new emerging viruses such as enterovirus 71 (EV71) and norovirus, and you have millions of water-borne infections and hundreds of thousands of deaths due to agents of viral origin in the world per year. In fact, despite all the water purification techniques utilized world-wide, it is estimated that up to 4.1% of the global burden of disease is from water-borne infections (WHO 2007).

Recently, iodine has been shown to be a potent antimicrobial agent. In emergency situations, iodine can be used in large quantities to make unsafe drinking water consumable. However, the iodine is present in the water at such high quantities that if consumed over a long period of time it is capable of causing thyroid dysfunction and disease. Ion-exchange resins have been loaded with iodine in order to still provide the killing power without consumption of the high levels of iodine. While the levels of iodine are less, iodine still leaches off these resins, and if enough water is consumed, thyroid disease can still result (Brion et al. 2004). Multiple companies have sold these iodinated products in the past, but now multi-national government bodies are beginning to ban these substances, as they have been shown to be harmful with long term use (EU 2009).

These persistent viruses are requiring a new set of antimicrobial agents in order to provide safe drinking water to the world. The research presented here is an attempt at identifying some of these new antimicrobial agents that can be used in the future. In Chapter 2, the basic innate properties of these compounds are examined. First the silver loaded ion exchange resins were evaluated. Since these resins were used in the form of a column, all of the components of the column were evaluated in order to ensure that silver was truly responsible for the viral inactivation. When possible, GAC, MB400, and resins that had not been loaded with silver were tested for their ability to inhibit viral replication. In doing so, the titers of all three viruses tested were the same as the titers coming out of the column that only contained either GAC, MB400 or a given resin. However, when you took a resin like Chelex-100 that had been loaded with silver and made a column consisting of Ag-Chelex100, GAC and MB400, the column then had biocidal properties. This clearly links silver to the apparent inactivation of these viruses and not any other component of the column. One shortcoming in this data is that it does not show whether the virus is being bound within the silver loaded resin or whether is it actually being rendered non-infectious. If it was being bound within the resin, like a filter, this would mean that there are still infectious particles within the column. The data presented does not directly address this question, and it will be further discussed during the summary of Chapter 3. Additionally, when examining the resins, it is clearly shown that iminodiacetic acid resins out-perform phosphonic acid resins. In Figure 2.2, it is shown that Chelex-100, Dowex, and D5194 resins loaded with silver are all capable of completely inactivating all three viruses tested. All three of these resins are IDA resins. Conversely, the same figure shows that S930 and S939

are only capable of completely inactivating calicivirus, and not inactivating rotavirus and teschovirus. This data clearly supports the use of a silver loaded IDA resin, and not a phosphonic acid resin, in order to develop a commercially viable water purifier product.

With this data it is very clear that these resins have value in being able to sterilize water. However, there has been much debate about how exactly silver ions are capable of inactivating microorganisms. Some researchers have said it may be due to oxygen radical formation, while others claim that it directly interacts with proteins and even nucleic acid. The Western blot data presented in Figure 3.3 shows that after 5 to 7 minutes of exposure to the silver loaded column, native rotaviral capsid protein is no longer detectable. This does not tell us if the protein is being denatured or if it is simply being bound to the column like a mechanical filter. Previous data suggests that it does not bind to resins like Chelex-100. However, the D5194 resin used in the Western blot assays was provided to the lab only in the silver loaded form, and no unloaded resin was available for testing in the lab.

In addition, another Western blot was performed to shed light on this topic. In Figure 3.4, rotavirus was exposed to columns containing the silver loaded D5194 resin only. After ten minutes, the native protein was no longer detectable, as seen in lane 3. In an attempt to remove the silver and anything bound to it or the column, either 5M NaCl or concentrated HCl was washed over the column. In both reactions, silver chloride was formed and the column was removed of silver. In neither case was native protein detectable, further indicating that the virus does not bind the column. However, in order to determine if the silver salts formed interfere with the Western blot assay, columns were made and the silver was removed either by NaCl or HCl. The resulting effluent was then mixed with positive control virus and tested for the presence of native viral protein. In the case of HCl, native protein was not detectable, indicating that the low pH of HCl is probably denaturing the protein. The NaCl sample, however, did show a band for native rotavirus capsid protein. This would then indicate that high salt concentrations at neutral pH do not interfere with the Western blot procedure. Therefore, since no band was present when the virus was eluted off the column by NaCl, this would strongly suggest that rotaviral proteins are not being bound by the column and are instead being denatured by silver ions present in the ion-exchange column.

The data shown in the Western blot assays support the visual data obtained by TEM. In Figure 3.1c, an image is presented that is representative of what effluent fluids look like. In this

image, very few intact virions are found and a high amount of proteinaceous matter is present. As a whole, the data presented here suggests that the silver is denaturing protein, which causes the puzzle like capsid structure to simply loose integrity and fall apart. A virus particle that loses its protein structure is a virus particle that is rendered non-infectious because it can no longer interact with cell surface proteins required to initiate infection of the cell. This data supports previous reports that silver has a tendency to interact with proteins.

The virucidal capabilities of metal based nanoparticles were also tested. The data presented here shows that the salt magnesium oxide did not have any biocidal properties and there was no mechanism of action for killing. However, when this salt is brominated, it becomes a potent killer. A few minutes of exposure to viruses, this nanoparticle is able to render virions non-infectious. As seen in Figure 2.3, within only 2-3 minutes of exposure, this nanoparticle can inactivate up to 100,000 infectious virions per milliliter. This is around ten times the recommended limit by the EPA for a substance that can be used in water purification. This nanoparticle has a huge upside in that it is very potent, but its ultimate effectiveness will be determined upon its apparent mechanism of action. While there was no apparent alteration in the viral proteins upon interaction with this nanoparticle, there was the loss of the ability to detect the VP7 gene of rotavirus. After 45 minutes of interaction with the nanoparticle in solution, the VP7 gene was no longer able to be amplified by the sensitive RT-PCR protocol, as shown in Figure 3.5. This is significant, as it indicates that somehow the virus is being inactivated through destruction of its genome.

The question still remains on how exactly it is happening, though. It is obvious that bromine is plays a huge role, as the MgO counterpart is shown to be inert. So, in order to determine if the bromine remains attached to the nanoparticle or if it dissociates, the experiment in Figure 3.1 was performed. It is shown here that a yellow color change occurs when KI is added to MgOBr supernatant fluids. In addition, since iodine levels are high within the solution, what is probably happening is that iodide is being converted to iodine by way of bromine. Since dilute amounts of iodine are yellowish in solution, this explains the color change. Regardless, the color change is indicative of free bromine being present in the supernatant solution. This supports the idea that bromine dissociates from the MgO nanoparticle in solution. This also implies that the bromine is most likely responsible for the inactivation of the viruses through degradation of the viral genome.

| Agent | Efficacy | Proposed Mechanism |
|---|---|---|
| Silver loaded ion exchange resins | Up to 10,000 virions killed in minutes | Denaturing of surface proteins |
| MgO Nanoparticles | No observed killing | No observed killing |
| MgOBr Nanoparticles | Up to 10,000 virions killed in minutes | No RNA replication |
| $TiO_2SiO_2Mn$ Nanoparticles | Up to 10,000 virions killed over 2 hours | No RNA replication |

**Table 4.1 Summary of killing efficacy and proposed mechanism of action**

Lastly, the biocidal properties of the $TiO_2Mn$ nanoparticle were evaluated. These nanoparticles were once again found to have biocidal properties, albeit to a lesser extent compared to the MgOBr nanoparticles. While rotavirus and calicivirus were shown to be inactivated by the $TiO_2Mn$ nanoparticles slowly over a 2 hour period, perhaps oddly it showed no ability to inactivate teschovirus. For the viruses that it was able to inactivate, it did so in a slow and monotonous fashion. It would appear on the surface that the mode of action would have to be different, as compared to the silver loaded resins and the MgOBr nanoparticles. When observed using TEM as shown in Figure 3.2d, few virions were found alone. Instead they were almost always found associated with clumps of $TiO_2Mn$ nanoparticle material. This indicated that the virus is actually being bound by the nanoparticles and the $TiO_2Mn$ compound is not itself virucidal. Viral proteins were detectable by Western blot after as much as 60 minutes of interaction with the nanoparticles. However, the data does not show if the virus is freed back into solution by the centrifugation step used in the Western blot. After 60 minutes of interaction, the VP7 gene was not detectable by RT-PCR. This would then indicate that virus is not being released in the centrifugation step and, rather, that the nanoparticles are destroying viral RNA. It is not shown, however, whether the $TiO_2Mn$ nanoparticles themselves are capable of interfering with the RT-PCR process. Overall, these results indicate that the virus is being inactivated by the nanoparticles by destroying viral protein.

In summary, the data collected and presented here indicates that silver loaded ion exchange resins are very capable of inactivating viruses. This is done through silver interacting with available surface proteins. In the case of the viruses used in this study, they were all non-

enveloped nucleocapsid viruses and the alteration of the protein by the silver is proven fatal for the viruses. The nanoparticles appear to inactivate viruses through a different mode of action. Here, the MgOBr and $TiO_2Mn$ nanoparticles both inhibit amplification of viral genes by RT-PCR. This would indicate that the viral genes are being disrupted. If such a virus was able to enter a cell, it would not be able to replicate its own genome, or make viral proteins. This virus would not be able to complete the cycle of infection, and this is likely why the virus is non-infectious.

## Future Directions

The data provided in Chapter 2 concerning the effectiveness of silver loaded ion exchange resins is a good start to understanding how these viruses can be inactivated through such a column. There are many factors that still need to be looked at. For example, all samples tested were with water that was already relatively clean. The limitations of the column need to be further addressed. For example, data needs to be provided showing how the column would perform in the presence of higher salts concentrations, in the presence of dirt, or just in the presence of other ions generally associated with "hard" water. The data shown in Chapter 3 would indicate that at a high enough salt concentration, silver will dissociate from the column in the form of silver salts and pass through as part of the effluent. In some parts of the world, the water consumed by many does not even pass the old standards of clean water, turbidity and smell. In the regions where this kind of water is consumed are also the regions where higher levels of disease can be found, and so it is important that if such a column is able to reduce the global burden of disease that dirty water parameters must be looked at. The question that needs to be addressed is at what concentration of salt or other non-purities can the column be exposed to and still keep enough silver loaded in order to be efficacious and have silver leaching levels below EPA and international standards.

Another parameter that should be addressed is determining how many liters can be passed through a column while the column still has the ability to kill the EPA required 10,000 viruses/ml. This parameter must be determined for all types of water. Clean water, water with high ion concentrations, and water with high levels of dirt must be evaluated. This is important, once again, from a commercial standpoint. If the column is only able to be used for a few liters, the worth of the device is somewhat limited. This work goes somewhat hand in hand with

determining what salts can be passed through the column in order to maintain its efficacy. For example, if only a few liters of dirty water are passed through the column and it still can kill bacteria; but then when 100 liters are passed through and it loses its efficacy, then it may not be an overall useful column.

In Chapter 3, the mechanism of action for the silver loaded column is hinted at. From the data it is shown that native viral protein is no longer present after interaction with the column. When the column was salted in order to remove the silver and anything bound to it, no detectable viral protein was found. This indicated that there was no virus bound to the silver. In Chapter 2, resins are used that did not have any silver loaded onto them. These resins contained no inherent virucidal capabilities, but empty D5194 was not tested. Since this is the resin used during all of the Western blot experiments, we cannot say for sure if the virus is still bound to the resin or not. Unfortunately, the manufacturer of the resin had only sent us silver loaded D5194 and they are now unwilling to give our collaborators, Safe Water, any further resin to use. If the day came where this resin was readily available, it would be important to show that the virus is not binding to the resin in order to say for sure that the viral proteins are denatured after interacting with the resin.

Also in Chapter 2, it is shown that $TiO_2Mn$ and MgOBr nanoparticles have some inherent biocidal capabilities. The $TiO_2Mn$ nanoparticles have a slow effect on the viruses, and in the case of teschovirus, it does not show any virucidal capabilities. It is debatable as to whether this nanoparticle would have any usefulness in water purification. The question does remain as to whether any parameters could be used in order to enhance the virucidal capabilities it does have. Things such as temperature, pH, and possible light activation of the nanoparticles would be good parameters to look at in order to determine if the nanoparticles can kill viruses quicker.
Since the MgOBr nanoparticles have such a quick mode of action, it may be possible to apply their use into real world applications. However, since it is shown in Chapter 3 how it is likely that bromine dissociates from MgO in aqueous solution, the application of this nanoparticle into a column where water passes through would not be the best way to use the nanoparticles. If this were the case, the bromine would simply just pass through and out of the column. Since bromine is the reason why these nanoparticles can inactivate viruses so quickly, such a column would lose efficacy rapidly. Instead, maybe it would be possible to add these nanoparticles or bromine alone to an ion exchange resin. In this case, a basic resin would be needed in order to

chelate the bromine or bromide. Several publications are present showing how bromide can be added to a resin, but none are currently present on the use of these ion exchange beads as a mode to kill microorganisms.

Finally, the mode of action of the MgOBr and $TiO_2Mn$ nanoparticles needs to be further looked at. Some basic work is shown in Chapter 3 showing how there is no longer any viral RNA left for amplification using RT-PCR. This work is only done with rotavirus, which is a double stranded RNA virus. Teschovirus and calicivirus are single stranded plus sense RNA viruses, which could potentially behave differently. So, PCRs would be need on these viruses too. Also, it may be useful to us Q-PCR in order to determine how much viral RNA is present. This would be useful showing how quickly the process happens and would also support previous work.

The work presented show much promise for helping prevent water-borne diseases, but much more work is needed in order to turn these product from something sitting on a laboratory shelf into a product that can be used by the people afflicted by these devastating diseases. Not only that, but spinoff applications from this work are abundant. First, there is silver. Silver has been previously shown to help prevent bacterial infections. This work confirms that viruses can also be inactivated by silver, and so useful products can be designed to prevent viral infections as well. Additionally, ionic bromide as a disinfectant is a somewhat unexplored world. It is well known that bromide compounds such as ethidium bromide intercalates into DNA, and so it makes sense that this compound would be a good antimicrobial agent. However, as mentioned earlier, applying it to a useful product will be the challenge.

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In accordance with Article 4(2) of Regulation (EC) No 2032/2003, biocidal products containing active substances for which a non-inclusion decision was taken shall be

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