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Virus Inactivation by Silver Doped Titanium Dioxide Nanoparticles for Drinking Water Treatment

by

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ABSTRACT

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UV disinfection of drinking water is becoming more common as water utilities attempt to control the formation of disinfection byproducts. While most organisms are readily inactivated by UV, certain viruses require a large fluence for adequate disinfection. In this study, photocatalytic silver doped titanium dioxide nanoparticles were investigated for their capability to enhance the UV disinfection of Bacteriophage MS2. The inactivation kinetics were compared to the base TiO₂ material and silver nanoparticles. Inactivation of MS2 was enhanced by doping TiO₂ with 8 and 10 wt. % silver, while no enhancement was observed with 4 and 6 wt. % silver. In order to determine the inactivation mechanism, alcohol scavengers were employed to eliminate the effects of hydroxyl free radical. When nAg/TiO₂ was used as the catalyst, the alcohols significantly decreased the inactivation rate, but did not completely eliminate the virucidal activity. When P25 TiO₂ was used, no virus inactivation was observed.
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INTRODUCTION

As a fundamental requirement for life, the importance of a clean, reliable source of drinking water cannot be understated. The effective disinfection of drinking water is one factor that has resulted in the relatively long life expectancies found in industrialized societies. However, many developing nations lack an adequate clean water supply, and water borne diseases are still prevalent [1]. Even where adequate water disinfection is commonplace, other problems, such as the formation of disinfection byproducts (DBPs), become a concern. DBP formation is of greatest concern when free chlorine is used to disinfect waters of high organic content. However, other disinfectants, such as chloramines and chlorine dioxide have been shown to produce DBPs as well [2]. A wide variety of compounds are formed as DBPs, and some such as trihalomethanes and haloacetic acids are known carcinogens [2].

Due to this, the USEPA has recently established regulations controlling the acceptable levels of DBPs for drinking water [2]. This in turn has increased the usage of UV disinfection systems, as no DBPs have been found to be formed when using this method of treatment. Several point of use disinfection systems based on UV technology are also becoming commonplace (SteriPEN®, Trojan UV MAX™). A major limitation with UV disinfection is that some viruses are highly resistant, especially Adenoviruses. Adenoviruses have been used by the EPA to set the UV fluence requirements for 4 log removal of viruses from drinking water to 186 mJ/cm² from 40 mJ/cm² [3]. The new high fluence requirement requires significant energy consumption which translates into a high treatment cost. Therefore, improving the viral inactivation efficiency of the UV disinfection process is a critical need in the water treatment industry. The purpose of this
study is to investigate a photocatalyst based on silver doped titanium dioxide nanoparticles (nAg/TiO₂) for virus inactivation in order to increase the effectiveness of the UV disinfection process for drinking water treatment.
BACKGROUND

UV Disinfection

UV radiation principally harms microorganisms by damaging the genetic material. UV radiation at 254 nm specifically causes dimerization of thymine and uracil residues [4]. Other wavelengths of UV radiation are known to be absorbed by proteins, which may cause damage to critical enzymes. While chlorine is ineffective at inactivating protozoa, UV radiation efficiently inactivates these organisms along with bacteria. However, some viruses, which are readily inactivated by chlorine, are highly resistant to UV disinfection. Adenoviruses in particular are highly resistant.

Mechanism of TiO₂ Based Photocatalysis

One method of increasing the efficiency of the UV disinfection process is to utilize a photocatalyst, such as TiO₂, that generates excited electrons and holes that can drive redox reactions when exposed to UV radiation. This technique has already been incorporated into several commercial water purification systems (Wallenius AOT®, Purifics®), but usage is not widespread. TiO₂ is an attractive material for water purification in that it is resistant to corrosion and is nontoxic when ingested [5]. The mechanism of TiO₂ photoactivation is shown in Figure 1. When the semiconductor absorbs a photon with energy equal or greater than the bandgap energy, an electron (e⁻) is promoted from the valence band to the conduction band, which leaves a positively charged hole (h⁺) in its place. After charge separation, several fates are possible for the excited electron and hole. Ideally e⁻ and h⁺ will migrate to the semiconductor surface and
either directly reduce or oxidize solution components, or become trapped on the particle surface and subsequently engage in redox reactions. Alternatively, the separated charges can recombine, generating heat and reducing the photocatalytic efficiency of the material.

![Figure 1. Mechanism of charge generation and transfer in TiO₂ photocatalysis, Adapted from Hoffman et al. (1995) [7].](image)

In a typical environmental application of TiO₂ photocatalysis, oxygen is present to readily accept exited electrons. Adsorbed oxygen is first reduced to superoxide anion (O₂⁻), from which hydroxyl free radical (HO⁻) and several other ROS species are subsequently produced as detailed in reactions (1) – (11) [6].

\[
\begin{align*}
O_2 + e^-_{cb} &\rightarrow O_2^- & \text{(1)} \\
O_2^- + H^+ &\rightarrow HO_2^* & \text{(2)} \\
HO_2^* + HO_2^* &\rightarrow H_2O_2 + O_2 & \text{(3)} \\
O_2^- + HO_2^* &\rightarrow HO_2^- + O_2 & \text{(4)} \\
HO_2^- + H^+ &\rightarrow H_2O_2 & \text{(5)}
\end{align*}
\]
\[ O_2^* + H_2O_2 \rightarrow HO^* + OH^- + O_2 \] (6)

\[ H_2O_2 + e^- \rightarrow \cdot H_2O_2 \] (7)

\[ O_2^- + \cdot H_2O_2 \rightarrow HO^* + OH^- + O_2 \] (8)

\[ H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \] (9)

\[ H_2O_2 + 2h^+ \rightarrow H^+ + O_2 \] (10)

\[ H_2O_2 + h\nu \rightarrow 2HO^* \] (11)

The holes vacated by the excited electrons in TiO2 are another source of ROS. Holes are capable of directly oxidizing adsorbed compounds, or becoming trapped as Ti-HO'. Holes can also oxidize H2O and OH' to form HO'. The HO' can either remain adsorbed on the TiO2 surface or migrate into the bulk solution and oxidize other solution components [7].

**Antimicrobial Properties and Mechanisms of TiO2 Based Photocatalysis**

The antibacterial properties of TiO2 have been well characterized in several studies [8-21]. The antibacterial activity is attributed to the generation of reactive oxygen species (ROS), especially hydroxyl free radical (HO') and hydrogen peroxide (H2O2) [14]. While fewer studies have investigated the antiviral properties of this material, its potential for inactivating viruses has been demonstrated [10, 20, 22].

Indirect evidence for the ROS responsible for microbial inactivation can be found through the addition of ROS specific scavengers to the reaction media. Alcohols, especially methanol and tert-butanol, have been used as HO' scavengers in several
photocatalytic studies [10, 23-25]. Reactions 12-14 detail the scavenging mechanism of these alcohols [24, 26].

\[ \text{CH}_3\text{OH} + \text{HO}^* \rightarrow \text{CH}_2\text{OH} + \text{H}^+ + \text{OH}^- \] (12)

\[ \text{*CH}_2\text{OH} + \text{HO}^* \rightarrow \text{HCHO} + \text{OH}^- + \text{H}^+ \] (13)

\[ (\text{CH}_3)_3\text{COH} + \text{HO}^* \rightarrow \text{*CH}_2\text{C(CH}_3)_2\text{OH} + \text{H}_2\text{O} \] (14)

There is some debate on whether methanol will scavenge surface bound holes. A study by Sun and Pignatello (1995) investigating the degradation mechanisms of 2,4-dichlorophenoxyacetic acid degradation by TiO₂ concluded that methanol (and tert-butanol) will not scavenge surface bound holes [25]. Two other studies claim that methanol will scavenge holes [10, 23] however the references cited in these studies lead back to Sun and Pignatello (1995). A useful difference between the two alcohols is that tert-butanol in some cases will not react with all surface bound HO⁻, while methanol will scavenge both surface bound and bulk HO⁻ [10, 24]. While one study has shown tert-butanol competitively adsorbs to TiO₂ [25], another demonstrated that it does not scavenge all surface bound HO⁻ when the concentration of the substrate to be degraded is higher on the TiO₂ surface than in solution [24]. Cho (2005) showed that that bulk HO⁻ is primarily responsible for the inactivation of MS2 by using methanol and tert-butanol as HO⁻ scavengers. This study also demonstrated enhanced MS2 inactivation with the addition of iron, which enhances HO⁻ formation via the Fenton reaction [10]. Another study demonstrated that MS2 inactivation by TiO₂ increases with the addition of ferrous sulfate [27]. A study investigating MS2 inactivation by fullerol found that singlet oxygen
and superoxide anion were the key ROS species responsible for viral inactivation by utilizing beta carotene and superoxide dismutase as scavengers for these ROS [28]. Therefore, other ROS besides HO' can result in inactivation of MS2.

**Metal Doped TiO₂ for Enhanced Photoactivity**

While the antimicrobial potential of TiO₂ has clearly been demonstrated, the material is limited by high rates of charge recombination. Recombination results in decreased photocatalytic activity and thus reduced inactivation kinetics. One strategy that has been used for enhancing charge separation is metal doping of the TiO₂ surface. Metal doping is thought to enhance photocatalysis by trapping excited electrons and preventing charge recombination (Figure 2) [29-32]. Electron trapping can occur if the dopant has a lower Fermi level than the excited electron.

![Figure 2](image)

**Figure 2.** Electron capture by a noble metal on a TiO₂ surface. Adapted from Iliev et al. (2006) [32].
Silver in particular has been shown to enhance the photocatalytic efficiency of TiO₂ for both contaminant degradation and bacterial inactivation [6, 17, 33-41]. However, there is limited information on the antiviral capabilities of this material [42]. Along with facilitating charge separation, silver is thought to enhance TiO₂ photocatalysis by providing more surface area for adsorption and directly interacting with microorganisms [43, 44]. However, one study demonstrating the enhanced activity of nAg/TiO₂ found that silver did not increase the surface area according to BET analysis [6]. Silver ions and nanoparticles have been shown to have antimicrobial properties themselves. Silver ions bind to the thiol groups of cysteine residues in microbial enzymes, hindering their functions, as well as producing damaging ROS, even without UV irradiation [45]. Silver ions have also been shown to damage bacterial cell membranes and inhibit DNA replication [46]. Silver nanoparticles have been shown to bind to external glycoproteins on HIV-1 viruses, inhibiting their infective mechanism [47]. A study by Morones et al (2005) suggested that three mechanisms are involved in the antibacterial action of silver nanoparticles against gram negative bacteria: 1. Particles from 1-10nm bind to the cell membrane and disrupt its function. 2. Particles enter the cell and disrupt the functions of sulfur and phosphorous containing compounds. 3. Particles release silver ions which have been shown to have antimicrobial activities [48].

Utilizing silver to enhance the antimicrobial action of TiO₂ photocatalysis allows several different inactivation mechanisms to work in concert. Therefore, it is possible that a synergism occurs between silver and TiO₂ when silver doped titanium dioxide (nAg/TiO₂) is used for inactivating microorganisms under UV radiation. While dissolution of silver may pose a challenge in implementing this technology, the reducing
power of activated TiO₂ may aid in keeping the metal in its zero valent state. This in turn may limit the effect of silver ions on microorganisms.

The optimum silver content for enhancing TiO₂ photocatalysis has been suggested in several studies. One study examining the simultaneous photochemical reduction of Ag⁺ onto Degussa P25 TiO₂ and degradation of sucrose and salicylic acid found that 2 atomic % of Ag was the optimum silver loading after testing a range of 0.5-20 atomic % Ag [6]. Two other studies that synthesized both TiO₂ and nAg/TiO₂ via the sol-gel route found that 5 atomic % of Ag was the optimum silver loading after testing a range of 0-10 atomic % Ag for Rhodamine B dye degradation [39] and 0-5 atomic % Ag for Rhodamine 6G dye degradation [38].

Multiple explanations have been given for an optimum silver content on TiO₂ [6]. High silver contents can decrease the amount of light reaching the TiO₂ surface and reducing the generation of excited electrons and holes [49, 50]. When applied above the optimum loading mass, the deposited silver can also act as recombination centers by capturing holes [51]. Smaller silver particles also may capture holes more readily than larger deposits, making them unavailable for oxidizing other solution components [51].

Effects of Solution Chemistry on TiO₂ Based Photocatalysis

For the application of TiO₂ based photocatalysis for water treatment, the effects of solution chemistry are an important consideration. In a study of the degradation of salicylic acid, aniline, and ethanol by P25 TiO₂, it was found that Cl⁻, PO₄³⁻, SO₄²⁻ decreased the degradation rate, while NaClO₄, NO₃⁻, and Na⁺ had no effect [52]. Bicarbonate is a known hydroxyl radical scavenger, and HCO₃⁻ was found to decrease the
degradation of NOM by TiO$_2$ [53]. In a study of MS2 inactivation by Degussa P25 TiO$_2$, NO$_3^-$, SO$_4^{2-}$, PO$_4^{3-}$, K$^+$, and Ca$^{2+}$ were found to decrease the inactivation rate, while Cl$^-$, Br$^-$, and Na$^+$ had no effect [54]. While the ions that reduce the efficiency of TiO$_2$ photocatalysis are common constituents in natural waters, their presence does not completely eliminate the photocatalytic activity.

**Bacteriophage MS2**

Bacteriophage MS2 is a single stranded (+) RNA virus with an icosahedral capsid about 25 nm in diameter [55]. MS2 is similar to some water borne pathogenic viruses and has been used as a surrogate in several disinfection studies [10, 56-58]. Compared to other bacteriophages, MS2 has been shown to be more resistant to UV disinfection [59]. In disinfection studies using chlorine and chloramines, MS2 was found to be comparable or more resistant than Hepatitis A virus [60] and Poliovirus [61]. MS2 has also been recommended by the EPA as an indicator for viral inactivation processes [62]. MS2 is particularly convenient to work with, as its propagation and enumeration are relatively simple when compared to procedures required with pathogenic human viruses.

**Inactivation of Bacteriophage MS2 by TiO$_2$ and nAg/TiO$_2$**

While bulk phase HO$^-$ has been shown to be responsible for MS2 inactivation [10], other studies have shown that the inactivation rate is directly proportional to the amount of MS2 adsorbed to the TiO$_2$ surface [57]. Increased adsorption may enhance the inactivation rate by placing the virus in close proximity to newly generated bulk HO$^-$. Since adsorption is an important factor, solution pH, which affects the electrostatics of
both TiO$_2$ and MS2, is an important variable in the system. The pH corresponding to a zero zeta potential on TiO$_2$ differs depending on crystal structure and synthesis procedure. For Degussa P25, one study found the zeta potential to be zero at approximately pH 5.5 [63], and another found it to be zero at approximately pH 6.8 [64]. The zeta potential of MS2 is zero at a pH of approximately 3.5 [65, 66]. The surface charge of MS2 has been calculated to be zero at a pH of 4 based on the ionizable surface amino acid residues [65]. A study investigating MS2 adsorption to Degussa P25 TiO$_2$ over a pH range of 3-10 found that the optimum pH for adsorption was be between pH 5 and 6 [57] where the TiO$_2$ carries little to no surface charge and MS2 has a negative surface charge.

In the nAg/TiO$_2$ system, silver may enhance adsorption of MS2 by interacting with the sulfur on cysteine residues. The primary sequence and three dimensional structure of the coat protein of MS2 are well known [65, 67, 68]. The capsid is made up of 180 copies of a single polypeptide [65]. There are two cysteine residues in each copy of the coat protein [67], however only one cysteine residue from each polypeptide is present on the capsid surface [65]. MS2 also contains a surface protein, denoted as the A protein, that contains 3 cysteine residues [69, 70]. This protein is required for viral infectivity [71] and is therefore an ideal target for inactivation of the virus. The 183 cysteine residues on the surface may interact with the nAg on nAg/TiO$_2$ to enhance adsorption and subsequent degradation.
MATERIALS AND METHODS

Synthesis and Characterization of nAg/TiO$_2$ and nAg

nAg/TiO$_2$ was synthesized in the Colvin Lab at Rice University. Briefly, Ag$^+$ from AgNO$_3$ was photochemically reduced via oxalic acid and UV 254 onto commercial TiO$_2$ nanoparticles. The base TiO$_2$ used in this study was Degussa P25 (approximately 70:30% anatase:rutile) and Alfa Aesar anatase. The silver content of the particles with P25 TiO$_2$ as the base material was adjusted to a nominal 4-10% by mass by varying the concentration of AgNO$_3$ used in the reaction. These particles were denoted 4%nAg/P25TiO$_2$, 6%nAg/P25TiO$_2$, 8%nAg/P25TiO$_2$, and 10%nAg/P25TiO$_2$. The particles synthesized using Alfa Aesar anatase TiO$_2$ were synthesized using only 10% by mass Ag, and were denoted 10%nAg/AATiO$_2$. The composite photocatalyst particles were dried to a powder after synthesis and stored covered and under vacuum. TEM and XPS analysis were also performed in the Colvin Lab. The silver nanoparticles (nAg) used in this study were manufactured by Novacentrix and have been well characterized in a previous study [48].

Particle size and electrophoretic mobility were measured using a Malvern Instruments Zen 3600 Zetasizer (Nano ZS). Particle size was measured using dynamic light scattering immediately after bath sonication and prior to addition to virus solutions for inactivation tests. The particle suspensions were diluted to 100 mg/L in ultrapure water (Barnstead E-Pure) to match the conditions of the viral inactivation experiments. The number based average data was used for analysis. For particle suspensions that were stable, four to ten measurements were taken for each analysis. For unstable suspensions,
only the first three measurements were analyzed, as they coincide with the approximately five minutes that the inactivation procedure was being undertaken. For each particle type, the size data obtained from each inactivation test were pooled into an aggregate average and the pooled standard deviations were determined. Separate particle suspensions dispersed by bath sonication in ultrapure water were made for electrophoretic mobility measurements. The measurements were taken at particle concentrations of 250-500 mg/L. A total of ten measurements were taken for each sample.

**Virus Propagation**

Bacteriophage MS2 (ATCC 15597-B1) and *E. coli* 15597 (ATCC 15597) were obtained from the ATCC. The virus stock solution used in the disinfection procedures was obtained by infecting 800 μL of an overnight incubation of the *E. coli* host with 200 μL of MS2 solution made by rehydrating the freeze dried MS2 pellet obtained from the ATCC. The mixture was briefly vortexed and then mixed with 3 mL of molten LB-Lennox (Fisher) media containing 0.7 % Bacto™ agar (Difco Laboratories), which was kept in a water bath at 45°C. This mixture was briefly vortexed and poured over a Petri dish containing solid LB-Lennox media with 1.5 % Bacto™ agar. After incubating overnight, 15 mL of sterile 100 mM bicarbonate (Fisher) buffer was added to the plate. The plate was then placed on a gently rocking table for 3 hours to allow the virus to diffuse into the buffer. The solution was withdrawn from the plate and then centrifuged at 10,900 x g for 15 min at 4°C in a sterile centrifuge tube to remove any cellular components and pieces of agar. The supernatant was then withdrawn and filtered through
a 0.22 μM PES syringe filter into a new sterile plastic 50 mL centrifuge tube. Sterile 100 mM bicarbonate buffer was then added to the tube to bring the total volume up to 40 mL. The virus suspension was then stored at 4°C and used without further preparation as the virus stock solution in the disinfection experiments. Initial quantification of the stock was preformed via the double agar layer method [72] using sterile bicarbonate buffer as the dilutant. The newly propagated MS2 stock measured \( \sim 7 \times 10^9 \) plaque forming units per milliliter (PFU/mL).

**Inactivation Procedure**

All materials that came in contact with the virus solutions, media, and reagents were sterilized by autoclaving, filtering, or purchased sterile. The first step of the inactivation process was to make 10-20 mL of 500 mg/L solutions of the TiO₂, nAg/TiO₂, or nAg nanoparticles in sterile ultrapure water. The particle suspensions were then bath sonicated for 30-45 minutes to suspend the nanoparticles. After sonication and before the UV inactivation experiment, particle size was analyzed by dynamic light scattering (DLS) using a Malvern Instruments Zen 3600 Zetasizer (Nano ZS) in order to determine if differences in particle size and thus surface area was responsible for any observed differences in viral inactivation.

The inactivation reactions were housed in 25 mL Pyrex Erlenmeyer flasks with ground glass stoppers in place. When testing TiO₂ and nAg/TiO₂, 16 mL of sterile ultrapure water (pH 5.5) was added to the flask followed by 25 μL of \( \sim 7 \times 10^9 \) PFU/mL stock virus solution, resulting in a \( \sim 7 \times 10^7 \) PFU/mL viral concentration. Immediately prior to placement in the photoreactor, 4.0 mL of the nanoparticle stock solution was
added to the flask, resulting in a 100 mg/L concentration of TiO$_2$ or nAg/TiO$_2$. When testing the nAg, 19.6 mL of sterile ultrapure water (pH 5.5) was added to the flask followed by 25 μL of ~7x10$^9$ PFU/mL stock virus solution. Immediately prior to placement in the photoreactor, 0.40 mL of the nAg stock solution was added to the flask, resulting in a final nAg concentration of 10 mg/L, which corresponds to the concentration of silver on the 10% nAg/TiO$_2$ particles when tested at 100 mg/L.

To investigate the effect of HO' scavengers on the MS2 inactivation rate, reactions were carried out in the presence of either 400 mM methanol (99.9%, Fisher spectranalyzed) or tert-butanol (Fisher, ACS Certified) with the P25 based nAg/TiO$_2$ (10 wt.% Ag) and P25 TiO$_2$. The amount of water used in the reactions was reduced accordingly to maintain a total volume of 20 mL. To account for any inactivation due to the alcohols, MS2 suspensions were made in ultrapure water and then sampled. Following this, methanol or tert-butanol was added to make a concentration of 400 mM. The solution was stirred for 10 minutes and sampled again. The titer of active viruses was then compared between the two conditions.

The photoreactor used in this study was a Luzchem model LZC-4V manufactured by Luzchem Research, Inc. The reactor was fitted with four 8W UV-A (315-400 nm) bulbs with peak emission at 350 nm. The bulbs were arranged in pairs and placed on the left and right sides of the reactor. The reaction flask was placed over the internal stirring device, which was set to stir during the reaction. The reactor was turned on at least 5 minutes before each inactivation procedure in order to allow the bulbs to warm and the light intensity to stabilize. A UV radiometer (Fisher Scientific) with a 350 nm sensor (NIST traceable) was used to measure the UV intensity in the reactor prior to each test.
Measurements were taken on the spot where the reaction flask was placed with the sensor facing both pairs of lights before each inactivation test. The average intensity of the pair of bulbs on the left hand side was 1.01 mW/cm², while the average intensity on the right side was 0.92 mW/cm². The reflected intensity from the top, back and front faces of the reactor measured approximately 0.2 mW/cm² each. Therefore, the reaction flask was exposed to a total intensity of approximately 2.5 mW/cm².

After the reactor bulbs had stabilized, the appropriate nanoparticle solution was then added to the reaction flask containing the diluted virus suspension. This was then allowed to stir for one minute in the dark, after which a 1 mL sample was taken representing the initial virus concentration. The reaction flask was then placed in the reactor and 1 mL samples were taken at 30 second intervals up to 2.5 minutes. All samples were immediately covered and refrigerated at 4°C to prevent further inactivation. The inactivation procedure was repeated a minimum of two times for each particle type and condition. When alcohol scavengers for HO⁻ were used, 100 µL samples of the virus suspensions were taken and immediately diluted into 100 mM bicarbonate buffer.

Adsorptive removal and dark inactivation by 10%Ag/P25TiO₂ and undoped P25 TiO₂ were also measured in this study. A suspension of ~7x10⁷ PFU/mL MS2 was made in 16 mL of sterile ultrapure water and stirred in the dark for 10 minutes, and a 1 mL sample was taken to determine the initial virus titer. After this, sonicated nanoparticles were added to the flask to make a 100 mg/L concentration, which was then stirred for another 10 minutes in the dark, and a 1 mL sample was then taken to determine the active virus titer with nanoparticles in suspension. 5 mL of the remaining solution was then removed and placed into a sterile centrifuge tube. The tube was centrifuged at 10,900 x g
for 15 minutes, and 1 mL of supernatant was then withdrawn to determine if sedimenting the nanoparticles with adsorbed viruses reduces the titer of active viruses in solution. To further investigate dark inactivation, the samples taken during the inactivation procedure, prior to UV-A irradiation, were kept at 4°C for 24 hours and enumerated again to compare with the virus titer that was immediately determined after inactivation tests.

**Virus Enumeration**

A serial dilution using 100 mM NaHCO₃ was performed on each sample, and the dilutions were quantified via the double agar overlay method [72] using an incubation of *E. coli* 15597 as the virus host. The procedure was performed on the same day the samples were taken. This procedure was performed in a biosafety cabinet with the overhead lights turned off. Following overnight incubation of the plates, the resulting plaques were counted and the corresponding PFU/mL determined. The survival ratios for each sample were determined and the results averaged from each of the tests. Control tests consisted of enumerating buffer solution to ensure that viral contamination was not present in any of the reagents.

**Data Analysis**

Kinetic inactivation data under UV-A irradiation was modeled using the Chick-Watson model as given by Equation 1, where *k* is the rate constant (s⁻¹) and *N* is the titer of active viruses in this case. For analysis in this study, *N₀* represents the virus titer after adsorption to the photocatalysts but before UV-A irradiation.

\[
\log \left( \frac{N}{N_0} \right) = -k \times t \quad \text{Equation (1)}
\]
RESULTS AND DISCUSSION

nAg/TiO₂ Characterization

The high surface area to volume ratio is a major factor responsible for the increased reactivity observed in nano-sized materials. In this application of nAg/TiO₂ photocatalysis, the increased surface area provides more sites for adsorption of MS2 to the catalyst surface. The surface charge of the particles is an important consideration for particle suspension stability and for adsorption of MS2 to the catalyst surface. At pH 5.5, MS2 carries a positive surface charge and adsorption will be enhanced to a negatively charged catalyst particle [65, 66].

The color of the four nAg/P25TiO₂ powders varied from sample to sample. The 10%nAg/P25TiO₂ powder was light tan while the 4%nAg/P25TiO₂ and 8%nAg/P25TiO₂ powders were reddish brown. The 6%nAg/P25TiO₂ was multi colored, with separate aggregates being purple, reddish brown, and tan colored. It is suspected that the reddish brown and purple colors are the result of oxidation or reactions of silver with light. The 10%nAg/AATiO₂ material was a light grey color.

Particle sizes of all nanoparticles (except nAg), displayed in Figure 3, were measured in ultrapure water using DLS immediately after sonication and prior to addition to virus solutions for inactivation procedures. P25 TiO₂, Alfa Aesar anatase TiO₂, 10%nAg/P25TiO₂ and 10%nAg/AATiO₂ all formed stable suspensions after sonication for at least 25 min, suggesting that these suspensions would be stable during the inactivation procedures (~ 5 minutes). The particle size data presented for these materials was taken over approximately 20-30 minutes. The P25 based nAg/TiO₂ containing 8, 6,
and 4 wt. % nAg were not stable during the course of the inactivation procedure, and settled out of suspension a few minutes after sonication. Due to this, only DLS data obtained in the first 5 minutes concurrent with the inactivation procedure is presented here.

Figure 3. Average diameter of photocatalyst particles used for inactivation of MS2. Data represents averages of all measurements taken for each inactivation test. Error bars represent one standard deviation.

The measured electrophoretic mobility of the particles is shown in Figure 4. The high values of the electrophoretic mobility observed for P25 TiO₂, 10%nAg/P25TiO₂, Alfa Aesar anatase TiO₂ and 10%nAg/AATiO₂ correlate with the small aggregate diameter and stability observed by these particles when suspended by bath sonication. The low values of the electrophoretic mobility observed with the other three photocatalyst samples correlates with large aggregate diameter and instability in solution after sonication. If the silver carries a negative charge, the particles with lower
concentration may be neutralizing the positive charge on P25 TiO$_2$, while the silver content on the 10% nAg/P25TiO$_2$ particles is high enough to reverse the charge. Alternatively, the 4, 6, and 8 wt% nAg/P25TiO$_2$ particles were not synthesized at the same time as the 10 wt% nAg/P25TiO$_2$ particles, and while the reported methodology was the same, it is possible that some change in the synthesis procedure was inadvertently made.

The measured electrophoretic mobility of P25 TiO$_2$ in this study was 1.97 $\mu$m cm/Vs at pH 5.5. This corresponds to a positive value for the zeta potential, which agrees with the results obtained by Sato et al. (2008) [64]. However, the study by Mandzy et al. (2005) found that the zeta potential of P25 TiO$_2$ at pH 5.5 was approximately 0 mV [63], which would correspond to an electrophoretic mobility of approximately 0 $\mu$m cm/Vs. Since the P25 TiO$_2$ in this study carried a positive surface charge, the negatively charged MS2 should be attracted to its surface more favorably than the other materials based on the difference in surface charges.
Figure 4. Measured values of the average electrophoretic mobility of photocatalyst nanoparticles used for MS2 inactivation. Error bars represent one standard deviation.

MS2 Adsorptive Removal and Dark Inactivation

MS2 removal from solution by pure adsorptive removal was tested using 10% nAg/P25TiO₂ and bare P25 TiO₂. Three samples were taken for each test: 1. virus suspension before the addition of nanoparticles, 2. suspension of virus and photocatalytic nanoparticles that had mixed in the dark for 10 min., and 3. the centrifuge supernatant of the mixed suspension of virus and photocatalytic nanoparticles. As shown in Figure 5, approximately 80% removal was observed with the nAg/TiO₂ in suspension, and another 10% was removed after centrifugation. The additional removal after centrifugation indicates that some of the adsorbed MS2 were still infective. Only approximately 35% removal of MS2 was observed with bare P25 TiO₂ in suspension, with no significant additional removal after centrifugation.
Figure 5. Adsorptive removal of MS2 by bare P25 TiO₂ (top) and 10% nAg/P25TiO₂ (bottom). Error bars represent one standard deviation.

The increased adsorptive removal by nAg/TiO₂ may be explained by the known affinity of silver for sulfur moieties. The 183 cysteine residues on the MS2 capsid surface contain sulfur groups that increase the probability of MS2 adsorbing to the nAg
on nAg/TiO₂. The limited difference between virus titers with nanoparticles in suspension and nanoparticle free centrifuge supernatant provides evidence that adsorption of MS2 to the nAg/TiO₂ and undoped TiO₂ surface either inactivates these viruses or sterically inhibits access of the MS2 A protein to the *E. coli* pili, where infection occurs. Since the MS2 A protein, which is required for viral infectivity, contains a cysteine residue, it is possible that viruses are binding at this location to nAg/TiO₂ and physically preventing infection through steric interference. However, with the A protein containing only 1 of 183 surface exposed cysteine residues, it is highly unlikely that adsorption randomly occurs to this specific cysteine residue at such a high rate. Additionally, the virus bound to P25 TiO₂ is also not infective, suggesting that other effects besides those from silver are responsible for inactivation due to adsorption. It is somewhat surprising that the adsorptive removal by P25 TiO₂ was so low considering that the nanoparticles and viruses are oppositely charged.

In addition to adsorptive removal, dark inactivation was investigated by re-enumerating the initial nanoparticle containing non UV-irradiated samples taken during the inactivation procedure after being stored at 4°C for 24 hours. No significant difference in virus titer was observed between the initial enumeration and that performed after 24 hours for either P25 TiO₂ or 10%nAg/P25TiO₂ materials.

**MS2 Inactivation by nAg/TiO₂ Under UV-A Radiation**

The inactivation of MS2 by P25 TiO₂, 10%nAg/P25TiO₂, nAg, and UV-A alone is shown in Figure 6. MS2 inactivation was significantly enhanced by the 10%nAg/P25TiO₂ as compared to plain P25 TiO₂. The rate constants (shown in Table 1)
for MS2 inactivation by 10%\textit{n}Ag/P25TiO$_2$ was 0.055 s$^{-1}$ vs. 0.013 s$^{-1}$ for P25 TiO$_2$. The silver doping on this material thus increased the reaction rate by \textasciitilde320\% as compared to the base TiO$_2$. UV-A alone and nAg resulted in negligible photocatalytic inactivation, suggesting that doping the TiO$_2$ with silver is enhancing the photocatalysis by facilitating charge separation resulting in more ROS generation and subsequent MS2 inactivation. MS2 inactivation by 10%\textit{n}Ag/P25TiO$_2$ shows a tailing effect after 60 seconds. This was not observed when MS2 was inactivated by the other materials. Since the reactions were undertaken in a sealed system, an oxygen deficit may be responsible for this effect. The tailing effect occurs after 4 LOG removal of MS2. No other material removed this much virus within the time frame used in this study. A tailing effect for other materials may have been observed if the reactions were allowed to continue longer.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{MS2 Inactivation by 10%\textit{n}Ag/P25TiO$_2$, P25 TiO$_2$, and nAg under UV-A irradiation, and UV-A irradiation alone. Error bars represent one standard deviation.}
\end{figure}
The inactivation of MS2 by 10%\text{nAg}/AATiO$_2$ was also enhanced when compared to the bare anatase TiO$_2$ material, as shown in Figure 7. The inactivation rate constant for 10%\text{nAg}/AATiO$_2$ was 0.024 s$^{-1}$ vs. 0.004 s$^{-1}$ for the plain anatase, which was not significant. Silver doping increased the inactivation rate by $\sim$500% as compared to the base TiO$_2$. In this case silver is again serving to facilitate charge separation in TiO$_2$ and thus resulting in more ROS generation and subsequent MS2 inactivation. When compared to 10%\text{nAg}/P25TiO$_2$, the inactivation rate obtained with 10%\text{nAg}/AATiO$_2$ is $\sim$2.3 times slower. P25 TiO$_2$ inactivated MS2 $\sim$3.2 times faster than the anatase TiO$_2$. This result is consistent with the known high reactivity of P25 [73].

**Figure 7.** MS2 inactivation by 10%\text{nAg}/AATiO$_2$, Alfa Aesar anatase TiO$_2$, and nAg under UV-A irradiation, and UV-A irradiation alone. Error bars represent one standard deviation.
In order to determine the optimum silver content for MS2 inactivation by nAg/TiO$_2$, the silver content on nAg/P25TiO$_2$ was varied from 4-10 nominal wt. %. The MS2 inactivation data obtained using these materials is shown in Figure 8.

![Graph showing MS2 inactivation](attachment:graph.png)

**Figure 8.** MS2 inactivation by P25 based nAg/TiO$_2$ with varying nominal nAg contents under UV-A irradiation. Error bars represent one standard deviation.

The 10% nAg coverage performed the best, followed by 8%, 4%, and 6%, with rate constants of 0.055 s$^{-1}$, 0.035 s$^{-1}$, 0.017 s$^{-1}$, and 0.010 s$^{-1}$, respectively. With the exception of the 6% nAg material, the inactivation rate increases with silver content. The data obtained using 6 wt% nAg/TiO$_2$ is peculiar in that the virus titer increased for the first minute of the reaction before decreasing. The initial titer measured approximately 1 LOG lower (~1x10$^6$ PFU/mL) than expected after dark adsorption (as measured using 10%nAg/P25TiO$_2$). If the initial virus titer used for calculation of the rate constant is 1x10$^7$ PFU/mL, the rate constant becomes 0.014 s$^{-1}$, which is comparable for that obtained with the 4% Ag coating (0.017 s$^{-1}$). Neither the 4% or 6% nAg/P25TiO$_2$
samples show a significant enhancement when compared to undoped P25 TiO₂, with a rate constant of 0.013 s⁻¹. However, the particle diameters of these nAg/TiO₂ samples were ~1,500 nm, while the P25 TiO₂ measured ~100 nm. Assuming a monodisperse suspension, spherical particle geometry, and identical density, the catalyst surface area of the 4% and 6% nAg/TiO₂ suspensions is ~15 times less than that of the P25 suspension. If the available surface area is truly an important factor, these nAg/TiO₂ materials could be said to be more active than undoped TiO₂ after taking account of the decreased surface area. However, using the same assumptions, there are ~1.3 x 10⁻⁷ particles/mL in these nAg/TiO₂ suspensions vs. 4.5 x 10⁻¹⁰ particles/mL in the P25 TiO₂ suspensions. Since the virus concentration used was ~10⁷ PFU/mL, there is still an ~1:1 catalyst particle to virus ratio with these nAg/TiO₂ suspensions, which may make the decreased surface area a negligible consideration.

One possible explanation for the strange data obtained with the 6%nAg/P25TiO₂ is that the nAg on nAg/TiO₂ is severely oxidized in these particles and silver oxides may have greater adsorptive affinity for MS2 than silver metal. In this case, the excited electrons produced from UV irradiation are initially reducing the silver oxide back to silver metal. During this process, ROS is not produced via the reductive pathway and infective MS2 is released from the particle surface. Evidence against this hypothesis is that the solution color does not turn tan (the color of the 10%nAg/P25TiO₂ suspension), but remains a reddish brown.
**Table 1.** Inactivation rate constants and correlation coefficients for MS2 inactivation fitted to the Chick-Watson model. *Rate determined using initial and final virus titer

<table>
<thead>
<tr>
<th>Material</th>
<th>Rate Constant (s⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Ag/P25TiO₂</td>
<td>0.055</td>
<td>0.91</td>
</tr>
<tr>
<td>8%Ag/P25TiO₂</td>
<td>0.035</td>
<td>0.99</td>
</tr>
<tr>
<td>6%Ag/P25TiO₂</td>
<td>0.014*</td>
<td>-</td>
</tr>
<tr>
<td>4%Ag/P25TiO₂</td>
<td>0.017</td>
<td>0.97</td>
</tr>
<tr>
<td>10%Ag/AATiO₂</td>
<td>0.024</td>
<td>0.99</td>
</tr>
<tr>
<td>P25 TiO₂</td>
<td>0.013</td>
<td>0.98</td>
</tr>
<tr>
<td>Alfa Aesar Anatase TiO₂</td>
<td>0.004</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**Effects of HO’ Scavengers on MS2 Inactivation**

The addition of methanol and tert-butanol scavengers for HO’ to the reaction medium was employed to assist in elucidating the mechanism of MS2 inactivation by the photocatalysts used in this study. The scavenging mechanism of these alcohols is shown in Equations 12-14. Methanol is known to scavenge both bulk and adsorbed HO’, while tert-butanol scavenges the bulk and only some adsorbed HO’ [10, 24]. These reactions were undertaken using 10%Ag/P25TiO₂ and undoped P25 TiO₂ (Figure 9).
Figure 9. MS2 inactivation by 10% nAg/P25TiO₂ (top) and P25 TiO₂ (bottom) under UV-A irradiation in the presence of methanol and tert-butanol HO' scavengers. Error bars represent one standard deviation.
Both alcohols significantly reduced the inactivation rate of MS2 by 10% nAg/P25TiO₂ and completely stopped inactivation of MS2 when plain P25 TiO₂ was used as the catalyst (Figure 9). These results suggest that HO' is responsible for MS2 inactivation by P25 TiO₂, but that other species play a significant role when nAg/TiO₂ is used. There is no significant difference in reaction rates when either methanol or tert-butanol were used as scavengers, suggesting that bulk, not adsorbed HO', is responsible for MS2 inactivation. Previous studies have shown singlet oxygen and superoxide anion can inactivate MS2 [28], but these species are also produced with plain TiO₂, making it unlikely that they are responsible for the observed difference. However, it is possible that the increased adsorption observed with nAg/TiO₂ allows other ROS to come into contact and inactivate MS2. Scavengers for these ROS may be employed in future studies to determine if they indeed have some effect.

When nAg/TiO₂ was used, the addition of both methanol and tert-butanol depressed the count of active viruses before irradiation, but this effect diminished after 30 seconds of irradiation, and the active titer then decreased. Since the depression of initial virus concentration was not observed with P25 TiO₂, this effect when nAg/TiO₂ was used is attributed to the interaction of the alcohol scavengers with the silver and the subsequent changes in viral adsorption capacity. The increased adsorption of MS2 by nAg/TiO₂ may facilitate direct oxidation by electron holes on the catalyst surface. This may be the reason that the alcohol scavengers do not completely stop the inactivation of MS2 with this material, as neither alcohol is likely to scavenge holes [25]. The tests of alcohol toxicity to MS2 in ultrapure water without nanoparticles did not result in any decrease in virus titer for either 400 mM methanol or tert-butanol.
CONCLUSION

This study has demonstrated that silver doping titanium dioxide nanoparticles is an effective way to increase their photocatalytic activity for virus inactivation. A synergism between TiO$_2$ and nAg is apparent when 10 and 8 nominal mass % nAg/TiO$_2$ catalysts are used. While the best results were obtained with 10%nAg/P25TiO$_2$, the strange characteristics (possible oxidation) of the particles containing lesser silver coverage makes it difficult if not impossible to draw a conclusion as to what is the best silver coverage on TiO$_2$ for virus inactivation when using this data for analysis. The differences in particle sizes between the catalysts containing different silver contents also makes drawing a definitive conclusion more difficult. XPS and/or XRD analysis need to be performed on these materials to determine if silver is present in an oxidized state. In a realistic application of this material, oxidation is bound to occur. Therefore, an investigation into the effects of silver oxidation is an important topic that requires further study.

The application of the HO$^-$ scavengers clearly showed that hydroxyl free radical is the primary ROS responsible for MS2 inactivation by TiO$_2$, however inactivation, while to a lesser degree, still occurred when nAg/TiO$_2$ was used as the photocatalyst. This suggests the HO$^-$ is the primary ROS responsible for inactivation, but that other species may be responsible for some inactivation. The increased adsorption of MS2 to these particles may facilitate increased direct hole oxidation. Further studies using scavengers for superoxide anion, singlet oxygen, and hydrogen peroxide are warranted to more clearly discern the suite of ROS species responsible for MS2 inactivation by this material.
Since employing nanoparticles for actual drinking water treatment poses a new problem, removal of the particles themselves, it is likely that a thin film coating on surfaces within a UV reactor would be a more feasible delivery mechanism. Additionally, coating transparent piping or shallow open channels with this material and exposing them to sunlight may be another employment method, as several studies have demonstrated increased visible light activation of nAg/TiO$_2$ as compared to plain TiO$_2$ [38, 74]. Another consideration for actual usage of this material is the potential for silver ions to migrate off of the catalyst and into the drinking water.

Since natural waters contain a large amount of other constituents (i.e. dissolved solids, natural organic matter, etc...), an investigation of the effects that common ions and NOM have on the photocatalytic efficiency is necessary. The primary motivator for this research is to overcome the resistance of infectious Adenovirus to UV disinfection. Therefore, testing nAg/TiO$_2$ for inactivation of Adenovirus, and exploring the inactivation mechanism, are critical research needs for this material.
REFERENCES


APPENDIX

MS2 Inactivation by Nominal 1 wt. % nAg/TiO$_2$ in 100 mM NaHCO$_3$ Buffer

This data was obtained using a different photoreactor (Rayonet model RPR-200) than that used for the data presented in Figures 5-9. The nAg/TiO$_2$ was synthesized using P25 TiO$_2$ as the base material. The silver nanoparticles used in this experiment were synthesized in the Colvin Lab, and have an average diameter of 8 nm with an 18% rsd.

![Graph showing MS2 inactivation](image)

**Figure A1.** MS2 inactivation by P25 based 1 wt. % nAg/TiO$_2$ under UV-A irradiation. Error bars represent one standard deviation above and below the mean.

The nAg/TiO$_2$ produced a tailing effect in this experiment, similar to that observed with the 10% nAg/TiO$_2$ in Figure 5. While the initial inactivation appears enhanced as compared to undoped P25 TiO$_2$, the results are not significant due to the large standard deviations. This data does show that both photocatalysts are effective with a high concentration of bicarbonate and sodium in solution.