

The Effect of Nanoparticles on Bacteriophage Infections

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ABSTRACT

As nanoparticles are being introduced into the ecosystem, in paint and clothing, there is massive concern for their ecological impact. This research focuses on the impact of chemically benign apatite nanoparticles on bacteriophage infection. While nanoparticles were tolerated by bacteria, even at high concentrations, the mixture of phage and nanoparticles seemed to increase the infection efficacy of bacterial strains. We have tested several bacterial strains to confirm our findings and the results have indicated a statistically relevant correlation between infection rates in the presence and absence of nanoparticles.

Keywords: nanoparticles, bacteriophages, *Escherichia coli*, *Mycobacterium smegmatis*

1. INTRODUCTION

Nanoparticles (NPs) are of great scientific interest because they serve as effective bridges between bulk materials and atomic or molecular structures and are increasingly used as critical components in new technologies and commercial products. Unintended consequences of nanoparticles introduced into the environment may be a cause for concern. Iron-doped apatite nanoparticles (~100 nm), which are non-toxic to microbes even at high concentrations [1] can be easily detected, tracked, and manipulated due to their simultaneously magnetic and fluorescent properties. [2,3]

Bacteriophages are viruses that infect bacteria by injecting their genetic material into the cytoplasm of a bacterial host cell. At approximately 10^{31} , bacteriophages are the most numerous organisms on the planet. [4,5] These phages can have a double-stranded DNA-genome, such as Macho and Second phage, a single-stranded RNA-genome, such as MS2 phage. Phages have specific host ranges which infect *Mycobacterium smegmatis*, such as macho, second phage, and Bxz1 or *Escherichia coli*, such as MS2 and T4. They can also replicate through different life cycles. A temperate phage replicates through both the lytic and lysogenic (Macho and Second phage) cycle while there are some phages that replicate only through the lytic (Bxz1 and T4). [6] In the ocean, there are estimated to be 10^{25} phage infections per second. [7] Since phages and bacteria both live in the ocean, if there was an increase in number of phage infections this would lower the number of bacteria and possibly have a major downstream effect. Filter

feeding organisms, such as sponges and mollusks, which eat bacteria could decrease in abundance and that could affect carnivores and omnivores including sharks and humans. Any effect of nanoparticles on bacteriophage infection could translate to significant effects on the microbial ecosystem.

2. MATERIALS AND METHODS

2.1.1 Synthesis of Fe-NPs

30% iron-doped nanoparticles were synthesized at 40°C using calcium hydroxide, Ca(OH)₂ and potassium dihydrogen phosphate, KH₂PO₄ (both analytical grade from Sigma-Aldrich, USA). Double de-ionized (ddI) water (Milli-Q unit, Millipore, USA) was used to prepare all the solutions/suspensions in the present study. 0.260 g Ca(OH)₂ was suspended in 100 ml ddIH₂O to which 0.243 g FeCl₃ dissolved in 100 ml ddIH₂O was added. This was followed by 0.288 g of citric acid (C₆H₈O₇). Lastly, 0.408 g KH₂PO₄ dissolved in 50 ml ddIH₂O was added drop wise bringing the total solution volume to 250 ml. The pH of solution was adjusted to 7.0 by adding a few drops of 0.5 M NaOH. The Fe doped NPs were magnetically stirred for 7 days at 40°C. The resulting colloid was autoclaved before use in subsequent experiments.

2.1.2 Synthesis of Fe-NP Precursors

Fe-NP precursors were made by mixing 0.260 g Ca(OH)₂ in 60 ml of ddIH₂O, 0.243 g FeCl₃ in 60 ml ddIH₂O, 0.288 g C₆H₈O₇ in 60 ml of ddIH₂O and 0.408 g KH₂PO₄ in 60 ml of ddIH₂O. Each solution was placed in a separate Erlenmeyer flask and autoclaved. Before each experiment, 2 ml of each of the four sterile chemicals were mixed together and 0.5M NaOH was added to bring the pH to 7.

2.2 Characterization of Fe-NPs and Fe-NP Precursors

2.2.1 Transmission Electron Microscopy (TEM)

Transmission electron microscopy was performed at the EMTRIX Electron Microscopy Facility in Missoula, MT employing the Hitachi H-7100 TEM. One microliter of each Fe-NPs and Fe-NP precursor were placed on carbon-coated grid and left to sit on the grid for 2 minutes. The samples were washed off and treated with ~40 µl of uranyl acetate which was wicked off after 20 seconds.

2.3 Growth of Bacterial Strains

2.3.1 *Mycobacterium smegmatis* cells

M. smegmatis (mc²155) were streaked from a frozen stock that was maintained at -80°C onto a 7H10 plate and grown in an incubator at 37°C for 2 days. When colonies formed on the plate, a single colony was picked into 1-3 ml of the mixture of 7H9 with 0.5% Tween 80. This solution was incubated at 37°C at 250 revolutions per minute (RPM) for 2 days. To grow *M. smegmatis* for everyday use, 100 µl of the *M. smegmatis*/7H9/tween solution was subcultured into 100 ml of 7H9/ADC/CB/CHX/CaCl₂ mixture. The cells were incubated in the shaker/incubator at 37°C at 250 (RPM) for 2 days. The cells were stored at 20°C on the lab bench and used as needed.

2.3.2 *Escherichia coli* A λ cells

E. coli A λ cells were streaked from a frozen stock that was maintained at -80°C onto a LB plate and grown in an incubator for 37°C for 1 day. When colonies formed on the plate, a single colony was picked into 25 ml of LB/0.1 M CaCl₂ broth. The mixture was incubated in the shaker/incubator at 37°C at 250 RPM for 1 day.

2.3.3 *Escherichia coli* B cells

E. coli B cells were purchased from Carolina Biological Supply Company and kept at 37°C. The cells were streaked from the *E. coli* slant onto a LB plate and grown in the incubator at 37°C for 1 day. When colonies formed on the plate, a single colony was picked into 25 ml of LB/CaCl₂ broth. The mixture was incubated in the shaker/incubator at 37°C at 250 RPM for 1 day.

2.4 Dilution of Phages

The phages were diluted in phage buffer, which contained 10 ml 1 M tris, pH = 7.5, 10 ml 1 M MgSO₄ and 4 g NaOH. After autoclaving 0.1 M CaCl₂ was added.

2.5 Infection of Phages, Bacterial Cells and Fe-NP's

Into 54 clearly labeled sterile test tubes, 250 µl of the appropriate cells were added. In half of the test tubes (27), 250 µl of Fe-NP's were added while the other half contained 250 µl sterile water. The test tubes were incubated at room temperature, 20°C, for one hour before phage infection. To the appropriate test tubes, 5 µl of diluted phage were added to the NP/cell and NP/ddIH₂O solution. Middlebrook Top Agar (MBTA), which was made by adding 4.7 g 7H9 broth and 7 g bacto agar for a final concentration of 0.35%. To MBTA before use,

solution was made by melting a full bottle of MTBA was and adding equal amounts of 7H9 with 2 ml of CaCl₂ were added. The melted MBTA (2.75 ml) was placed into each test tube and poured onto the appropriately labeled split 7H10 agar plate. All plates were incubated overnight at 37°C and the phage plaques were counted. Each experiment was performed multiple times for statistical purposes.

2.6 Infection of Phages, Bacterial Cells, Fe-NPs, and NP Precursor

Into 72 clearly labeled sterile test tubes, 250 µl of the appropriate cells were added. To a quarter of the test tubes (18), 250 µl of Fe-NP's were added, to another 18 250 µl of NP precursor was added. The other 36 contained 250 µl sterile water. The test tubes were incubated at room temperature, 20°C, for one hour before phage infection. To the appropriate test tubes, 5 µl of diluted phage were added to the NP/cell, NP precursor/cell or ddIH₂O/cell solution. Eighteen of the test tubes with cells and ddIH₂O served as the control. Middlebrook Top Agar (MBTA) solution was made by melting a full bottle of MTBA and adding equal amounts of 7H9 with 2 ml of 0.1 M CaCl₂. The melted MBTA (2.75 ml) was placed into each test tube and poured onto the appropriately labeled split 7H10 agar plate. All plates were incubated overnight at 37°C. The following day, the phage plaques were counted. Each experiment was performed multiple times for statistical purposes.

3. RESULTS AND DISCUSSION

Selected results of phage/NP or phage/NP precursor on phage infection are described below.

3.1.1 Interaction of Fe-NPs and Second Phage

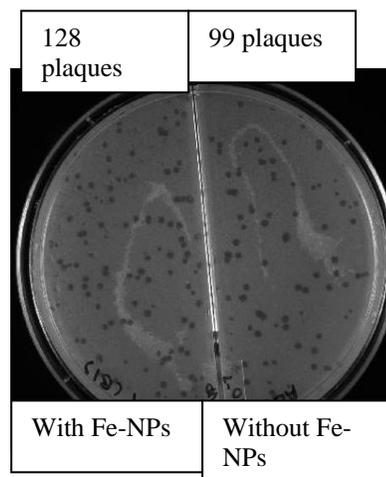


Figure 1: 7H10 plate with Second phase plaques. The side with NPs contains 128 plaques and the side without contains 99 plaques.

Total plaques with NPs	Total plaques without NPs
4065	3635

Table 1: Aggregate results from four experiments with Second phase and Fe-NPs.

- There was an 11.8% increase in phage plaques with NPs compared to without.

3.1.2 Interaction of NP Precursors and Second Phase

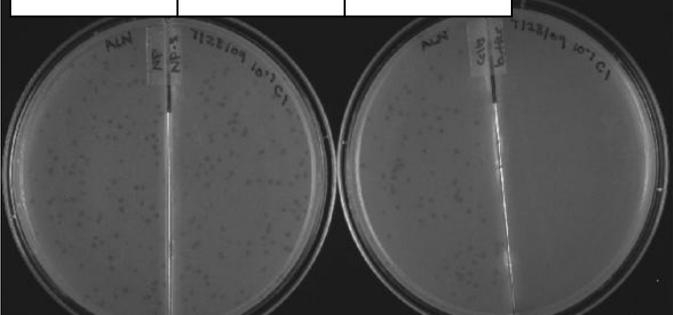
148 plaques	135 plaques	138 plaques	0 plaques
			
With Fe-NPs	NP Precursor	Without Fe-NPs	Control

Figure 2: 7H10 plates with Second phase plaques. The side with NPs contains 148 plaques, the side with NP precursor contains 135, and the side without contains 138 plaques.

Total plaques with NP's	Total plaques with NP precursor	Total plaques without NP's	Total plaques with no phage
2165	1904	1907	0

Table 2: Aggregate results from two experiments with Second phase, Fe-NPs, and NP precursor.

- There was a 14% increase in phage plaques with NPs then without as well as with the NP precursor.

3.2.1 Interaction of Fe-NPs and Bxz1 Phage

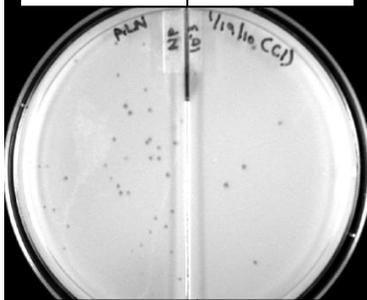
50 plaques	10 plaques
	
With Fe-NPs	Without Fe-NPs

Figure 3: 7H10 plate with Bxz1 phage plaques. The side with NPs contains 128 plaques and the side without contains 99 plaques.

Total plaques with NPs	Total plaques without NPs
2949	2541

Table 3: Aggregate results from three experiments with Bxz1 phage and Fe-NPs.

- There was a 16.1% increase in phage plaques with NPs compared to without

3.2.2 Interaction of NP Precursors and Bxz1 Phage

76 plaques	46 plaques	67 plaques	0 plaques
			
With Fe-NPs	NP Precursor	Without Fe-NPs	Control

Figure 4: 7H10 plates with Bxz1 phage plaques. The side with NPs contains 76 plaques, the side with NP precursor contains 46, and the side without contains 67 plaques.

Total plaques with NP's	Total plaques with NP precursor	Total plaques without NP's	Total plaques with no phage
1533	1232	1348	0

Table 4: Aggregate results from three experiments with Bxz1 phage, Fe-NPs, and NP precursor.

- There was a 14% increase in phage plaques with NPs compared to without and a 24% increase with the NP precursor.

3.3.1 Overall Results from all Phages and Fe-NPs

Total plaques with NPs	Total plaques without NPs
17808	15981

Table 5: Aggregate results from five phages including Macho, Second, Bxz1, and MS2 with NPs and without NPs

- Overall, there is an 11.4% increase in phage plaques with NPs compared to without
- Using T-test to statically analyze the data, P=0.002

3.3.2 Total Results of all Phages, Fe-NPs, and NP Precursor

Total plaques with NP's	Total plaques with NP precursor	Total plaques without NP's	Total plaques with no phage
7806	6620	6714	0

Table 6: Aggregate results from three phages using NPs and the NP precursor. The phages included are Second, Bxz1 and MS2.

- Overall, there is a 16% increase in phage plaques with NPs compared to without and an 18.6% increase with the NP precursor.
- Using T-test to statically analyze the data, P=0.002

3.4 TEM Images

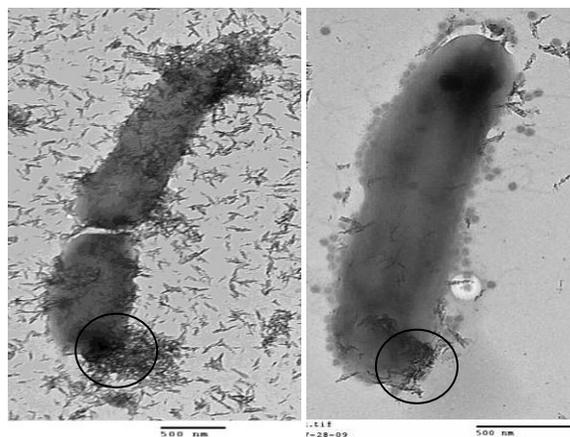


Figure 5: size bars = 500 nm. Representation of *M. smegmatis* and Fe-NPs on left and *M. smegmatis*, Fe-NPs and Second phage on right.

- Hypothesis: NP's accumulate towards poles of cell possibly causing receptor sites to be more available for phage infection and absorption.
- NP's affect the outer layer of the cell making the cell less resistant to phage infection.

4. REFERENCES

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