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Laboratory Experiment

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Magnetic Microorganisms: Using Chemically Functionalized Magnetic Nanoparticles To Observe and Control Paramecia

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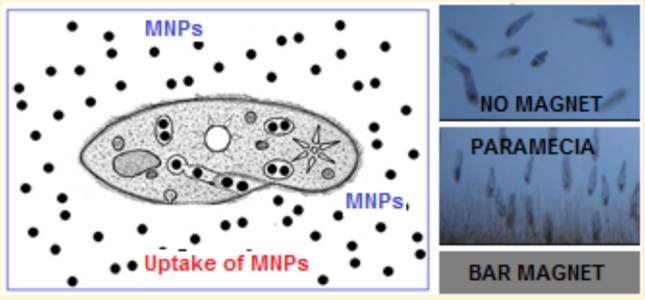
S *Supporting Information*

ABSTRACT: With the growing use of nanotechnology in everyday life, there is a developing need to study the fate and potential impact of nanomaterials in the environment. This multiday laboratory experiment introduces students to magnetic nanoparticles (MNPs) and to the uptake of both uncoated and coated MNPs by microorganisms. To highlight the possible uses and potential consequences of the uptake of MNPs, a procedure was developed to "feed" small magnetic particles to paramecia, a process that can be observed using an optical microscope. During extensive laboratory analysis, uptake of the MNPs by these microorganisms was noted with all three species of paramecia examined: *Paramecium aurelia*, *Paramecium bursaria*, and *Paramecium caudatum*. The paramecia clearly ingested the iron oxide MNPs, but the amount of magnetic material per microorganism varied. In some cases, we were able to manipulate the "magnetized" *P. aurelia* and *P. bursaria* using a magnetic field. Uptake of uncoated MNPs led to microorganism death within the initial 12−18 h observation window. However, paramecia that ingested MNPs coated with the polymer poly(vinylpyrrolidone) (PVP) were still alive 1 week after ingestion. Included in this report are the procedures for the synthesis of the iron oxide (Fe_3O_4) MNPs and their functionalization with PVP, along with an analysis of the chemistry that occurs when uncoated MNPs are present within a cellular environment. Designed for high school students, this engaging experiment integrates chemistry, biology, and physics into the observations of a traditional science laboratory activity, providing a hands-on learning experience.



KEYWORDS: *Nanotechnology, Magnetic Properties, Biological Cells, Materials Science, Environmental Chemistry, Hands-On Learning/Manipulatives, Interdisciplinary/Multidisciplinary, General Public, Elementary/Middle School Science, High School/Introductory Chemistry*

INTRODUCTION

The goal of this laboratory experiment is to introduce nanotechnology to high school students in a project that integrates the subjects of nanoparticle synthesis (magnetic nanoparticles), nanoparticle functionalization (with an organic polymer), and the impact of nanoparticles upon the environment (uptake by unicellular microorganisms) into their curriculum. Nanotechnology is a multidisciplinary subject that involves the manipulation of matter at the nanoscale and encompasses chemistry, biology, physics, and engineering.[1] The exploration of nanosized objects has significant potential for generating student enthusiasm and can be a useful addition to a secondary student's curriculum. Furthermore, by integrating the observations of various size regimes (i.e., nanoparticles, microorganisms, millimeter-sized sample areas) within an engaging project, this experiment provides not only an understanding of scale but also an opportunity to raise awareness of the use of nanotechnology (and its relative component sizes) for purposes such as magnetic memory or biomedical applications.[2] The development of the experimental protocols used in this interdisciplinary laboratory experiment involved students both in a high school setting and in a university research laboratory.[3−7] While incorporation of high-profile research topics into required educational materials can sometimes be difficult, the project outlined in this report provides an exciting and attention-grabbing method to introduce the subject matter of recent scientific journals in lessons focusing on nanotechnology and microorganisms/

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protists. This experiment also provides an opening for discussions about environmental concerns associated with nanotechnology and the fate of nanoscale materials upon their uptake by microorganisms.

Nanomaterials, which consist of particles and other types of matter with at least one dimension in the range of 1 to 100 nm, can be found in numerous everyday items including sunscreens, stain-free clothing, makeup, stained glass, and medicines.[1,6,8] The nanotechnology revolution has occurred due to the distinctive physical properties of these materials caused by their small size and high surface-to-volume ratio. Examples include the antibacterial properties of nanosilver or the special magnetic properties (superparamagnetism) of iron oxide magnetic nanoparticles (MNPs).[7,9−11] There is also a significant body of research where MNPs have been explored as components of biosensors, as contrast agents, and as carriers for drug delivery. When used as a contrast agent, iron oxide (Fe_3O_4) particles are typically coated with a polymer or other organic molecule to render them biocompatible. In fact, most of the MNPs currently used in biomedical applications (i.e., theranostics) are coated for easy functionalization and biocompatibility. The magnetic properties of these MNPs provide the ability to activate the particles remotely using an external oscillating magnetic field, which can cause the MNPs to generate heat in the vicinity of the particle, possibly releasing a payload at a targeted destination within the human body. In contrast to these particulate forms of nanotechnology being tested in the biomedical field, scientists in the oil industry are seeking to engineer nanorobots that are sufficiently small to be undetected and that can carry a load to a desired location.[4,12] These investigators have been modeling nanorobots after microorganisms for their motility, means of energy consumption, and size.[9,12] But this raises the question: Instead of engineering nanorobots, what if we could find a way to make microorganisms perform the tasks of a nanorobot? To accomplish this goal, we need a means of providing for remote control, and we need subjects that can be manipulated without causing them injury or death.

Eukaryotic organisms have been used for years in research laboratories for the study of photodynamic therapy,[13] toxicology,[14,15] bioaccumulation,[16] and environmental impact.[7,17] While much previous research has examined the uptake of gold or silver nanoparticles by such organisms, an exploration of the uptake of magnetic nanoparticles, such as Fe_3O_4 particles, is sorely lacking.[7,18,19] The ability of paramecia, in particular, to phagocytize particulate matter makes them attractive targets for this study. The three species of paramecia acquired for this project included *Paramecium aurelia*, *Paramecium bursaria*, and *Paramecium caudatum*. These ciliated unicellular microorganisms are popular for use in educational laboratories for three reasons: (1) they can be easily visualized using an optical microscope, (2) they are relatively easy to handle and maintain, and (3) they are relatively inexpensive.

The longer-scale project (e.g., nanoparticle synthesis, investigation of various surfactant or polymer coatings, and the development of methods of paramecium manipulation) described in this report can be carried out by a mix of high school and graduate students, while the two-day high school experiment (see the Supporting Information for a detailed outline) is designed to be carried out as a part of a student's regular laboratory in a high school setting. In the two-day experiment, students functionalize (coat with a polymer) the previously synthesized Fe_3O_4 MNPs and then add them to tubes holding paramecia in distilled water. The students then observe the ingestion of the uncoated and coated MNPs with an optical microscope at 40×, 100×, and 400× magnification. Additionally, the use of a microscope enables observation of the cilia movement of the paramecia, their organelles, and the ingested MNPs. The students can document the uptake of MNPs by the paramecia and study their fate using a camera attached to the microscope. Notably, in the case of *P. aurelia* and *P. caudatum*, the students can try to manipulate the movement of the "magnetized" microorganisms using a bar magnet (depending on the amount of ingested MNPs and the strength of the bar magnet). Furthermore, we found that the *P. aurelia* can maintain their magnetic properties for up to 1 week without cell death, when fed judiciously coated MNPs and provided with an appropriate food source.

LEARNING OBJECTIVES

As noted above, the goal of this experiment is to introduce high school students to nanotechnology via the synthesis and functionalization of MNPs and their subsequent uptake by paramecia. Students will become familiar with the use of nonaqueous solvents and molecules for the functionalization of MNPs, and the use of an optical microscope to observe the uptake. Students will also learn the following as they maneuver through this laboratory: (1) how to conduct research and read scientific literature, (2) how to use a sonicator and centrifuge during the washing process, and (3) what methods and materials are needed to maintain and magnetically manipulate the paramecia.

The Texas Essential Knowledge and Skills (TEKS) are the standards that define what the high school students should know and be able to do in any particular subject in the state of Texas. Provisions §112.31 (science) and §112.35 (chemistry specifically) provide the objectives of scientific inquiry, an important component of learning chemistry. In the activities outlined here, the students use experimental, descriptive, and comparative methods to investigate the synthesis and functionalization of MNPs and their subsequent uptake by paramecia. During this laboratory, the students use scientific processes and demonstrate safe laboratory practices (including being aware of hazards and using material safety data sheets). The students get an opportunity to engage in critical thinking and scientific reasoning that leads to scientific explanations; they also apply scientific information extracted from various sources, such as published journal articles, and communicate the observations and conclusions in their laboratory reports (in the case of students who do the two-day experiment) and posters (in the case of those who pursue the longer project). For AP chemistry courses, the College Board also emphasizes science practices that involve correlating lines of evidence to the specific phenomena that allow the students to reach their conclusions. In addition, the American Chemical Society has published *Chemistry in the National Science Education Standards* (*CNSES*),[20] which provides models for learning in high school chemistry classrooms. There are two chapters that focus on scientific inquiry and the link between chemistry and other disciplines. The laboratory experiment reported here is an excellent example of the use of scientific inquiry and multidisciplinary research in chemistry, biology, environmental science, and physics.

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EXPERIMENTAL SECTION

Materials and Equipment

P. aurelia, *P. bursaria*, and *P. caudatum* were purchased from Carolina Biological Supply Company and used as purchased. Daily aeration was necessary to keep the organisms alive. All other chemicals were purchased from Sigma-Aldrich and used without any further purification. Several magnets were purchased from online vendors including a grade N42 bar magnet and a smaller rare-earth magnet. Necessary equipment included a centrifuge capable of holding either test tubes or small centrifuge tubes, and an optical microscope with 40×, 100×, and 400× magnification. Plastic 1.5 mL microtubes, plastic 5 mL disposable pipettes, and glass slides with cover sheets were also used during this experiment.

Synthesis of Fe_3O_4 MNPs

The Fe_3O_4 MNPs were synthesized by following a modified version of a procedure reported by Deng et al.[21] For this modified procedure, a round-bottomed flask with a stir bar was charged with $FeCl_3{\cdot}6H_2O$ (2.0 g), which was then dissolved in 15 mL of ethylene glycol, followed by the addition of sodium acetate (5.4 g). Upon stirring, a rapid change in the color of the solution from orange to brown was observed and the solution was then stirred for an additional 30 min. The colored solution was injected all at once into a round-bottomed flask containing a vigorously stirred solution of polyvinylpyrrolidone (PVP; 0.60 g) in 60 mL of ethylene glycol. This flask was then heated to 180 °C and vigorously stirred for 8 h. After cooling the solution to room temperature, the resulting magnetic black precipitate was collected using a bar magnet. The MNPs were then purified by repeated cycles of washing and redispersing in ethanol and Milli-Q water. By adjusting the amount of the iron precursor, these Fe_3O_4 particles can be tuned to produce MNPs with diameters less than 10 nm up to several hundred nanometers. The shape and size of the MNPs were observed using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). As an alternative to the in-house synthesis of these particles, Fe_3O_4 MNP solution can also be purchased from Sigma-Aldrich (product number 725358). These MNPs employed in this laboratory experiment were used in two forms: (1) as-is with no coating (i.e., uncoated) and (2) after coating with PVP as described below.

Experimental Method Developed for Coating the MNPs

A solution containing MNPs at a concentration of ∼100 mg/mL was prepared. An aliquot (10−20 μL) of this MNP solution was then added into two 1.5 mL microtubes, followed by addition of poly(ethylene oxide) to one microtube and 10 mg of polyvinylpyrrolidone to the other. We then added distilled water to the fill line of the microtube and thoroughly mixed the solution by shaking it for 3 min by hand. After 5 min of centrifugation at the highest speed, we used a grade N42 bar magnet to hold the particles at the bottom of the microtube while pipetting out the supernatant. The washing process (i.e., add water, shake, centrifuge, remove supernatant) was performed three times to ensure that all polymer not attached to the MNPs was completely removed. After the third wash, we removed the excess liquid by careful decantation, leaving the PVP-coated MNPs at the bottom of the microtube.

Exploration of Procedures To "Magnetize" the Microorganisms

Initial attempts to magnetize the paramecia by attaching magnetic nanoparticles to their exterior cell wall using small Fe_3O_4 MNPs (5−10 nm) failed. These MNPs were functionalized with various electrostatically charged ligands, including poly(ethylenimine), poly(diallyldimethylammonium chloride), and poly(allylamine hydrochloride). Unfortunately, these experiments failed to consistently produce paramecia with surface-attached MNPs that enabled manipulation using a magnetic field. Several factors might have caused these failures, such as insufficient surface charge density for successful surface attachment or simply the small number of attached MNPs did not provide a magnetic field of sufficient strength. Moreover, the complexities and time involved with functionalizing the particles using these methods posed significant challenges for practical application.

The alternative approach of "feeding" the MNPs to the microorganisms also presented problems. As noted in the Introduction, ingestion of uncoated Fe_3O_4 MNPs causes these microorganisms to die. The fact that uncoated Fe_3O_4 MNPs are cytotoxic has been noted in several publications.[1,15,22] Our modified approach was based on encapsulating the MNPs to make them safe for ingestion and also mimic the MNPs that are being used in various applications; however, experimentally, there was a concern over whether the paramecia would (or even could) ingest a sufficient quantity of the coated MNPs necessary to produce a magnetic field capable of enabling magnetic manipulation. Initial attempts to manipulate the paramecia after ingesting MNPs coated with citrate or polyethylene glycol (PEG)[23] were unsuccessful. Fortuitously, a concurrent project that involved the coating of MNP clusters with polyvinylpyrrolidone (PVP) provided an opportunity to determine whether paramecia would ingest MNPs coated with PVP, which they did. The ease of preparation of the PVP-coated MNPs and their compatibility with the paramecia led to our abandoning all other strategies and adopting the latter approach.

Uptake of MNPs by Paramecia

To the PVP-coated MNPs, we added on day one approximately 1.5 mL of microorganism solution to each of the microtubes and allowed sufficient time (overnight) for the paramecia to internalize the MNPs. On day two, we pipetted a few drops of the incubated solution onto a glass slide and observed the microorganisms under an optical microscope. Bar magnets were used to control the *P. aurelia* and *P. bursaria* while on the glass slide and in the Petri dish. In some instances, the grade N42 bar magnet proved to be too strong, pulling the nanoparticles out of the organisms, which caused their death. A digital camera was used to take pictures of the microorganisms. After all observations were made, soap and water was used to clean the work area. All contact surfaces where the microorganisms were present were cleaned with a dilute bleach/water mixture.

HAZARDS

All chemicals used were listed as irritants and were handled with appropriate personal protective equipment (e.g., goggles, lab aprons, and gloves) as indicated in the safety data sheets (SDSs). Labeled waste containers were made available for any waste solutions. The bar magnets used for magnetic nanoparticle manipulation are powerful and should be handled with care to avoid pinching. The microorganism solutions were disposed of properly, and all contact surfaces were washed with dilute bleach (25% bleach/water solution) to prevent illness caused by microorganisms. For additional safety and hazard

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information for each of the chemicals and organisms, please refer to the SDSs provided by the vendors.

RESULTS AND DISCUSSION

We prepared monodisperse, 100 nm Fe_3O_4 MNPs using a solvothermal reduction method involving sodium acetate mediated reduction of $FeCl_3$ by ethylene glycol,[21,24] a high-boiling reducing agent. This synthetic strategy can be used to tailor the size of the MNPs at a chosen precursor concentration by varying the reaction temperature and reaction time. The reaction parameters affect the nucleation and growth processes and consequently the size of the MNPs. To obtain a monodisperse sample, the nucleation and growth processes are decoupled.[25] The size and shape of our MNPs after 8 h at 180 °C were confirmed using SEM and TEM (see Figure 1).

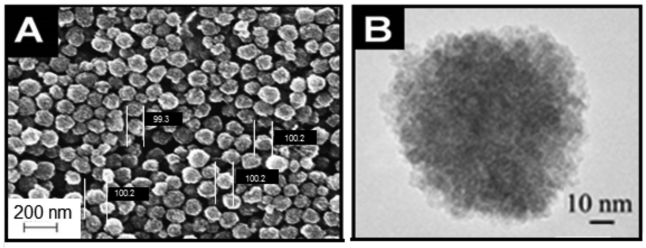

Figure 1. SEM (A) and TEM (B) images of Fe_3O_4 nanoparticles.

Functionalization (or "coating") of nanoparticles is typically required to ensure colloidal stability and biocompatibility. The nonionic polymer PVP is commonly used for this purpose.[26] Studies have shown that PVP coating of Fe_3O_4 MNPs occurs via a coordinative interaction between the Fe atoms in the MNPs and the O atoms of PVP.[11,27] The presence of the PVP prevents aggregation arising from van der Waals attraction between the MNPs.[26,28] PVP became the coating of choice after several exploratory attempts with other agents described in the Experimental Section. The process for coating with PVP was simple and thus appropriate for this laboratory experiment designed for high school students. Importantly, PVP-coated MNPs provided the students with MNPs that could be "fed" to the paramecia without toxic side effects.

Paramecia, like all protists, are single-celled microorganisms. Each of the paramecium species eats bacteria, algae, or smaller protozoa and moves with the aid of cilia. *P. aurelia* and *P. caudatum* are spindle-shaped, while *P. bursaria* are shorter and smaller. *P. bursaria* are green due to the presence of symbiotic green algae within them. Following the procedure described in the Experimental Section, after 12 h in the presence of both uncoated and coated (with PVP) MNPs, each paramecium species was observed under a microscope. Uptake of MNPs was readily visible in each species. The Supporting Information includes videos demonstrating the uptake. The simultaneous recording of observations and collection of pictures of moving paramecia proved challenging; therefore, a quieting agent (Protoslo) was sometimes used that slows the movement without killing the microorganisms. Figures 2A and 2B show the optical microscope images of *P. aurelia* after internalization of the PVP-coated Fe_3O_4 MNPs at 12 h and after 4 d. Figure 2F is an SEM image of the same. Following the positive results with *P. aurelia*, the experiment was repeated using the two other paramecium species. Figures 2C, 2D, and 2E show the paramecia 18 h after introducing them to the coated MNP

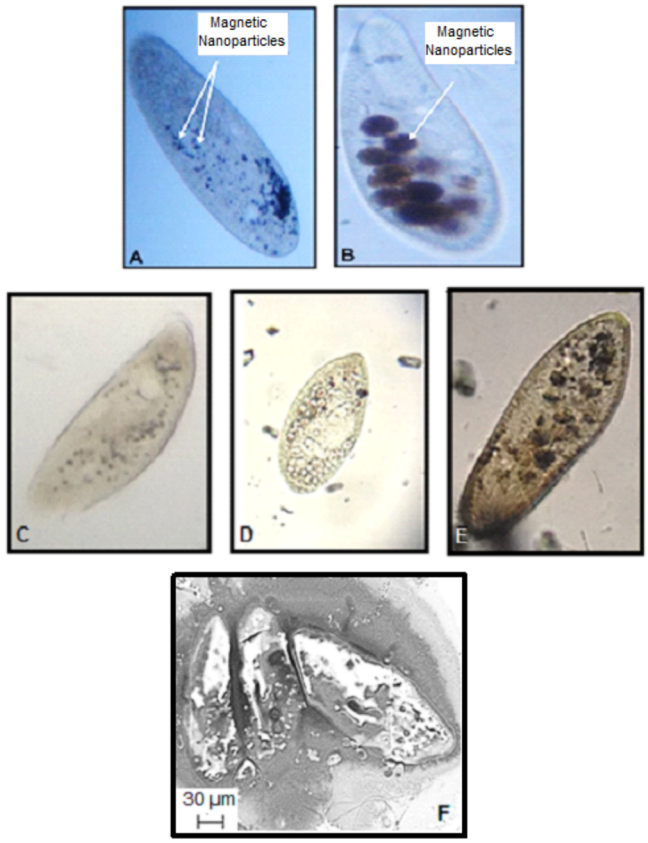

Figure 2. Viewed with 400× power on an optical microscope after exposure to and internalization of Fe_3O_4 MNPs: (A) *P. aurelia* at 12 h, (B) *P. aurelia* at 4 days, (C) *P. aurelia* at 18 h, (D) *P. bursaria* at 18 h, and (E) *P. caudatum* at 18 h. (F) A scanning electron microscopic (SEM) image of *P. aurelia* with ingested MNPs.

environment. After 1 d, the Fe_3O_4 MNPs appear to have aggregated inside the microorganisms in the food vacuoles created after uptake.

While the uptake of MNPs coated by PVP was achieved for each species studied, the uptake of uncoated MNPs (at 0.004 M) led to the rupture of the cell wall of the microorganism. Shoup et al.[29] studied the effect of the presence of iron on the respiration of paramecia and found that iron(III) oxide was toxic to these microorganisms at 0.001 M at 5.9 pH, leading to a 60% loss in respiration rate. More recently, Singh et al. studied the toxicity of Fe_3O_4 MNPs and the likely mechanisms that cause a toxic response.[22] Exposure to these uncoated MNPs has been associated with inflammation, DNA damage, impaired mitochondrial function, and generation of reactive oxygen species (ROS). In our experiments, following internalization of uncoated Fe_3O_4 MNPs, the MNPs degrade into ferrous ions that likely reacted with the H_2O_2 and O_2 produced by the mitochondria to form highly reactive hydroxyl radicals. It is believed that these radicals are capable of destroying the lipid membrane. For the paramecia examined in this report, cell death after ingestion of uncoated MNPs was frequently accompanied by a sudden rupturing of the cell wall: a stunning event to witness through a microscope.

In addition to evaluating the uptake of MNPs by the paramecia, we wished to determine if we could control the movement of our "magnetic" paramecia. If we could use the ingested MNPs to move the paramecia with a magnet, then it might be possible to make them perform tasks, like a

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nanorobot. To our delight, the microorganisms were attracted to a bar magnet (Figure 3), which caused the paramecia to drift

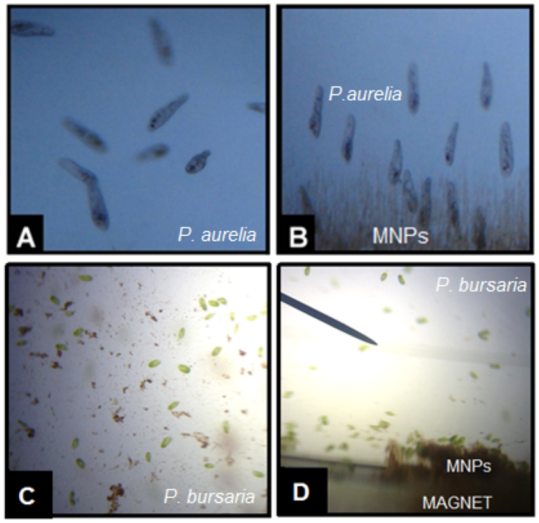

Figure 3. *P. aurelia* and *P. bursaria* viewed under an optical microscope at 40× power: (A, C) swimming freely and (B, D) being controlled with a magnet located at the bottom of the picture. Nanoparticle waste is also attracted to the magnet as seen in B and D.

slowly toward it. In some instances where we had observed uptake of MNPs, the paramecia were not attracted to the magnet, except those that got carried away by the fluid flow initiated by the bulk of the MNPs moving toward the magnet. In these cases, considering that the saturation magnetization of the MNPs was ∼70 emu/g, and based on the size of the food vacuole that housed them, there was likely an insufficient number of MNPs to generate movement of the paramecia toward the magnet.

The experiment outlined in the Supporting Information was performed using *P. aurelia* by 40 groups of 4 to 6 Advanced Placement (AP) biology students in their high school. The long-term research involving several additional experiments of functionalization and evaluation with all three species were performed by two high school students in a university research laboratory.

CONCLUSIONS

Using the results obtained and experimental procedures developed in these studies, nanotechnology can be introduced and utilized in a basic experiment that most students can perform. To develop the longer-scale research project, two high school students worked with chemistry techniques while performing MNP synthesis and functionalization, and used the techniques of sonication, centrifugation, and magnetic separation. With the outlined two-day experiment, students in the high school laboratory were able to functionalize the previously synthesized MNPs, and observe and document the uptake of MNPs by microorganisms (paramecia) using an optical microscope. Additionally, the students were able to witness the influence of MNPs on microorganisms as they manipulated the "magnetic" paramecia with a strong magnet. When implemented in a high school setting, many students described this experiment as "cool" and questioned how they might use magnetic nanoparticles in other ways or what would happen in the presence of different types of MNPs. The idea of using paramecia as nanorobots also generated interest among the students. The uptake of uncoated MNPs, which led to cell rupture and microorganism death, was a significant observation and enabled discussion of the potential negative impacts of nanotechnology and the important role that chemistry plays in living systems.

Most of the MNPs currently used in various biomedical applications are coated for easy functionalization and biocompatibilty; as such, this aspect of the project also enables a discussion about how toxic materials can be made safe for specific purposes. Furthermore, the subject matter of this experiment provides a forum for the discussion of the appropriate means of waste disposal and the possible health-related consequences of nanoparticles getting into the food web. As a whole, this experiment not only offers an appealing platform for introducing nanotechnology to high school students, but also provides an excellent opportunity to introduce the standards of scientific inquiry while encouraging the study of overlapping disciplines. For this assignment, students can be assessed using the high school National Science Education Standards (NSES), the College Board, and the TEKS.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.6b00222.

Instructions for the students and notes for the instructor (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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