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Article

Liposome and chitosan coated iron oxide nanoparticles pass a model blood–brain barrier to kill bacteria

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SUMMARY

Bacterial meningitis is treated with intravenous antibiotics, which contributes to antibiotic resistance when prescribed widely. The U.S. Centers for Disease Control and Prevention has predicted that one person will die from antibiotic-resistant bacteria every 3 seconds by 2050, representing a significant healthcare crisis. To avoid the use of antibiotics to fight infections, we sought to design, engineer, and characterize iron oxide nanoparticles embedded in liposomes and coated with chitosan to pass the blood–brain barrier to kill bacteria. To create such nanoparticles, the following processes were used: co-precipitation of iron oxide, liposomal encapsulation, and the addition of a chitosan coating. Chitosan was used due to its antibacterial properties. The nanoparticles were characterized by size using dynamic light scattering and tested for their ability to pass through a model blood–brain barrier (filter paper coated with a lipid bilayer) and kill *Escherichia coli* bacteria. We conducted experiments both in the presence and absence of a magnet to magnetically drive the iron oxide nanoparticles embedded in liposomes and coated with chitosan through a model blood–brain barrier. We observed that the nanoparticles effectively killed bacteria after 12 hours, suggesting that iron oxide nanoparticles embedded in liposomes and coated with chitosan should be further studied for treating bacterial meningitis without resorting to antibiotic use.

INTRODUCTION

Meningitis is inflammation of the meninges, which are the protective membranes surrounding the brain and spinal cord (1). Inflammation is usually caused by an infection, and it can be life-threatening if not promptly diagnosed and treated (1). There are two types of infections, namely viral and bacterial. Viral meningitis does not usually require intervention. In contrast, for bacterial meningitis, immediate hospitalization and the administration of intravenous antibiotics is needed (1). Without proper treatment, bacterial meningitis can result in serious neurological complications. Research in 2021 estimated at least 1.2 million cases of bacterial meningitis occur annually (1). According to the World Health Organization (WHO), around 1 in 10 people who contract bacterial meningitis die, even with treatment (1). The CDC reports that the death rate can be as high as 70% without treatment (2).

Bacterial meningitis is caused by a variety of bacteria. The most common are *Streptococcus pneumoniae*, *Neisseria meningitidis* (*meningococcus*), *Haemophilus influenzae type b* (Hib), *Listeria monocytogenes*, and *Escherichia coli* (*E. coli*) (2). Bacterial meningitis can be passed through respiratory droplets, making it contagious (1). Outbreaks of bacterial meningitis are considered a medical emergency due to the disease's ability to progress extremely fast and lead to consequences like brain damage, hearing loss, or even death.

To prevent severe health crises from bacterial meningitis, antibiotics are quickly deployed (1). Antibiotics are a class of medication used to treat bacterial infections by either killing the bacteria or preventing their growth; however, bacteria can quickly mutate and become resistant to overused antibiotics, thus rendering such antibiotics useless (2). This has led to the CDC and Maternal Health Surveillance (MHS) literature predicting that 10 million people will die in 2050 due to antibiotic resistance; that is, one person every 3 seconds, representing a significant healthcare crisis (2). This predicted death from antibiotic-resistant bacteria trumps the estimate for deaths from cancer (today's leading cause of death), predicted at 8.2 million deaths in 2050 (2). Antibiotic resistance occurs when a mutation renders a bacterium the ability to withstand the effects of current-day antibiotics, allowing infections to thrive. Antibiotic resistance is an example of natural selection. First, a random bacterial gene mutates to make that bacterium immune to antibiotics. This resistance gene will be passed to daughter bacterial cells (vertical gene transfer) and can also be shared with other bacteria in the population via horizontal gene transfer (2). When antibiotics are used to kill the surrounding bacteria, those with the resistance gene will be left behind. These bacteria will reproduce and spread within the same host or to other beings. For this reason, antibiotic resistance is a significant global health threat.

The second problem faced in treating bacterial infections in the brain (like meningitis) is getting medications to pass through the blood–brain barrier (BBB), a highly selective barrier between the brain and blood vessels (1-11). The function of the BBB is to act as a physical barrier preventing the entry of toxins, pathogens, and large molecules into brain tissue. The BBB is composed of endothelial cells, astrocytes, and pericytes (3). Recent studies have demonstrated that nanoparticles, if created correctly, can pass the BBB, supporting the possible utilization of nanoparticles to treat central nervous system diseases (3). Nanoparticles can be optimized to pass the BBB by altering their surface properties, size, charge, chemistry, coatings, geometry, and/or composing such nanoparticles out of chemistries that respond to external stimuli to magnetically drive them through the BBB (such as for iron oxide) (3).

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Various biocompatible materials can be coated onto iron oxide nanoparticles, such as those as small as 10–100 nm in diameter, and driven by an external magnetic field to pass the BBB (4). Iron oxide nanoparticles can be coated with a wide range of chemistries to improve passage through the BBB and integrate antibacterial properties (4). Iron oxide can be functionalized with -OH, $-NH_3$, and CH_3 to alter the surface charge, which will influence passage through the BBB (4). Further, iron oxide nanoparticles can be coated with antibiotics (such as cephalosporins (7-9)) and/or chitosan, which is a well-known naturally occurring antibacterial protein to impose antibacterial properties (3). While promising, coating iron oxide nanoparticles with such biomolecules remains largely uninvestigated for the treatment of meningitis. Past research has focused on using unmodified iron oxide nanoparticles for treating meningitis but has not focused on using them to deliver biomolecules with antibacterial properties (3, 4).

Moreover, phospholipids can be used to create a liposomal coating around iron oxide nanoparticles to pass the BBB by a process called carrier-mediated transport (5). Further, iron oxide nanoparticles by themselves can be partially antibacterial due to their chemical structure and metal ion release (6). Specifically, Fe^{3+} first combines with H_2O_2 and then is reduced to Fe^{2+}, which can then react with H_2O_2 to produce free hydroxyl radicals, as shown with the Fenton equations below (Eq. 1–4). The free hydroxyl radicals produced kill the bacteria. This reaction system continues even after the death of the bacteria, thus preventing further bacteria activity in that system (6).

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{3+}H_2O_2 \quad (1)$$
$$Fe^{3+}H_2O_2 \rightarrow Fe^{2+} + \bullet HO_2 + H^+ \quad (2)$$
$$Fe^{3+} + \bullet HO_2 \rightarrow Fe^{2+} + O_2 + H^+ \quad (3)$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \quad (4)$$

Considering the antibiotic properties of nanoparticles and their ability to cross the BBB, the objective of the current study was to utilize iron oxide nanomaterials to develop an antibiotic-free treatment for bacterial meningitis. We hypothesized that iron oxide nanoparticles, iron oxide nanoparticles coated in liposomes, and iron oxide nanoparticles coated in liposomes and chitosan would be able to traverse a simulated BBB and kill K-12 *E. coli*, our model organism for bacterial meningitis. Chitosan was chosen since it possesses antibacterial properties (7, 8). *E. coli* is the most common Gram-negative bacillary organism that causes meningitis, which is why it was chosen for the present study (9). We showed that all combinations of iron oxide nanoparticles formulated in the present study (uncoated, as well as coated with lipids and chitosan) were able to cross the simulated BBB and completely kill K-12 *E. coli* after just 12 hours of culture. Such results suggest that the present iron oxide nanoparticles should be further studied for a wide range of neurological diseases, including meningitis.

RESULTS

The objective of the present study was to formulate iron oxide nanoparticles embedded in liposomes and coated with chitosan that can magnetically cross the BBB to kill bacteria that cause meningitis. For this, iron oxide nanoparticles were first synthesized through co-precipitation, then embedded in liposomes, and finally coated with chitosan. We determined the size of the nanoparticles using dynamic light scattering (DLS). Nanoparticle diameter can be an indicator of a successful coating, with an increased diameter more likely indicating a successful coating. Uncoated iron oxide nanoparticles averaged a diameter of 48 nanometers (nm) (**Table 1**), falling in the range of the average size of nanoparticles from a few

	Iron Oxide Nanoparticles	Iron Oxide Nanoparticles Coated with Liposomes	Iron Oxide Nanoparticles Coated with Chitosan
Diameter (nm)	48 ± 2.3	122 ± 10.4	273 ± 18.9

Table 1: Dynamic light scattering (DLS) results for the iron oxide nanoparticles, iron oxide nanoparticles coated with liposomes, and iron oxide nanoparticles coated with chitosan. N = 3. Results = Mean ± standard deviation.

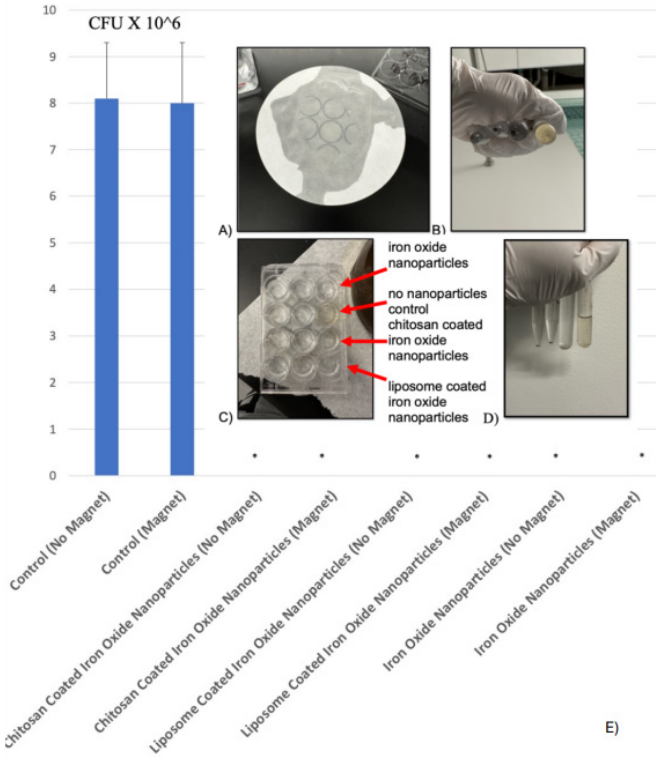

Figure 1: Experimental results exposing *E. coli* to iron oxide nanoparticles with the model BBB filter. A. The setup used in the model BBB trial. A sheet of filter paper coated with a phospholipid bi-layer guarding entry to a case of wells, each containing the bacteria. B. A bottom view of each row centrifuged. Going left to right, iron oxide nanoparticles coated with chitosan, iron oxide nanoparticles coated with liposomes, iron oxide nanoparticles, and the control can be seen. C. The wells containing bacteria after contact with the nanoparticles. The four sets of wells are iron oxide nanoparticles, control, iron oxide nanoparticles coated with chitosan, and iron oxide nanoparticles coated with liposomes. D. A front-facing view of each row centrifuged. Going left to right, iron oxide nanoparticles coated with chitosan, iron oxide nanoparticles coated with liposomes, and iron oxide nanoparticles, then the control can be seen. The black iron oxide nanoparticles can be clearly seen at the bottom of the respective centrifuge tubes. E. Colony forming units (CFU) of K-12 E. coli cultured in the presence of 10 mg/ml of iron oxide nanoparticles coated with chitosan, iron oxide nanoparticles, and controls (no nanoparticles) for 12 hours with/without a magnet. Data = mean +/- SEM; N = 3 (in triplicate), * $p < 0.01$ compared to controls.

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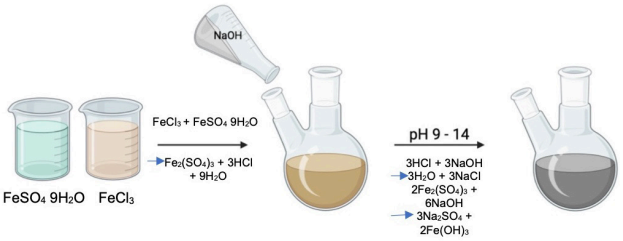

Figure 2: The process used to create the iron oxide nanoparticles. To formulate the iron oxide nanoparticles, ferrous sulfate and ferric (III) chloride were added to distilled water. Once fully mixed, sodium hydroxide was added, and the solution was allowed to settle for 1.5 hours. After pouring and pipetting unreacted chemicals, the precipitated iron oxide nanoparticles were placed in an oven at 200 °C for 1 hour and collected as a powder.

nanometers to around 100 nanometers (6). Nanoparticles coated in liposomes averaged 122 nm in diameter, whereas iron oxide nanoparticles coated in chitosan were larger at an average diameter of 273 nm (**Table 1**).

The next step was to determine whether the formulated nanoparticles could pass through a simulated BBB to inhibit bacteria growth. This barrier was made up of a filter paper approximately one micron thick and coated in a lipid bilayer (setup shown in **Figure 1A**). While still an approximation, this bilayer simulated some properties of the BBB. Many studies found in the literature either have not used a model BBB, such as the filter used here or have created a cell-based BBB, which was not feasible in the present study (3, 4). After laying the simulated BBB filter over each of the wells, the nanoparticles were placed over their respective wells. Then, a magnet was placed on the other side of the wells, creating an external magnetic force for 12 hrs. The nanoparticles were then added and exposed to a magnet with bacteria underneath, and bacteria growth was determined. Results showed that the nanoparticles passed the model BBB, as initially shown by centrifugation (**Figure 1B-D**), and killed bacteria, as confirmed through quantified CFU assays (**Figure 1E**). This observation supports our conclusion that bacteria grew only in the control and not in the environment containing nanoparticles. These results indicated that the various iron oxide nanoparticles formulated here can cross the model BBB filter to kill bacteria and, thus, should be further studied to evaluate their potential use in the treatment of neural bacterial infections.

DISCUSSION

Here, we sought to design, fabricate, and test iron oxide-based nanoparticles that can cross the BBB to kill bacteria responsible for meningitis. We synthesized uncoated iron oxide nanoparticles, iron oxide nanoparticles embedded in liposomes, and iron oxide nanoparticles embedded in liposomes and coated with chitosan and tested their ability to cross a model BBB filter to kill underlying *E. coli*. Iron oxide nanoparticles were successfully synthesized and characterized using DLS. Liposomes are commonly used to encapsulate nanoparticles to avoid immune system clearance, and chitosan was chosen since it is an antibacterial agent. An increase in nanoparticle size, as measured by DLS, confirmed the coatings. Afterward, each nanoparticle's ability to kill bacteria and pass a simulated BBB was tested and confirmed.

Each iron oxide nanoparticle, coated or not, killed the bacteria. Furthermore, we examined the capacity of the nanoparticles to pass the simulated BBB under the aid of an external magnetic field. All tested nanoparticle types passed the BBB and killed the bacteria in the wells. We speculated that all nanoparticles, since they were composed of iron oxide, produced intracellular iron, which successfully killed and stopped further growth of the bacteria (16).

The goal of the current study was to create nanoparticles with coatings that had sizes tailored to passing the BBB, with the additional functionality of antibacterial activity. Our results showed that this was successful, but there are several limitations that should be considered. First, while DLS confirmed the size of iron oxide nanoparticles, we did not characterize the chemistry of the nanoparticles. While we followed standard techniques to create iron oxide nanoparticles and demonstrated that they were magnetic, it is plausible, although remote, that another nanoparticle chemistry was created in this study. Further, GNC TOTAL LEAN® Chitosan with Glucomannan capsules were used as the source of the chitosan, so there is a possibility that other ingredients in the capsules may have caused confounding results.

Additional limiting factors of the present study include the size of pores in the filter paper, the bacteria used, and the pills the chitosan was extracted from. Pores in the filter paper used were 1 micron thick, while BBB pores span from 4-6 angstroms in width. Thus, further study is required to determine whether the present nanoparticles would also be able to pass through the pores of the BBB. Further, this experiment used K-12 *E. coli* bacteria, which are non-pathogenic and do not cause meningitis. Although *E. coli* has been shown to cause meningitis (2), the specific strain we used (K-12) is a safe alternative, approved by University Environmental Health and Safety guidelines, that can be used in a laboratory setting. Therefore, more experimentation would be needed to determine the bactericidal activity of our nanoparticles against other bacteria that can cause meningitis. However, other strains of *E. coli* and other bacteria that cause meningitis (*Streptococcus pneumoniae*, *Neisseria meningitidis* [*meningococcus*], *Haemophilus influenzae type b* [Hib], and *Listeria monocytogenes*) should be susceptible to death by the same process of intracellular iron release.

This study provided tentative evidence regarding the utility of nanoparticles for the treatment of bacterial meningitis. This study suggested that these nanoparticles can kill bacteria and, in theory, pass the BBB. The next step would be to decide on the best form of nanoparticle delivery. Current ideas include co-delivery with transport enhancers, focused ultrasound,

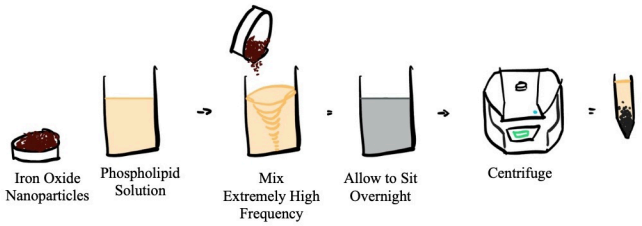

Figure 3: The process used to coat the iron oxide nanoparticles with liposomes.

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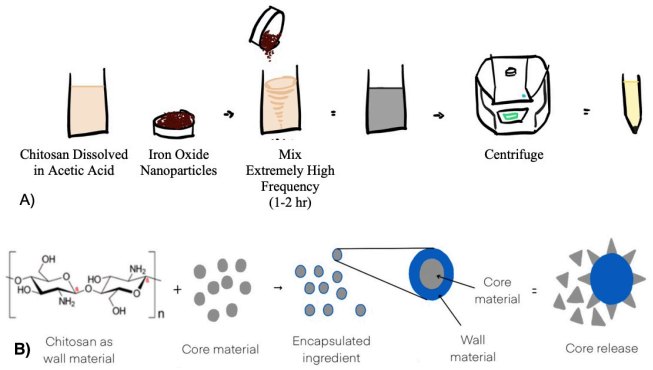

Figure 4: A. The process used to coat the iron oxide nanoparticles in chitosan. B. Representation and applications of materials coated in chitosan. At a 1:1 ratio (by weight), chitosan was added to the aforementioned iron oxide nanoparticles. The solution was stirred for one hour, centrifuged, and the chitosan-coated iron oxide nanoparticles were collected.

or external magnetic fields (16). External magnetic fields are particularly promising in the context of bacterial meningitis, as they address the concern of specific targeting, which would allow for targeting of solely the infected part of the brain.

Placing these results into perspective, it is important to note that in 2021, 1.2 million cases of bacterial meningitis were estimated to occur annually, with 1 in 10 people dying even with treatment and a death rate as high as 70% without treatment (1, 2). This study continued the investigation of iron oxide nanoparticles coated with liposomes and chitosan for treating bacterial infections. It showed that iron oxide nanoparticles encapsulated in a liposome and coated with chitosan could be synthesized and cross a simulated in vitro BBB filter to kill a prominent bacteria strain associated with meningitis.

MATERIALS AND METHODS

Iron oxide nanoparticles

Protocols used in this study followed those previously reported in the literature (10, 11). The procedure for nanoparticle production is shown in **Figure 2**. To formulate the iron oxide nanoparticles, 2 M ferrous sulfate, 1 M ferric (III) chloride, and sodium hydroxide at pH 13 were used (all chemicals from Sigma Aldrich). First, 10.647 g ferrous sulfate and 8.276 g ferric (III) chloride were added to distilled water. Once fully mixed, sodium hydroxide was added to reach a pH of 12.9, and the solution was allowed to settle for 1.5 hours. After pouring and pipetting unreacted chemicals, the precipitated iron oxide nanoparticles were placed in an oven at 200 °C for 1 hour and collected as a powder.

Liposomal iron oxide nanoparticles

To coat the iron oxide nanoparticles for easier passage through the BBB, a liposomal vitamin C procedure was used (12). Lecithin (i.e., the phospholipid) from CVS Health Lecithin Softgels was removed from the capsules and fully dissolved in distilled water. Then, lecithin was added at a 4:1 ratio (by weight) to the iron oxide nanoparticle solution (**Figures 3A** and **B**). The solution was stirred for 30 minutes, and the mixture was allowed to sit overnight.

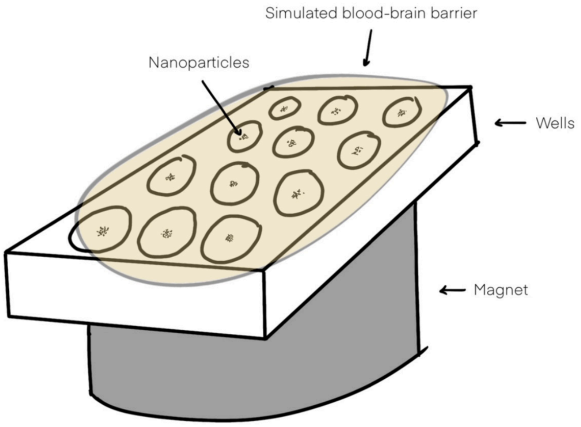

Figure 5: A diagram of the setup used in the model BBB trial. .

Chitosan coating

Chitosan was the next proposed coating due to its ability to kill bacteria (13, 14). At a 1:1 ratio (by weight), 0.5 g of chitosan removed from GNC TOTAL LEAN® Chitosan with Glucomannan capsules (dissolved in 0.1 M HCl) was added to 0.5 g of the aforementioned iron oxide nanoparticles. Since it is possible that the HCl used to dissolve the Glucomannan capsules may interfere with the generation of iron oxide nanoparticles, we confirmed the magnetic properties of the iron oxide nanoparticles after completing this step. The solution was stirred for one hour and centrifuged at 500 rpm for 5 minutes to collect the chitosan-coated iron oxide nanoparticles (**Figures 4A** and **B**).

Nanoparticle characterization

The size of the nanoparticles was characterized using dynamic light scattering (DLS). DLS experiments were completed in triplicate. For this, all iron oxide nanoparticles were added to deionized water in cuvettes and subjected to DLS measurements according to the DLS manufacturer's instructions (Zetasizer Pro; Malvern Panalytical).

E. coli killing assays and colony forming unit (CFU) counts

A BBB filter paper model was used and coated with a lipid bilayer. To create the lipid bi-layer, phospholipids from CVS Health Lecithin Softgels were dissolved into deionized water, creating a thick solution capable of coating the filter paper. *E. coli* (K-12, ATCC) was used as a model bacterium. The experimental setup is shown in **Figure 5**. Although there are no perfect simulations of the BBB for in vitro models and in vivo testing is the only way to truly test BBB passage, any type of filter modeling the BBB is better than traditional studies that have not used any filtering (3, 4). For the present study, filter paper coated with a lipid bi-layer was used to model the BBB due to its ease of availability, low cost, presence of pores, and chemical similarity. The apparatus was left still for 12 hours to allow the 10 mg/mL nanoparticle solution to pass through the filter paper and then migrate into a 10^6 CFU/mL K-12 *E. coli* solution. In some experiments, a magnet (0.2 Tesla, Fisher Scientific) was placed below the well plate to cover the well

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completely and to promote iron oxide nanoparticle passage through the filter paper. After 12 hours, CFU assays were completed by taking the bacteria and spreading and plating them on agar plates, as well as counting the number of CFUs according to standard procedures (16). All experiments were completed in triplicate, and standard statistical tests (student t-tests) were performed to determine differences between means.

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