Encapsulating Various Proteins Within Silica Nanoparticles

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INTRODUCTION

Over the past decade, the interest and studies on nanoparticles is substantial. The drive behind such vast research is the influence of using nanoparticles in modern medicine. Nanoparticles in themselves act as vehicles for proteins, enzymes, drugs, etc. and carry them throughout a system to a specific target. In this specific project, silica nanoparticles will be used. Silica nanoparticles have been shown to be biocompatible in humans and have a lot of biological significance, and this reasoning is why this research focusing on this type of nanoparticles over others. The silica nanoparticles that will be synthesized are mesoporous which allows the nanoparticles to be biocompatible due to its low hemolytic activity.5 Since they are biocompatible, silica nanoparticles can be used for things such as drug delivery, biosensors, imaging, and more. All of these functions occur at the nanoscale.

It is significant that nanoparticles are being used because the nanoscale is the level in which biology occurs. Being at such a small scale, this allows the particles to be more precise in their targeting and less invasive overall. Also, nanoparticles tend to form a spherical shape, and the smaller it is, the greater is its surface area to size ratio. With a greater surface area, this ratio allows the nanoparticles, like in encapsulation, to have more activity and absorbance overall. Encapsulation allows the nanoparticles to create a spherical shape around the protein while also having many more benefits from the encapsulation of things such as proteins.

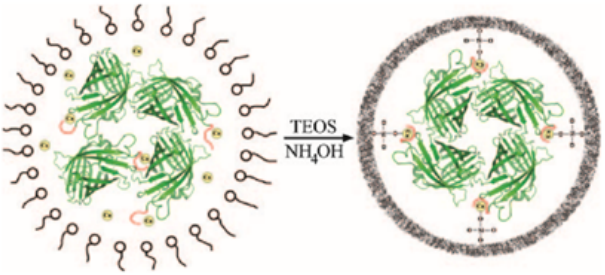
Encapsulation of proteins gives many benefits through its protection of the proteins. Through encapsulation, proteins are protected, in this case, by a silica shell. This shell prevents the protein from denaturing from heat, acidity, or metabolism.4 All the while, the drug or enzyme that is encapsulated should still maintain its function and interact with a target site because the silica shell contains small pores that allow such reactions.

The theory behind this experiment is that the silica nanoparticles can greatly aid in the delivery of drugs and proteins throughout the human body. Proteins are vital to the normal functions of the body such as growth and metabolic regulation, and having the silica shell around proteins like these can benefit protein therapies that are used like in the treatment of people with diabetes.

MATERIALS & METHODS

The procedure used to encapsulate proteins within silica nanoparticles is a water in oil reverse microemulsion process.1 A microemulsion is a water in oil mixture, typically with a surfactant that allows things such as water and oil, that typically do not mix to be able to mix and form some type of precipitant.3 The reverse microemulsion process gives an advantage in the encapsulation process, allowing the silica nanoparticles to encapsulate negatively charged proteins (a Isoelectric point less than seven), whereas negative proteins are typically not able to be encapsulated. This process should work for both positively and negatively charged proteins.

This novel method was developed by Cao, and the procedure began with the preparation of a one mg/mL solution of $CaCl_2$. The calcium chloride solution is very important to the encapsulation process in that the calcium ions create coordinate bonds linking the silica shell to His-tagged proteins, creating a hollow silica shell (seen in the figure below). The microemulsion mixture was started by adding 35 µL TEOS, 2.5 mL cyclohexane, 0.6 mL Triton X-100, 0.6 n-hexanol, and 100 µL of a protein solution in a 0.2 mL of the $CaCl_2$ solution in a five mL micro-vial. Then 20 µL of a 25% ammonia solution, prepared beforehand, is added to the solution. The solution is left stirring for 24 hours. After stirring, 6.5 mL of acetone is added to the microemulsion solution. The nanoparticles are then abtained by centrifugation at 12,000 rpm. Lastly, the nanoparticles are washed several times with DI water and ethanol. Once the nanoparticles are obtained, various characterization experiments will be run on the nanoparticles to confirm the synthesis. This same basic procedure was used for each of the three proteins used in this project: myoglobin, green fluorescent protein, and chymotrypsin.



The formation of the encapsulation of GFP through the coordinate bonds formed between Ca2+, the negatively charged oxygen on silicate, and the his-tagged protein

Myoglobin

Despite having en isoelectric point higher than seven, this reverse micromulsion should still work, according to the literature, for positively charged proteins. Myoglobin was chosen as a starting block to the encapsulation process because it was relateively cheap and readily available, and former experiments shows that myoglobin has been successfully encapsulated within silica nanoparticles in the past.3 For these reasons, it was mainly used as practice for the general synthesis as well as practice for finding different characterization techniques.

A myolobin solution was prepared for this experiement by weighing out 6.1 mg of myoglobin and putting it in 10 mL of the original CaCl2 solution, giving it a concentration of 3.5714x10-5M which is about the same concentration of the green fluorescent protein (GFP) solution that was ordered. Once the synthesis and washing is complete for the myoglobin encapsulated particles, the absorabance was taken of the myoglobin solution using a PS-2600 Pasco spectrometer and compared to the absorabance that was taken earlier during the synthesis before ammonia was added to induce polymerization.

The absorbance peak around 410 nm was observed and compared from both spectra, and Beer's Law8 was used to determine the concentration of myoglobin before and after synthesis. This measurement would show that if less myoglobin was present after the synthesis then it can be assumed that the myoglobin was indeed encapsulated by not showing up in the absorbance spectra. Some nanoparticles were also dried out over night and weighed to get its dry weight.

Green Fluorescent Protein

Green fluorescent protein is a more expensive option and was used for additional practice and characterization. The same synthesis procedure was used for the GFP encapsulation solution using 100 µL of the GFP solution. The absorbance was also taken of the GFP solution before and after synthesis to again try to find the concentration of each. Lastly, the GFP encapsulated silica nanoparticles were viewed under a fluorescent microscope to view the fluorescence of GFP while being encapsulted and possibly count the particles, depending on the fluorescent microscope's ability.

Chymotrypsin

he final protein used for encapsulation was chymotrypsin. Chymotrypsin was chosen to test the function of the enzyme while still being encapsulated within the silica shell. Before synthesizing chymotrypsin encapsulated silica nanoparticles, a control experiment was run on chymotrypsin and how it reacts with its substrate, N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE).

The control experiment began by firstly preparing an 80 nM Trizma-base buffer at a pH of 7.8 at room temperature. The Triza-base was diluted using DI water, and 1M sodium hydroxide was added to reach the desired pH. One hundred mL of the 1.18 nM BTEE solution was then prepared by weighing out about 37 mg of BTEE, adding 63.4 mL of methanol, stirring, and lastly bringing the final volume of the BTEE solution to 100 mL. A 2 M CaCl2 solution was then prepared by adding 2.94g of CaCl2 in 10 mL of DI water. Next, a 1,000 fold dilution of 1 M hyrdochloric acid solution was prepared using DI water. Lastly, immediately before the experiment was run, the ezyme solution was prepared by adding 2-5 chymotrypsin units in cold HCl solution.

The solutions created in each test were immediately mixed together by inversion, and the absorbance of each solution was taken at 256 nm for about 5 minutes using a Diode Array Sepctrophotometer. Results were written down from each test.

After the control experiment, this same experiment was run on chymotrypsin encapsulated within silica nanoparticles. The encapsulated chymotrypsin was synthesized using the same procedure as with the encapsulation of myoglobin and GFP. In the second experiment, the encapsulated chymotrypsin replaced the normal enzyme solution. Again, the absorbance was taken at 256 nm for 5 minutes, and the results were written down.

Other

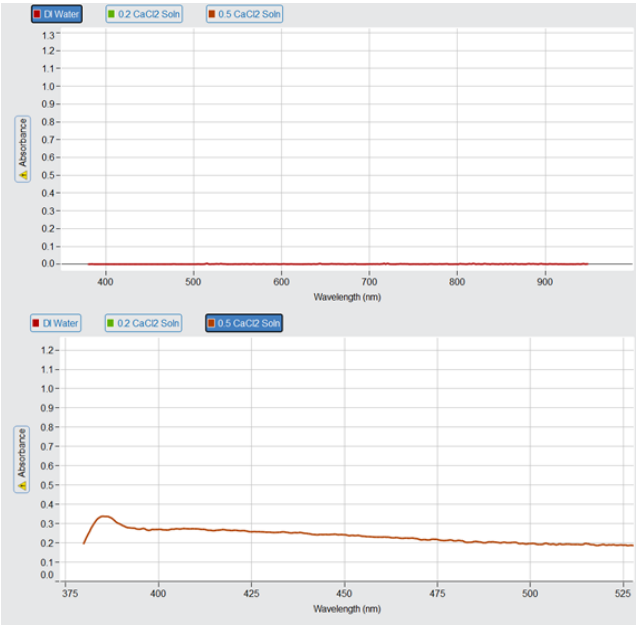
Other experiments were run to firsly prove that nanoparicles were present in a synthesis solution and to secondly follow the same synthesis procedure without encapsulating any proteins. To show that nanoparticles were present in the solution, an absorbance spectrum was taken of just the CaCl2 solution as a control, and another absorbance spectrum was taken of a batch of silica nanoparticles. Next, to check to make sure the results of encapsulated proteins are unique, an experiment was

run in which the same synthesis procedure was done but without adding any protein to the solution. Observations and results were recorded.

All of the encapsulated protein solutions (even the blank encapsulation noted above) were sent off to the Virginia Commonwealth University to be looked at under an elctron microscope. The results from VCU would allow a closer look at our particles to see their shape and average size.

RESULTS & DISCUSSION

The first experiment that was run was to confirm that silica nanoparticles were being formed. Absorbance spectra was taken comparing a control of DI water with that of the CaCl2 solution. The spectra are seen below.

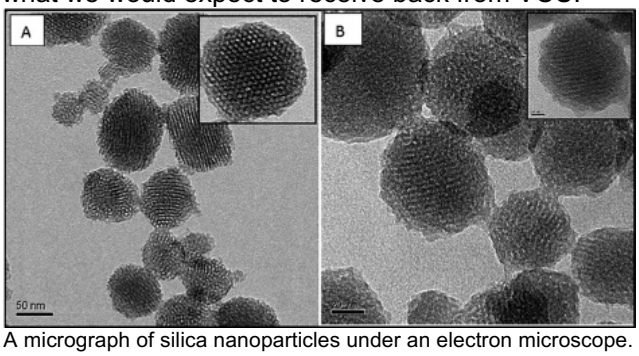


Comparison between the absorbance of the control (top) of DI water and the absorbance of one of the experimental groups (bottom) to test for the presence of nanoparticles.

The results were as expected. The only difference between the two spectra is that one is raised higher than the other. This difference can be explained in that the silica nanoparticles are not absorbing more light but reflecting a lot of the light, and therefore, the spectra shows more absorbance. This result is expected with silica nanoparticles.

The results of the synthesis of the blank (with no proteins) nanoparticles was very interesting. During the synthesis stage, the solution remained very cloudy whereas in the normal synthesis with proteins the solution remained quite clear. The blank solution also had a black precipitate that formed that did not form in the normal synthesis with proteins. The black precipitate is most likely some polymer that formed in the mixture, and the cloudiness of the solution confirms that this synthesis is a template reaction. Being a template synthesis, the reaction would need the protein in order to occur and work the way it is supposed to. Since the solution was cloudy, the silicate was not able to form around the proteins and is still suspended in the solution, making it appear cloudy.

Each protein that was encapsulated, and the blank synthesis, was sent to VCU for evaluation under an electron microscope. These results have still not been received. What these results should show is the average size of the particles and possibly the size of the pores. These pictures would also help count many of the particles. These pictures are a great indicator as to if the proteins were indeed encapsulated or not, and below is a representation of what we would expect to receive back from VCU.

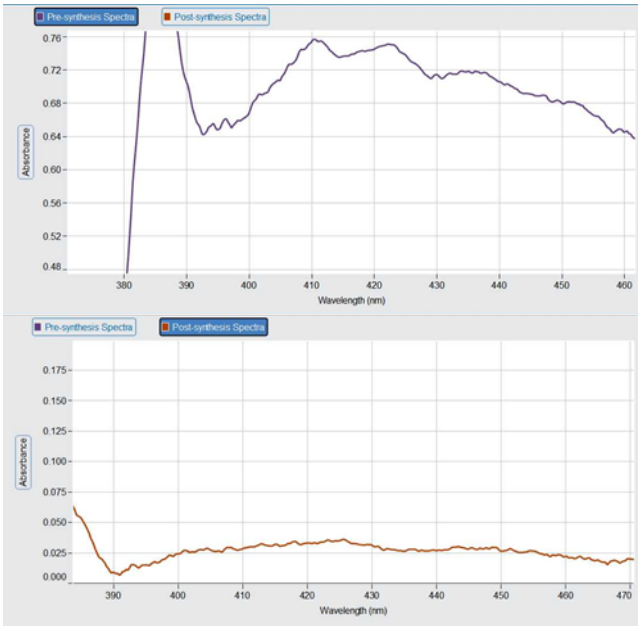


A micrograph of silica nanoparticles under an electron microscope.

Myoglobin

The dry weight of myoglobin encapsulated nanoparticles was also taken using an analytical balance. The empty Eppendorf tube weighed 0.9771g, and the weight of the tub with the dry nanoparticles within it weighed 0.9778g. Therefore, the weight of the particles was 0.007g. There is no direct significance to the dry weight, because it was not sure what weight was expected. Even if the desired weight was known, it would be difficult to have that outcome due to many switches of glassware and possibly human error.

The absorbance at the myoglobin peak of around 410 nm was compared before and after the synthesis of the myoglobin encapsulated particles, and the spectra are seen below.



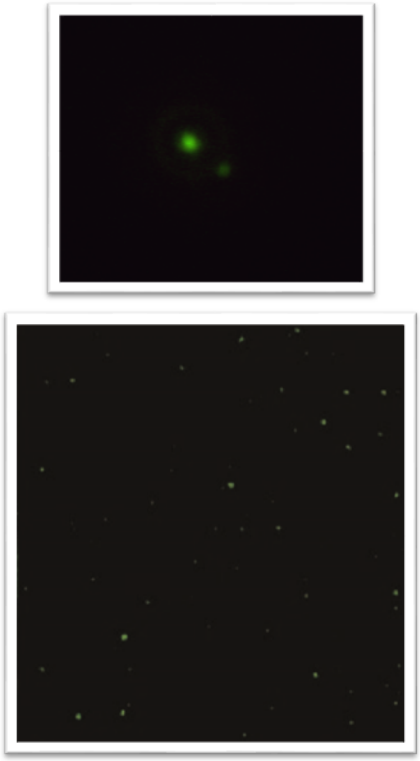
The comparison of the pre-synthesis solution (top) and the supernatant fluid after synthesis (bottom)

Seen in the figures above, the absorbance is clearly much less after the synthesis. Using Beer's law, the concentrations of each were calculated using a molar absorptivity constant of 315,564 molar absorptivity units: 8.873x10-7M myoglobin pre-synthesis and 7.922x10-8M post synthesis. Cleary the concentration of myoglobin for pre-synthesis is about ten times greater than the concentration seen in the post synthesis supernatant fluid after centrifugation. These results are significant in that they show that the myoglobin had to go somewhere. If it is not in the supernatant fluid, then the myoglobin must be encapsulated within the silica nanoparticles left in the bottom of the Eppendorf tube after centrifugation.

Green Fluorescent Protein

Absorbance spectra was also used to observe the pre-synthesis and post-synthesis solutions of the GFP encapsulated silica nanoparticles. However, this experiment was difficult to do because, GFP does not has as much of a distinguished peak as myoglobin. Therefore, the results were inconclusive.

Since GFP fluoresces, another experiment was run to look at the GFP encapsulated silica nanoparticles under a fluorescent microscope (seen below).



Comparison between one of the pictures from the fluorescent microscope in this experiment (top), compared to the results from Cao (bottom).

Compared to the literature, the results certainly match, but not nearly as many GFP fluorescent spots were found as in comparison to the literature. However, in the literature the scientists had access to confocal microscopy, making it much easier to find more GFP encapsulated nanoparticles. Again, these results help confirm that the protein was indeed encapsulated.

Chymotrypsin

The goal of the chymotrypsin experiments was to show that things such as enzymes and drugs will still be functional while being encapsulated. Before testing the encapsulated chymotrypsin, a control experiment was run to test chymotrypsin's reaction with BTEE without the silica nanoparticles involved. Unfortunately, kinetics was not able to be run due to a lack of time. So, the absorbance of N-benzoyl-L-tyrosine, which is the product of BTEE and chymotrypsin, was taken at 256 nm. The results from the control experiment are seen in the table below.

Absorbance at 256 nm	Test 1 (A.U.)	Test 2 (A.U.)	Test 3 (A.U.)	Test 4 (blank) (A.U.)
	2.1941	2.1633	2.1676	1.8425

The absorbance results from each Test in the control chymotrypsin experiment.

These results clearly show a difference in the tests with the chymotrypsin solution and the blank control solution. This confirms that there is a presence of the N-benzoyl-L-tyrosine product in the tests while there is not really any in the control. But, it is unusual that the absorbance does not increase as the amount of enzyme increases.

Next, the same experiment was run but with the chymotrypsin that has been encapsulated within silica nanoparticles. These results are seen below.

Absorbance at 256 nm	Test 1 (A.U.)	Test 2 (A.U.)	Test 3 (A.U.)	Test 4 (blank) (A.U.)
	1.5609	1.578	1.5574	1.5087

The absorbance results from the encapsulated chymotrypsin experiment.

This experiment shows a slight increase of the product in the test solutions, but overall, it is much less than in the control experiment. These results most likely arise from human error from a long process of synthesis and possibly a different amount of chymotrypsin used in the control experiment versus the amount of encapsulated chymotrypsin used in the latter experiment. But again, there still is the possibility that the encapsulated chymotrypsin does not react as well with the BTEE compared to the normal chymotrypsin. More experiments need to be run.

CONCLUSION & FUTURE DIRECTIONS

The results from most of the experiments point towards a successful procedure to encapsulate proteins within silica nanoparticles. The blank synthesis procedure showed that there were indeed silica nanoparticles in the solution. Next, the myoglobin concentration greatly decreased after the synthesis, proving that the myoglobin was encapsulated within the nanoparticles. With green fluorescent protein, the results are similar to those in the literature in that the GFP can still fluoresce while being encapsulated. The results from the chymotrypsin experiments are more difficult to interpret. Being crunched for time, kinetics experiments were not able to be done but rather just a record of the absorbance at 256 nm after 5 minutes was kept. Also, because of the lack of time, we were unable to rerun the chymotrypsin experiments to try to achieve better results. It will also be very helpful once the results return from VCU to evaluate how the procedure worked and if any changes need to be made.

In addition to rerunning the chymotrypsin experiments and running kinetics experiments on the chymotrypsin, there are many more possible future directions that this experiment could take. Other proteins and enzymes could be examined and attempted to be encapsulated. An enzyme with a more targeted approach could be used to continue testing the function of these nanoparticles. A magnet could be incorporated within the nanoparticles and can make them easily retrievable. Experiments could also be run on microparticles, which would be much easier to characterize due to their bigger size. The possibilities are endless in which direction this experiment could go. The final results from VCU will determine if this procedure needs to be changed and redone or if it is viable and maybe needs some finetuning and more practice.

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