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The Use of Dynamic Light Scattering to Determine Mineral Precipitation in Bacteria.

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The Use of Dynamic Light Scattering to Determine Mineral Precipitation in Bacteria.

By Isabella Manning

Abstract

Particulate calcium carbonate (PCC) is an important filler in industrial products, including paints, which are obtained through mining and sintering (heating to 1,100°C), which is responsible for up to 2% of global CO_2 emissions. A potential solution to reduce these emissions is the use of calcium carbonate produced by bacteria from atmospheric CO_2, through a process called microbially induced carbonate precipitation (MICP). One of the primary uses of PCCs is in paint, requiring them to be a specific size (~700nm). When we have been collecting samples of PCCs produced by bacteria, which demonstrate clumping, making them too large for use in paint. I wanted to see if this was occurring in actively growing cells or if this was the result of centrifugation to collect the PCCs. To test this, I used dynamic light scattering (DLS). My data demonstrated that DLS allows us to view the PCCs as they accumulate in culture, with large particles forming in solution, suggesting clumping was occurring. To see if I could prevent this, I began looking at surfactants to see if they might prevent the PCCs from creating aggregates. The data suggested that the interactions of cells with particulates in culture is complicated and must be resolved prior to the commercialization of MICP in industrial filler production, but DLS is an effective method to examine PCC production in culture.

Introduction

Biomineralization, the chemical alteration of environments through microbial activity resulting in the precipitation of minerals, is a common process in bacteria (Anbu, Kang, Shin, *et al.* 2016.). In *Escherichia coli,* microbially induced calcite precipitation (MICP) is an important part of emerging industrial processes involving carbon sequestration. Calcium is toxic to bacteria in high concentrations, so they must find a way to detoxify excess calcium in the environment. One method is to precipitate excess calcium extracellularly, in the form of calcium carbonate ($CaCO_3$) crystals. Bacteria have an antiporter called ChaA, which allows them to export excess calcium (Ca^{2+}) into the environment. Carbonate ions (${CO_3}^{2-}$) are produced during metabolism, which can combine with the carbonate ions to produce the insoluble calcium carbonate crystals (Anbu,, Kang, Shin. *et al.* 2016.). Calcium carbonate can be used in many ways in the industry. It is advantageous for use due to its high purity, white color, and well-ordered particle size and morphology. Currently, it is used as a filler in products like paper, paint, plastics, rubbers, and drugs (Jimoh, Otitoju, Hussin, *et al* 2016). Using calcium carbonate in paint allows producers to control many different properties of the paint like scrub resistance, water uptake, and gloss (Alvarez and Paulis 2017). Calcium carbonate is important to the gloss; the larger the crystals, the more disrupted the surface and the less reflective it is. However, there is an optimal size for calcium carbonate filler in paint, which is about 700 nm (Bodurtha, Matthews, Kettle, *et al* 2005).

Currently, the precipitated calcium carbonate (PCC) used for paint is obtained from mining and sintering, which is responsible for up to 2% of global emissions of CO_2. This process occurs by first mining calcium carbonate from the ground, which is common in limestone. The

rocks are then crushed to a smaller size and calcium carbonate can then be produced through the recarbonation process. This involves using the dissolution of $Ca(OH)_2$ and CO_2 to produce the calcium carbonate simultaneously. This process can be carried out in different reaction conditions to achieve the desired morphology and size of particles (Jimoh, Otitoju, Hussin, *et al* 2016). CO_2 gas is released in both the steps of mining and purification. This gas that is released gets trapped in the atmosphere, keeping the heat close to Earth's surface. This results in a rise of temperature, and contributes to our changing climate, leading to occurrences like extreme weather events, rising sea levels, and shifting wildlife populations/habitats. CO_2 can stay in the atmosphere for thousands of years, meaning the frequent release of it will continue to cause greater accumulation in the atmosphere (Friedlingstein and Solomon 2005). However, using the calcium carbonate produced from bacteria would not require mining and sintering, fixes CO_2 to make the carbonate, and would be a more sustainable solution if these PCCs can be produced at the desired size for industrial use.

Initial experiments by Md Nabil used scanning electron microscopy (SEM) to look at microbially-precipitated PCCs that had been separated using centrifugation on a micrometer scale. This method showed clumping of the PCCs, which was thought to be due to spinning down samples when preparing them (Figures 1 and 2); however, as SEM uses a vacuum it can only be used to look at dry samples. To further study these results and see if the clumping was due to centrifuging samples or drying them, the samples needed to be looked at in active cultures. To do this, a technique called dynamic light scattering (DLS) was used which allows us to see the size of particles in solution.

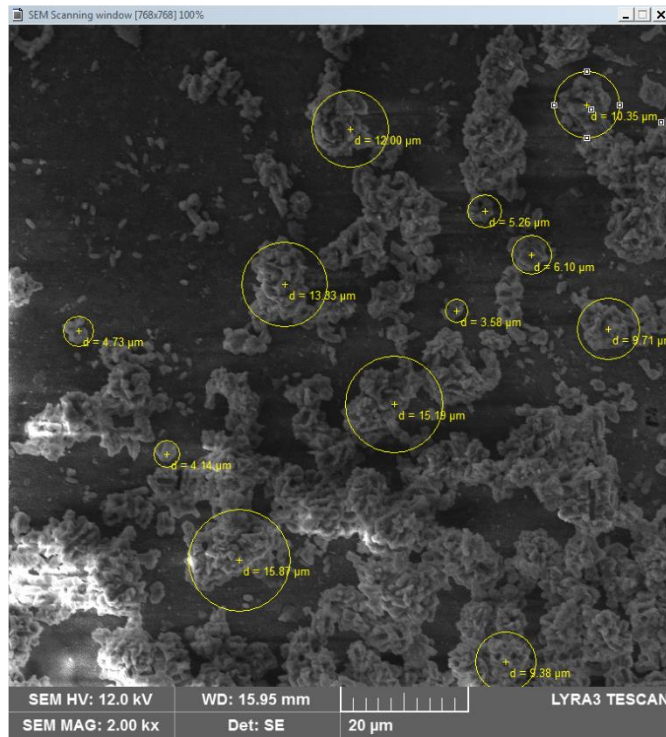

Figure 1. SEM results for culture sample centrifuged at 100rpm for 1 minute, 2kx magnification. Image collected by Md Nabil.

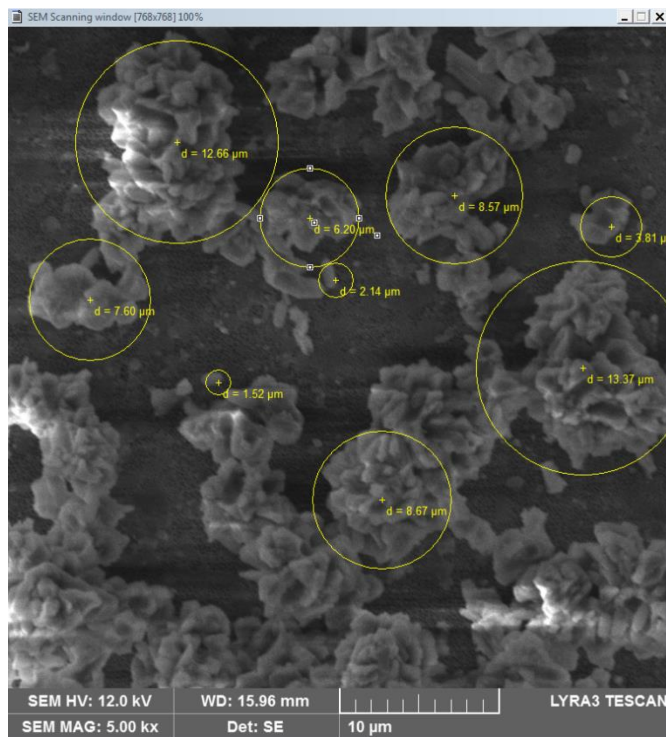

Figure 2. SEM results for culture sample centrifuged at 100rpm for 1 minute, 5kx magnification. Image collected by Md Nabil.

The goal of this project was to use DLS to determine if clumping of microbially produced PCCs was due to centrifugation or if it was occurring in cultures. By understanding this, it would be possible to prevent clumping, for example, by using surfactants. If particle size can be limited to non-clumping particles, these PCCs could be collected by differential centrifugation, with the heavier PCCs getting separated first as the heavy particle. The bacteria will settle slower because they are lighter, allowing us to just get the calcium carbonate (Livshits, Khomyakova, Evtushenko, *et al* 2015). The end goal after these steps is to then be able to use the calcium carbonate as filler in paint as a more sustainable approach to producing PCCs.

Methods

To produce the calcium carbonate for testing, liquid cultures were made using yeast extract media (deionized (DI) water and 2 g/L of yeast) containing calcium succinate to induce calcium stress. Flasks were set up in volumes of 50 mL (40 mL yeast extract media, 10 mL calcium succinate) with a stir bar at 200 RPM to allow for constant mixing during culture growth. 50 µL of *E. coli* was grown in tryptic soy broth overnight to an optical density of OD_{600}1.0 before using it to inoculate the culture.

The machine used for DLS was the BI-200SM Research Goniometer from Brookhaven Instruments (Holtsville, NY). To prepare samples for DLS, it was essential to ensure that the fluid used to suspend the sample contained no dust or other particles as this would skew the size results obtained. To do this, DI water and 100% ethanol were filtered through a 0.2 µm filter before being used for samples. Water was used as an internal control because it dissolves PCCs, allowing us to see size data that corresponds to the dividing stages of an *E. coli* cell. Ethanol does not dissolve the PCCs, allowing us to look at them with DLS. The run time for each sample

was 5 minutes, with the machine being set to the proper suspension fluid being used which ensured the correct viscosity number was used when calculating size. The laser power was also set to ensure a count rate of ~500 particles for each sample.

The first experiment was performed by running a liquid flask to 75 hours, with samples taken from the flask at 25, 30, 48, 54, and 75 hours. Two 50 µL samples were taken at each time point, and one sample was placed in 10 mL of water while the other was placed in 10 mL of ethanol. In the second experiment, a liquid flask was run to 72 hours, and different amounts of sample were placed in 10 mL of water including 0 µL, 50 µL, and 100 µL to look at the effect of different amounts of sample on DLS. Following these experiments, surfactants were incorporated with samples to see if there was an effect on particle size. To test this, a flask was set up to run for 72 hours, and 5 samples of 50 µL were then taken to run DLS on. One sample was placed in just 10 mL of ethanol, and the remaining samples were then placed in ethanol and a surfactant. To do this, ethanol and surfactants were first combined, and the sample was added to the falcon tube after. These were then vortexed and placed in the glass vials for DLS. The surfactants used were Triton X-100 and Tween 80. Surfactants were both used in concentrations of 0.1% and 0.01%, as 1% was shown to be too lethal to bacteria. The final experiment was performed by letting a flask run to 94 hours, and two 50 µL samples were taken at this point. One sample was placed in 10 mL of water and the other sample was placed in 10 mL of ethanol to see if PCCs can still be seen using DLS after an extended period of time.

Results

DLS measures the Brownian motion of particles in a dispersion to determine the hydrodynamic size. The hydrodynamic size is the size of a sphere that diffuses at the same rate as the particle that is being measured; smaller particles move faster, and larger particles move more slowly. To measure this, the particles suspended in the medium are illuminated by a laser and the laser is reflected back to a detector. This detector will create an intensity signal which is compared back to the original laser signal. The Stokes-Einstein equation ($d_H = \frac{kT}{3\pi\eta D}$) can then be used to calculate the hydrodynamic size (Stetefeld, McKenna, and Patel. 2016). The size data can be analyzed by looking at the 1st quartile, median, and 3rd quartile sizes calculated to give you a range of particle sizes. However, the data for size produced is only reliable if an acceptable correlation curve is produced. The correlation graph shows the similarity between the original signal and the newly measured signal. The DLS machine takes an intensity signal at the beginning of the run time and then takes more intensity signals as the sample is run. The two samples are then compared. An accurate correlation graph should produce a curve at the top that sharply declines to zero over time (in µs) as the intensity signals become less similar as the particles, in this case the PCCs, diffuse in solution (Berne and Pecora 1976). Correlation graphs produced from the experiments ran all showed a lower correlation value. This can be attributed to the polydispersity of the samples run as the carbonates are seen along a large size range.

In the first experiment, I ran samples from the culture up to 75 hours in water and ethanol. Figures 4 and 5 show results from the flask run to 75 hours with samples run in water

and ethanol from 24, 30, 48, 54, and 75 hours. Looking at the correlation graphs obtained from the water samples, no samples produced a very robust intensity curve. However, looking at the correlation graphs from the ethanol samples, a proper correlation graph can be seen beginning at 75 hours.

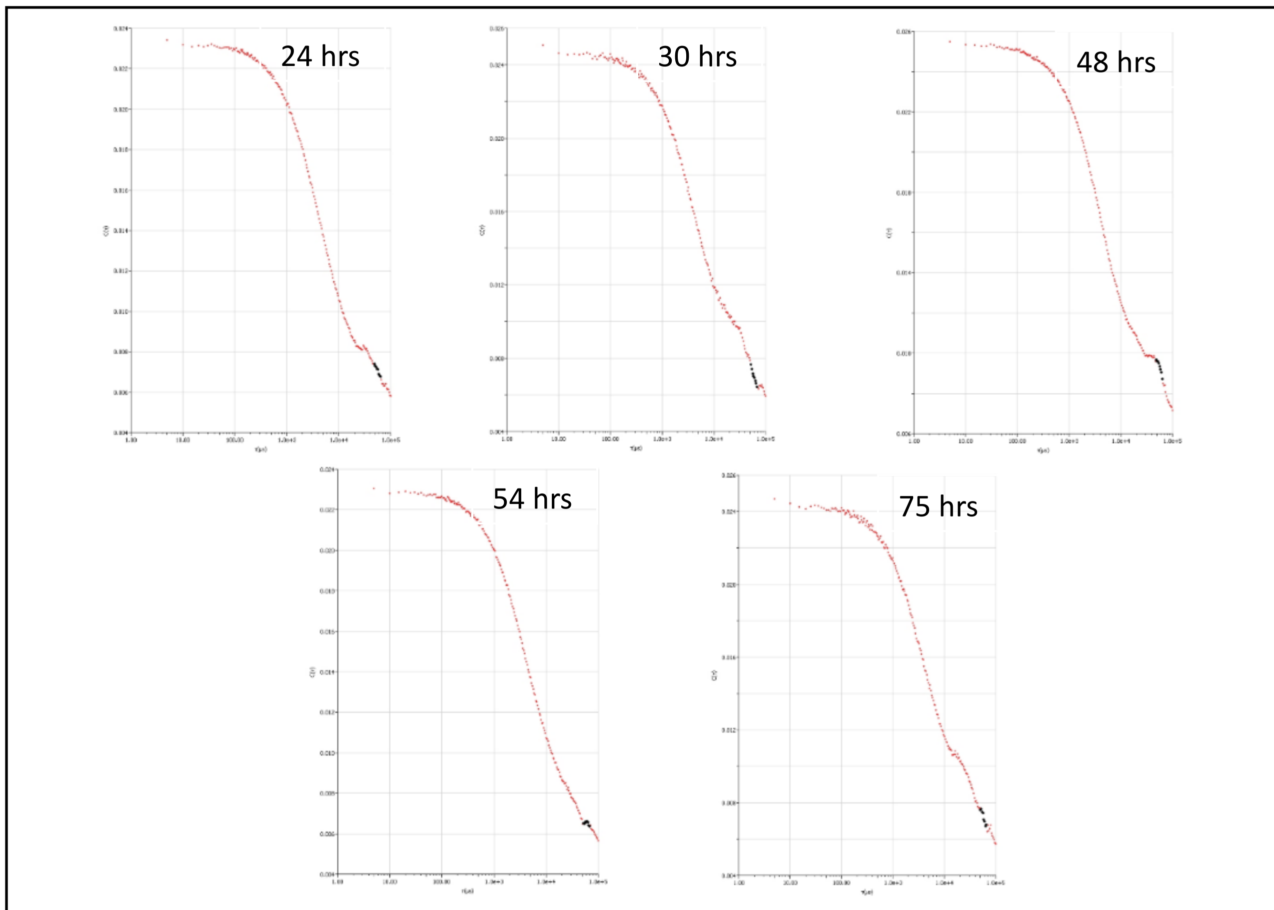

Figure 3. DLS correlation graphs for water

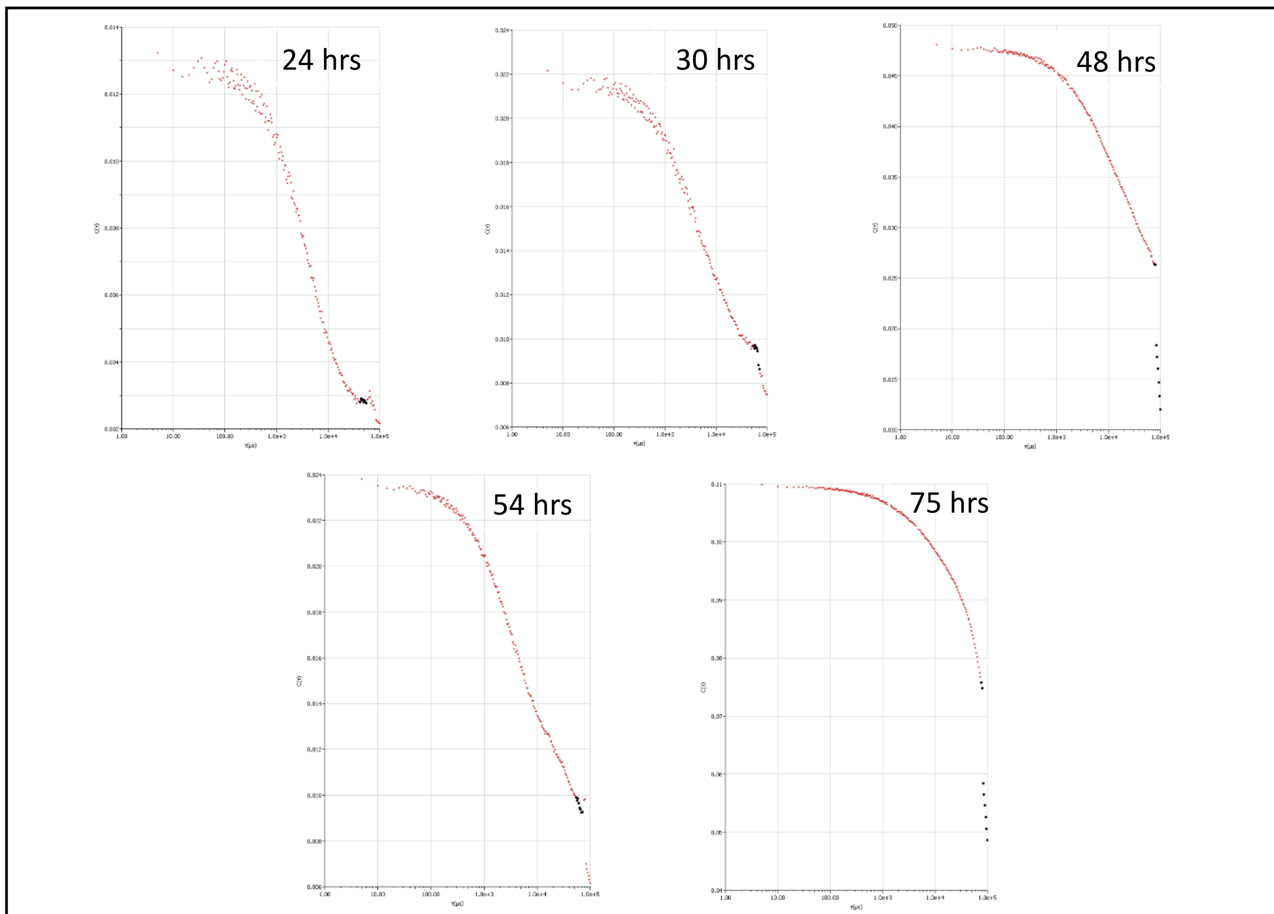

Figure 4. DLS correlation graphs for ethanol

Using the intensity curves, I obtained the particle size data calculated from these samples (Table 1). Halfway up the curve shows the 1st quartile size, the top of the curve shows the median size, and halfway down the curve shows the 3rd quartile size. This size data shows that particles are clumping in cultures as the median size for the 75-hour ethanol sample is ~11,000 nm, compared to the size of an *E. coli* cell which is around 1600 nm. A large range for particle size can also be observed with this sample, all the way up to ~40,000 nm, suggesting that the PCCs are clumping in culture.

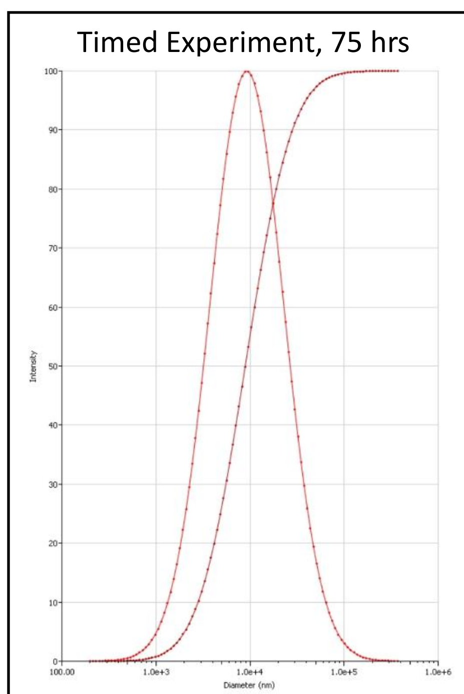

Figure 5. Intensity size curve for samples ran in ethanol from timed experiment (75 hrs)

Table 1. DLS size results for timed experiment

		1st Quartile (nm)	Median (nm)	3rd Quartile (nm)
Timed Experiment				
24 hrs	Water	756	1632	3478
24 hrs	Ethanol	513	1191	2601
30 hrs	Water	764	1676	3674
30 hrs	Ethanol	653	1444	3395
48 hrs	Water	746	1780	4247
48 hrs	Ethanol	1826	5672	16745
54 hrs	Water	756	1854	4266
54 hrs	Ethanol	572	1422	3536
75 hrs	Water	693	1700	4172
75 hrs	Ethanol	2628	10677	39245

Figure 6 shows DLS correlation graphs produced from running 72-hour samples of 0, 50, and 100 µL in water. Looking at these correlation graphs, 0 µL gives no correlation, and 100 µL shows to be too much sample as the graph is scattered at the top. Having no correlation for 0 µL is expected as there should be no particles to measure a signal from. Having a scattered correlation for 100 µL could be due to the concentration of the sample being too high, resulting in particles re-scattering the light from other particles, giving you inaccurate results. This suggests that using 50 µL for samples provides the most accurate results.

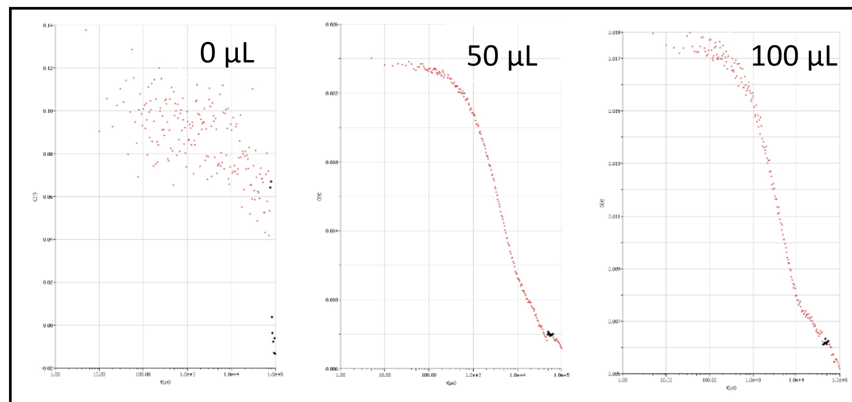

Figure 6. DLS correlation graphs for 0, 50, and 100 µL sample ran in water

Clumping was thought to be occurring due to the large particle sizes that were produced from the sample ran in ethanol at 75 hours. Surfactants are a potential solution to the problem of clumping. Surfactants are compounds that decrease the surface tension between a gas and a liquid, a solid and a liquid, or a liquid and a liquid. There are different kinds of surfactants depending on their properties which include anionic, nonionic, cationic, and amphoteric (Thiele 2005). They are commonly used in many items like cleaning products, shampoos, perfumes, paints, etc. because of the many properties they have (Karsa 2007). Looking at paint,

surfactants are used in paints as dispersants, wetting agents, and emulsifiers (Holmberg 1992). They are also used in paint to assist in the even distribution of calcium carbonate. The two surfactants used in these experiments were Triton X-100 and Tween 80.

Figures 7 show DLS correlation graphs produced from running 72-hour culture samples in ethanol and different concentrations of surfactants. None of the results with surfactants show a proper correlation graph. This suggests that the time window for enough precipitation to occur to run DLS is somewhere between 72 and 75 hours, suggesting that this experiment be performed again with samples taken at a later time to ensure enough PCCs have precipitated.

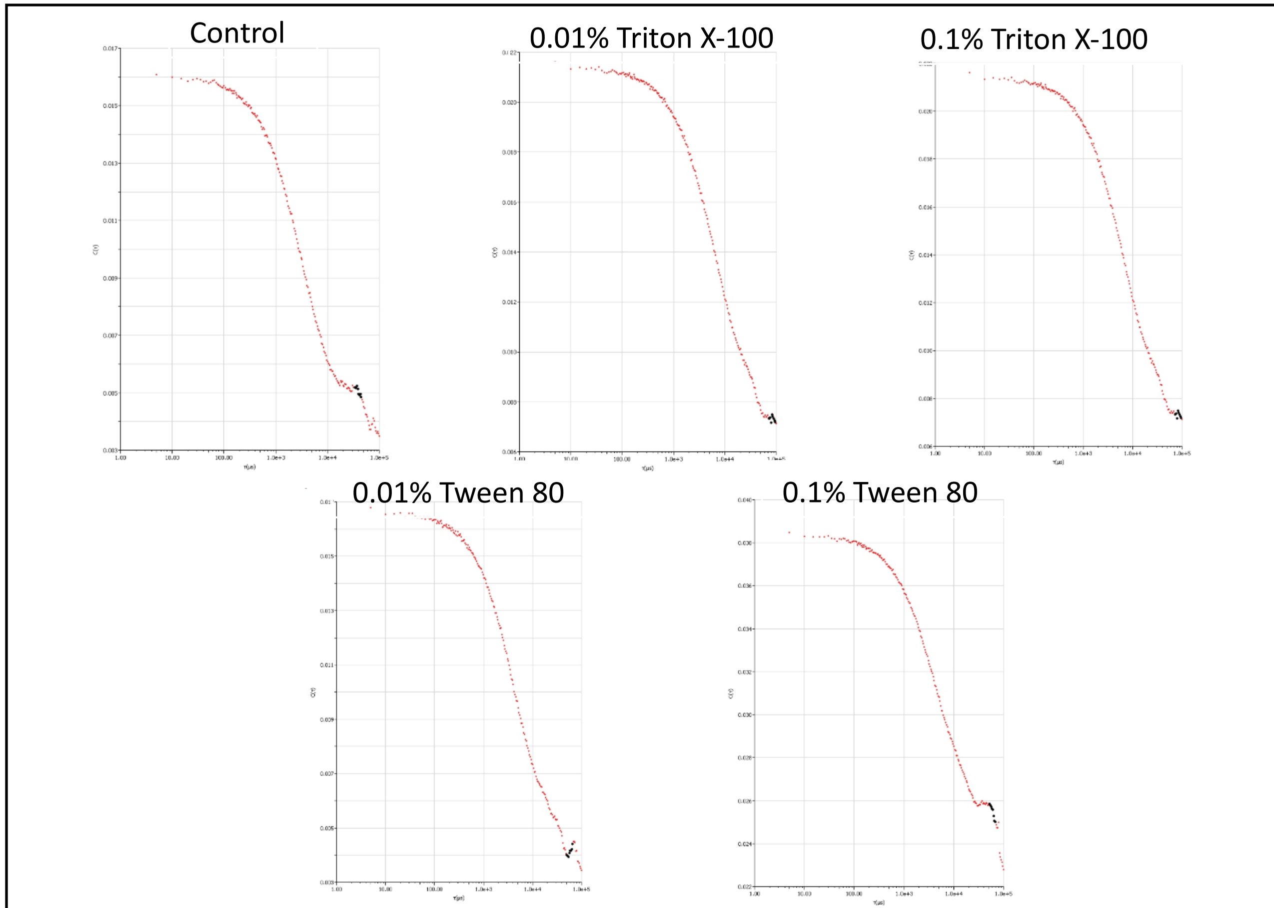

Figure 7. DLS correlation graphs for samples ran in ethanol and surfactants

Using the intensity curves, I obtained the particle size data calculated from these samples (Table 2). The particle sizes produced are smaller than the sizes seen from the sample run in ethanol at 75 hours; however, the size data obtained is not reliable because the correlation graphs produced were not reliable.

Table 2. DLS size results for surfactant experiment

		1st Quartile (nm)	Median (nm)	3rd Quartile (nm)
Surfactants				
72 hrs	Ethanol	456	946	1961
72 hrs	Ethanol + 0.1% Triton X-100	1051	2450	6091
72 hrs	Ethanol + 0.01% Triton X-100	580	1403	3392
72 hrs	Ethanol + 0.1% Tween 80	821	2443	5081
72 hrs	Ethanol + 0.01% Tween 80	660	1447	3175

A 94-hour run was performed to see if accurate results could still be produced after extended precipitation. These results show a poor correlation graph for water but show a proper correlation graph for ethanol. This suggests that particles can still be seen with ethanol and produce reliable results after extended precipitation of the culture, allowing us to obtain reliable size data for the sample in ethanol.

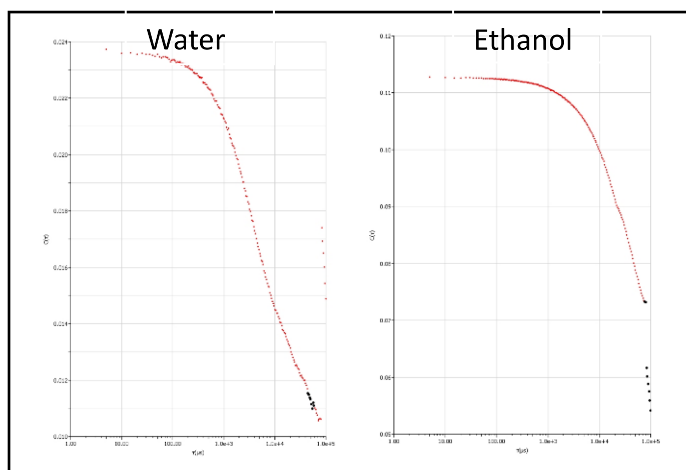

Figure 8. DLS correlation graphs for samples ran in ethanol and water at 94 hours

Table 3 shows the size data obtained from the intensity curves of these 94-hour samples. Results show that the median particle size (~10,000 nm) is similar to the size observed from the sample ran in ethanol at 75 hours (~11,000 nm). This shows that particles are still large and clumping in solution after extended precipitation. A large range of particle sizes is still observed as well, with particles up to ~28,000 nm observed.

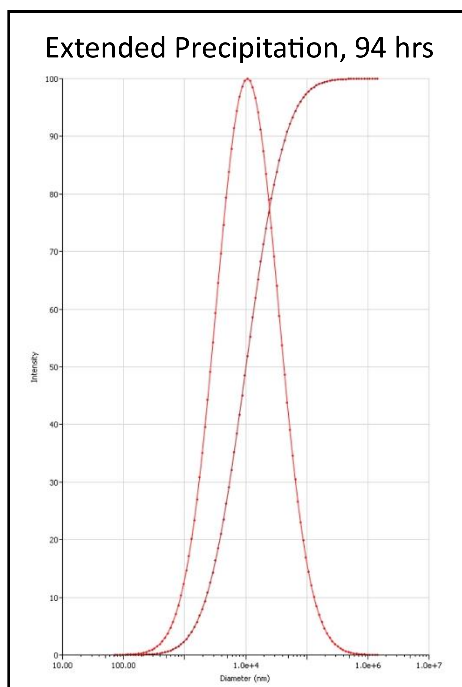

Figure 9. Intensity size curve for samples ran in ethanol from extended precipitation (94 hrs)

Table 3. DLS size results for extended precipitation

		1st Quartile (nm)	Median (nm)	3rd Quartile (nm)
Extended Precipitation				
94 hrs	Water	654	1712	3941
94 hrs	Ethanol	3060	9575	27769

Discussion

The SEM results in figures 1 and 2 show the sizes of PCCs obtained from the centrifuged sample. By looking at just the PCCs in the photos and not the sizes, the clumping of the calcite can be seen, which was thought to be due to centrifuging or drying of the sample. Looking at the sizes of the PCCs, the sizes can be seen to range from 1.52 μm all the way up to 15.87 μm, suggesting that these PCCs are too large to be used in paint.

Using DLS to study these particles in solution, the correlation graphs can show many things about the PCCs being produced. Water was used as an internal control because it dissolves PCCs, allowing us to see size data that corresponds to the dividing stages of an *E. coli* cell. The dissolution rate of calcium carbonate depends on the chemical undersaturation of the water; the more unsaturated the water, the greater amount of calcium carbonate dissolved (Palmer 1991). This can explain the observation of only bacteria being measured, as the water was undersaturated enough to dissolve the PCCs to a point where there was not enough in the sample to measure. The size of an *E*. *coli* cell ranges from 650-4000 nm, depending on what stage of division the cell is in, with a normal *E. coli* cell size being around 1600nm (Figure 10). The size data obtained before precipitation began matched this size (Table 1). When PPCs were produced, the DLS suggested median particle sizes of ~10,000-11,000 nm, up to a maximum size of 40,000 nm. This suggests that the PCCs are much larger than the size of an E. *coli* cell (Figure 10) and are in the range of sizes seen produced from SEM results (Figures 1 and 2). This suggests that the clumping of PCCs seen is not due to centrifuging or drying samples, because these large particles are also being produced in culture.

The size results from DLS suggest that these particles being produced are too large to be used in paint. Looking at the use of surfactants, there was not enough precipitation to get an acceptable intensity signal, showing that the time window for PCC production occurs after 72 hours. Adding more sample did not improve the signal and allow us to see PCCs earlier, as a 100 µL sample produced a poor correlation curve. The PCCs did produce a reliable intensity signal when suspended with ethanol, suggesting that this solvent prevents the dissolution of the PCCs particles in solution. Some outlying size data can be seen from the first experiment and the surfactant experiment. The 48-hour sample run in ethanol produced larger size results than expected, and the ethanol sample in the surfactant experiment produced smaller PCCs than expected. However, this may be related to the poor correlation graphs, and the size data cannot be considered reliable.

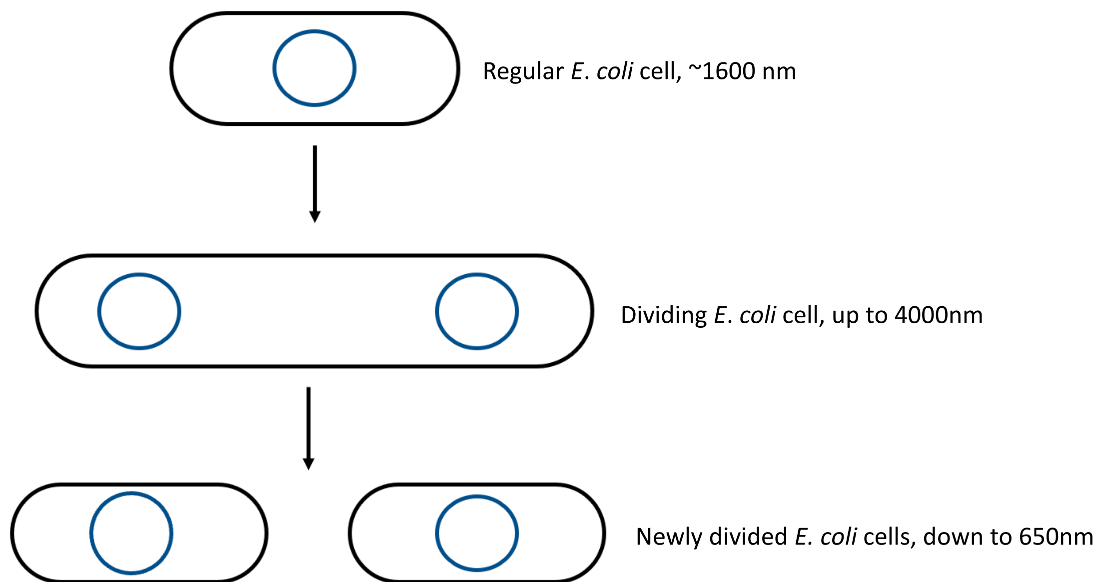

Figure 10. Phases of *E. coli* cell division with their sizes, data obtained from DLS results

To work towards the goal of using these PCCs in paint, further research needs to be conducted looking into the effects of surfactants on clumping. Samples were taken too early in the surfactant experiment performed, so the same experiment can be done at a later time point to ensure enough PCCs are in solution to give us a reliable intensity signal. If surfactants prove to be useful for reducing clumping and particle size, the next step would be to see if the PCC size can be reduced to 700nm, and differential centrifugation could then be used to separate these PCCs to be used as filler in paint.

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