THE USE OF TAILORED NANOPOARTICLES FOR THE INTERATION AND KILLING OF PSEUDOMONAS AERUGINOSA

By

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ABSTRACT

Pseudomonas aeruginosa, is an opportunistic pathogen that is currently compromising the lives of many, particularly individuals with suppressed immune systems. This is due to this pathogen's drug-resistant nature. With this in mind, the direction our research is to vary the concentrations of silver nanoparticle solutions mixed with Pseudomonas aeruginosa in hopes of disinfecting/killing the bacterium in the samples in their entirety. In doing so, zero hour and five hour tests will be performed in order to attain detection limits and log reduction ratios, which serve to show the relative number of live microbes eliminated from the surface by disinfecting.

1. Introduction:

Pseudomonas aeruginosa is a commonly found pathogen that can affect the health of both animals and humans. It is Gram-negative, aerobic, and has unipolar motility. It can be identified by its 'pearlescent' appearance, along with its unusual odor when in vitro, smelling somewhat like a tortilla. It flourishes in water, agricultural, and nearly all man made environments. Not only can this bacterium subsist in normal atmospheres, it can also survive in oxygen-deprived atmospheres, consequently making it easy for the bacterium to inhabit both natural and synthetic environments. [3]

P. aeruginosa is the quintessence of an opportunistic pathogen. It has great adaptability due to its ability to feed on many types of organic materials (can use more than seventy five organic compounds for growth). This gives it the advantage of being able to infect damaged tissues of humans with diminished immune systems. Although it seldom infects uncompromised tissues, it can potentially infect any type of tissue as long its defense system is compromised in some form or fashion.

Typical infections include urinary tract, gastrointestinal, soft tissue, kidney, and respiratory infections. Symptoms can be generalized by sepsis and severe inflammation. P. aeruginosa is a serious threat for those patients who are hospitalized with serious ailments such as cancer, cystic fibrosis, and severe burns. If colonization does occur, results can be deadly, with a fatality rate of almost fifty percent. [1]

Pseudomonas aeruginosa has little requirements for survival, particularly in the area of nutrition. Often times, it can be found growing in distilled water, which demonstrates how little nutrient-wise the bacterium need. They are also very tolerant when it comes to physical conditions, such as chemical environment and temperature. P. aeruginosa's optimal temperature is 37 ^{o}C; that of the human body. However, it can continue growth up to temperatures as high as 42 ^{o}C. In regards to habitat, P. aeruginosa is resistant to many frequently used antibiotics, dilute antiseptics, and high concentrations of salt. [3]

P. aeruginosa has an inclination for growth in wet, moist environments. As a result, this pathogen is frequently found on medical equipment (i.e. catheters), which tends to cause cross infections in hospitals, making it a nosocomial pathogen. As mentioned earlier, P. aeruginosa is an opportunistic pathogen and can be the culprit of

unremitting infections. These infections pose a severe problem for medical care in developed countries, primarily affecting immunosuppressed and elderly patients. These infections cannot be effectively treated due to their drug-resistant nature. This is thought to be a result of biofilms. A biofilm is a “structured community of microorganisms that are encapsulated within a self-developed polymeric matrix and are adherent to a living or inert surface”. These biofilms appear to protect the bacterium from harmful environmental factors. [1]

The purpose of this research is to address and find a solution to Pseudomonas aeruginosa’s drug-resistant nature, by inoculating the pathogen with different concentrations of silver nanoparticle solutions.

2. Experimental:

2.1 Aseptic Technique

Of great importance when performing the experiment is aseptic technique. This technique must be employed in order to avoid contamination. Aseptic technique is as follows:

1. Always have a flame going. This creates a negative pressure in the area.
2. Wear gloves especially when handling bacteria. (gloves are not considered sterile)
3. Always be conscious of the things you want to keep sterile (pipeter tips, flask contents, sterile media)
4. If ever unsure of whether something is contaminated or not, it probably is. Discard whatever it is and start a new one.

In regards to bottles and containers, always open slowly and with care so as not to touch the insides of the bottle, pouring lip or inside of cap. When the container is open, flame the pouring edge of the bottle and the inside of the cap. Always hold the bottle at an angle. If the bottle is plastic, do not hold it in the flame for extended amounts of time, so as to avoid melting. Instead pass it through the flame several times relatively quickly. Once completed, the liquid can be poured from the container. When finished the contained must be re-flamed, including the container lip and cap before recapping and tightening all the way.

When using measuring tools aseptic technique is just as important. A sterile tip must be used for pipeters. Avoid touching the sides of sterile containers with non sterile parts of the pipeter (anything but the tip). When using an electric pipeter, only sterile wrapped serological tips should be used. Unwrap the attaching end of the pipet and attach to the electric pipeter. Remove wrapping from pipeter in one motion. The pipet tip is sterile and should not be touched to anything. One should avoid touching the sides of containers with the pipet tip, and avoid sucking up too much liquid past the end of the pipeter. This will damage the inside mechanism. When using loops, a flame must be started and used to sterilize the loop by holding it in the flame until it is glowing red hot. At this point the loop is sterile and should not be touched to anywhere besides the plates. It is ok to set the handle of the loop on a prop of some sort while one does other things as long as the tip does not contact anything. It should not be left in open air for long periods of time. Lastly, when using glass spreaders, one must have a jar of 90% ethanol and a glass lid for smothering fires on hand. First start a flame, place the spreaders in alcohol and spread

alcohol around the inside of the glass lid. Dip your spreaders in alcohol again, and light with a flame; taking special care to hold spreaders at a downward angle so burning ethanol doesn't fall on hands. Use the lit spreaders to light the ethanol in the glass lid before setting spreaders inside circumference of the lid. Once the flame has died down, the spreaders are considered sterile. If left for extended period of time, re sterilize spreaders and lid. After spreading, return spreader to the alcohol. Once all spreaders have been used and have been placed back into the alcohol, remove them and flame them before setting them down in glass dish to allow to cool. Always allow time for spreaders to cool before use, so as to not kill the bacteria.

2.2 Preparation

TSA Plates

Begin with a 2 liter Erlenmeyer flask, in which 1 liter of TSA will be prepared. Mix the appropriate amount of powder (measure weight) with DI water in the flask (measure with graduated cylinder). Swirl the solution slowly increasing your speed until all clumps have dissolved completely. Once dissolved, cap the flask with a double layer of aluminum foil and label it with autoclave tape (Type of media, date, Initials). The flasks should be autoclaved on slow for 20 minutes. Before doing so, one should check the DI water level inside the autoclave, making sure it reaches the inner rim. Before removal of the flasks from the autoclave upon completion of the cycle, be sure the internal pressure is in the safe range, as shown on the pressure gauge. Use thermal gloves for removal and take caution of escaping steam.

Upon removal, lightly swirl the flasks until they are homogenous. Place the flasks in a preheated water bath (50^{o}C/min) and allow to cool for 20 to 30 minutes. Before removing the aluminum foil from the flask, a flame should be started, and one should use aseptic technique. Pour the agar into sterile Petri dishes until the bottom is 2/3 of the way covered. Re-cover the plate and swirl plate lightly to evenly cover the bottom. Re-flame the pouring edge of the flask briefly throughout the process. Once pouring is complete, allow the plates to set up for 30 minutes. Check the plates for solidification by lightly tapping the sides of each plate. If the center quivers, the plates have not yet solidified. Upon solidification, invert the plates so they are cover side down. This stops moisture from dropping onto the agar. To ensure complete solidification, place in a refrigerator and leave overnight.

Tryptic Soy Broth

Using 250 mL screw top flasks, mix 1 liter or desired amount of TSB according to directions on package. Use DI water to dissolve the powder completely. Measure out 100 mL of mixed TSB into 250 ml flasks. Cap each flask, but do not tighten the lid all the way, as this will break the glassware. The top should still turn freely. The flasks should then be autoclaved for 20 min on slow. After autoclaving, remove flasks with thermal gloves and set aside to cool. After cooling, refrigerate the TSB and use as needed.

Phosphate buffered saline (PBS)

Find a narrow mouthed, one liter bottle with cap. Obtain a 1 liter graduated cylinder and fill with 800 ml of DI water. The following should be weighed out and dissolve into the 800 ml of water: 8g NaCl, 0.2g KCL, 1.44g Na_2HPO_4, 0.24 KH_2PO_4 Once mixed pour into 1 liter bottle and adjust the pH to 7.4. The end volume should be adjusted to 1 liter. Invert and shake several times to mix until homogeneous. Sterilize by autoclaving on slow for 20 minutes (loosen cap and tape with autoclave tape, label), then remove and allow to cool before refrigerating.

DE Stop Solution

Following the instructions on the label, combine DI water and DE broth powder in a 1 liter bottle. Dissolve the DE completely, loosely cap the top and tape with autoclave tape to secure. Autoclave on slow for 20 minutes, then remove from autoclave and allow to cool before refrigerating and using.

Adjusting pH (Calibration and Testing)

The pH meter must be calibrated before use. Remove the probe from the initial solution and hold over an empty cup. Rinse the probe and dry lightly with chem wipes. Obtain 7pH (yellow) and 10pH (blue) standards for calibration of the machine. Place the probe in the 7 pH standard and press “calibrate”. It should read a value very close to 7.00. Remove fro m the standard, rinse the probe again and dry. Place the probe in the 10 pH standard and press the calibrate button. The read value should read around 10.01. Rinse the probe and move on to testing the sample. Place the probe in the solution that you wish to measure. The solution should be on the stir plate with a stir bar inserted and stirring continuously during the procedure. Use a dropper (Pasteur pipets) to add NaOH or H_2SO_4, one drop at a time. Allow a few drops to mix before adding more, so as to not overshoot the desired pH. Once desired pH is reached, remove the probe, rinse and dry before placing in buffer solution.

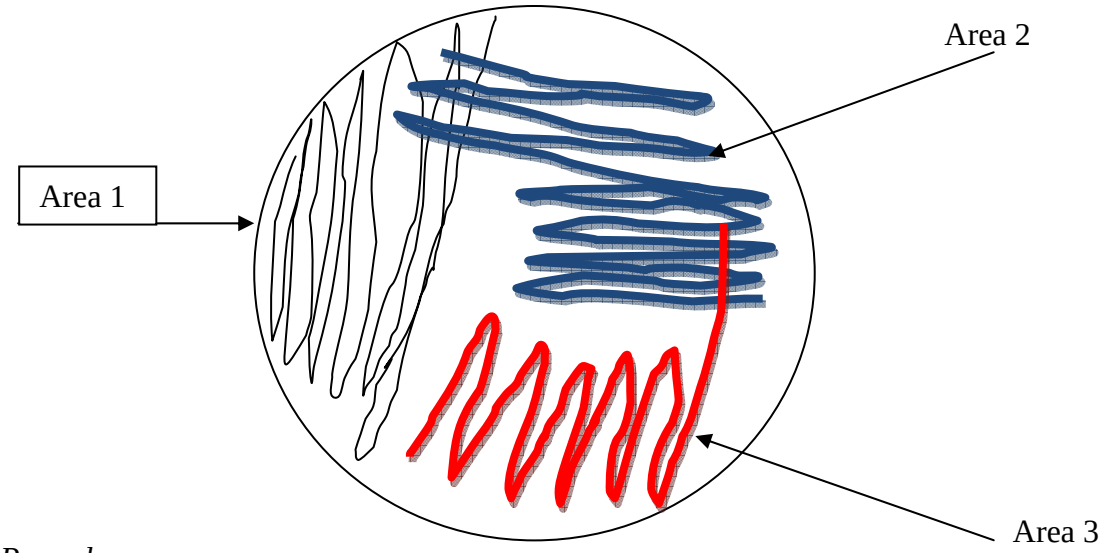
Dilution/ DE stop/ Turbiditity tubes

Fill a plastic beaker with as many Eppendorf tubes as will fit and cover with tin foil and mark with autoclave tape. Autoclave on fast because there is no liquid is in the tubes. Using aseptic technique, remove two corners of the tin foil and place sideways on work area. Some tubes will fall onto the foil. Close the tubes being careful not to touch the rims or edges of the fasteners, so as to avoid contamination. For dilution tubes, start a flame, fill a sterile 50 mL conical tube with PBS and use a pipeter to fill each tube with 900 µL of PBS. For DE stop tubes, the same process is involved, however DE solution is used instead. For Turbitidity tubes fill with 10 mL of PBS via an electric serological pipeter.

Quality Control for Bacteria

Obtain two TSA plates free of contamination and label them with bacterial species, strain number, and date. Using aseptic technique, sterilize a loop and allow to cool. Using Original QC plates, take one individual colony from the plate on one side of the loop. On a new plate, spread the same side of the loop across one area of plate as shown. For Area 1 it is ok to retrace over previous lines. When done re cover plate.

Resterilize loop and allow to cool. For the second section (Area 2), start a few cm into area one and drag a line outward, not using too much pressure. Trace backwards into area 1 twice more and then drag in parallel lines without touching area 1 as shown. The lines should not cross of this trace should not touch one another and should have no breaks (areas where you pick up the loop). Resterilize the loop and allow to cool. For the final area (Area 3), start a few cm into area 2 and draw outward but do not trace back into area 2. Drag parallel lines outward without touching area 1 or 2 and without crossing lines after initial streak. Do not pick up loop. Repeat this process for the duplicate plate and other bacterial species. Incubate the plates over night or until the spots reach desired size.



2.3 Procedure

Inoculating TSB

To begin start a flame, and sterilize the full length of the wire part of a metal loop, but not the metal handle. Allow to cool for 30 seconds. Using aseptic technique, collect a single large colony or several small colonies from QC plates with the sterile loop. Open a TSB flask next to flame and hold onto cap. Flame the mouth of the TSB flask. Carefully submerge the tip of the loop in TSB liquid being careful not to touch the sides of the container with handle. Stir the loop in the TSB until the clump of bacteria falls off in the solution. Remove the loop carefully, flame the mouth of the flask and cap the TSB flask loosely, securing it with autoclave tape. Sterilize the length of the loop, to avoid spreading the bacteria. Place the flask securely in a shaker in the incubation room. Turn on the shaker and leave overnight to grow.

Spinning down TSB

Remove from the shaker, after it has come to a full stop. Obtain two 250 mL sterilized plastic bottles that have already been autoclaved. Be sure that the bottles fit in the centrifuge receptacles. Using aseptic technique with a flame going, pour the contents of the flask into one of the sterile bottles. Cap the bottle after flaming the mouth. At this point the flask no longer needs to be treated aseptically. Use a balance to match the weight of the first bottle containing the broth with the second sterile bottle. This bottle does need to be treated aseptically. Fill the second bottle with DI water until just under the first bottles weight. Match the weights by adding drops of water with the squeeze bottle. Make sure to include the bottle cap on the scale when weighing. Place both sealed bottles in the rotor of the floor centrifuge. The bottles should be opposite one another. Place the rotor cap on top of the rotor and screw it down tightly. Test that the rotor is secure by attempting to lift it up. Close the centrifuge lid and use the following settings: 8000 RPM, brake: 4, temp: 22-33, and time: 15 min. Press start and place hands on the lid to feel for vibrations. If imbalance warning light illuminates, press stop of if the lid vibrates excessively, press stop. Continue holding with hands until the centrifuge reaches speed. After centrifuge has finished and lid has unlocked, remove the cap and retrieve bottles gently as to not disturb the bacteria pellet that formed during centrifuging. Using aseptic technique, slowly pour off the TSB into an empty flask, so as to not disturb the pellet. Fill the bottle containing the pellet with approximately 100 mL of PBS. This measurement does not need to be precise. Reflame bottle mouth and secure lid before shaking vigorously until pellet is resuspended. Rebalance the bottle and repeat the above steps for balancing the 2 bottles for use in the centrifuge. After the centrifuge stops, remove the bottles carefully and aseptically pour off the PBS from the bottle containing the pellet into a flask. Do not disturb the pellet. Aseptically measure out 10 mL of PBS with the serological electric pipeter and a sterile tip. Add this PBS to the bottle containing the pellet. Seal and shake vigorously to suspend the pellet.

Using Turbidity Meter

Turn on the turbidity meter and obtain a McFarland standard # 0.5. Clean a turbidity tube with alcohol and and chem. wipe. Ensure the PBS is free of debris. Place the tube in the turbidity meter and set it to a value of 0. Check Mcfarland standard after inverting it several times. It should be around 47%±1%. Using a pipeter and sterile tips, measure out 90µL from the bacterial concentrate and add to the turbidity tube. Cap the tube tightly and invert several times before measuring its turbidity. Re-zero and measure the McFarland standard again for comparison. Add smaller increments (10µL - 5 µL) of concentrate as you get closer to the correct turbidity. Once 47% or 48% turbidity has been reached, the bacterial concentration of the standard ($1.5x10^8$ cfu/milliliter) has been matched.

Preparing and inoculating testing tubes

Using aseptic technique, fill 50 mL conical tubes with 10 mL of PBS, each using a new serological pipeter. Mark tubes with appropriate labels. Using aseptic technique and a pipeter, inoculate each tube with 100µL of solution from the standardized turbidity tube. Vortex each tube after capping tightly. This yields a concentration of $1.5x10^6$ cfu/milliliter.

Taking "Zero Hour"

A true zero hour cannot be obtained because once silver is added, timing begins. In this experiment a "zero hour" is taken before adding silver. Next, vortex the inoculated tube and the aseptically pipet 100µL from each. Add this to a 900µl DE stop dilution tube and vortex the tube. Repeat the process for all samples.

Adding Ag Nanoparticles.

Shake vigorously to ensure that all nanoparticle samples have been re-suspended. At this point the experiment becomes time sensitive so the procedure must be quick and efficient. Add 20µL (or proper amount for 10 ppm silver) of nanoparticle solution aseptically to test tube. Seal the tube and then vortex it. Repeat for all test samples, making sure to change tips in between. Slightly loosen the caps on all samples and place in the tube rack. Tape down the caps to tube rack and place the rack on the shaker and secure with clamps. Turn on shaker and set it to 300 RPM. Be sure to note the start time of the experiment and return before your next time point.

Taking time points.

Stop the shaker and aseptically measure out 1 mL from each test tube. Place the sample in a 1 mL DE stop tubes, cap, and then vortex. Repeat this process for all samples before re-securing tube rack and caps and restarting the shaker.

Serial Dilutions

First vortex the 10^{-1} tube. Aseptically draw 100 µL from it and then place in the next 900 µL dilution tube. Be sure to cap and vortex. Change the tips and repeat out to the desired number of dilutions.

Plating dilutions

Pick the desired dilutions to plate and label plates appropriately. Follow aseptic technique for preparing sterile spreaders. The dilutions should be vortexed before aseptically drawing 100 µL solution from them and inoculating onto each plate. The tips should be changed when changing dilutions. Make sure the spreaders are cool by pressing them against the agar away from the solution on the plate. Spread solution over the plate slowly at first, turning the plate as you spread. Do this until the plate is no longer wet or the liquid has been sufficiently spread over the entire surface. Invert the plate before continuing. When done spreading, be sure to deposit the used spreader in alcohol and flame before reuse. Repeat this process until the desired plates are spread. Lastly,

incubate the plates in incubation room in stacks of plates on trays. Always keep plates inverted to prevent water contamination.

Counting Plates

Use a counter to count the number of spots. Look for and note any contamination. Contamination spots should not be counted. One should be able to differentiate between the P. aeruginosa and contamination, due to P. aeruginosa's pearlescent appearance. Use QC plates as a reference if necessary.

3. Results and Calculations:

3.1 Data

In regards to the results, the sample number and date are given when the samples were made. The next column shows the compounds contained in nanoparticle form (Ag, AgBr, AgCl etc.). A minus sign such as used in AgBr – 2.5% Cu denotes AgBr nanoparticles doped with 2.5% Cu. A plus symbol such as AgBr + 2.5% CuI indicates a mixture of AgBr nanoparticles and CuI nanoparticles. The next column is % silver by weight solution. The next two columns denote Amino acids and thiols used respectively. A dash in either column means no AA or thiol. PVP in the AA column refers to polyvinylpyrrolidone. The following column is a ratio of silver to AA to thiol. The pH is only given for some samples. The next two columns are log reductions of bacteria. A dash means an insignificant reduction. ND or X means no test was done. Any value greater than a negative sign means that the test exceeded the detection limit of our method (we have two plates at each dilution, so if we are on the 10-1 plates and detect one colony forming unit on one plate, that is our detection limit, 1 cfu per 200 microliters). Lastly, different organisms are indicated in the headings.

3.2 Log Reductions

Essentially we start with our counts from zero hour plates. For example if our counts were 250 and 260 for 10-3 dilution, 24 and 26 for 10-4 dilution, and 2 and 1 for the 10-5 dilution, one will then use the most accurate dilution, meaning the colony count in the hundreds. If the counts are below fifty they are not accurate enough. Therefore one must count the nest highest dilution. The average for the two plates for each dilution is then calculated to attain the most accurate dilution. In this situation the count would be 255, which comes from the 10-3 dilution. Next multiply by 10^3 to determine the concentration of the initial 10^1 plate.

The ultimate goal is to determine the concentration present in the test solution. Initially 100 microliters are taken from the test solution and combined into a 900 microliters DE stop solution. This yields a 1 to 10 dilution. If one multiplies by the concentration value from the 10-1 dilution plate by 10, one gets a concentration of 2.5E6 cfu/mL. This is done for all zero hour tests. Lastly, the average of the values yields the titer, which should be a value close to the mcfarland standard, which is 1.5E6. This completes the zero hour calculations.

Sample values for the five hour samples are 0 and 0 on the 10-1 dilution plates. These values are past the detection limit, so one determines the value for a single spot.

The average is taken between 1 and 0, which gives a value of 0.5. Again one multiplies by 10^1 to get a value of 5. Next multiply this value by 2 to calculate the value in the test tube. This is because at the five hour time point 1 mL is taken from the test tube and diluted in 1 ml of DE stop solution. This yields a 1 to 2 dilution. By multiplying by 2 one gets 10 cfu per mL.
For log reduction calculations, simply divide the zero hour titer concentration by the five hour concentration. For example, if the zero hour average was 3.5e6, dividing by 10 gives a value of 3.5E5. Finally take log10 of this to get a logarithmic value. The value is now 5.54, which our log reduction. Because this value was computed assuming 1 spot was present onboth plates, this is the detection limit. Since the actual values were 0 and 0 on the plates, one cannot be 100% sure of the actual log reduction, only that it is greater than the detection limit, therefore one reports it as a log reduction of >5.54 logs.

4. Conclusion

Unfortunately at this time, we have not succeeded in 100% effectively killing the Pseudomonas aeruginosa. However, do to varying the concentrations of the silver nanoparticles added to the P. aeruginosa bacterium, we are slowly deducing the correct concentration of silver needed to successfully kill in its entirety this drug-resistant pathogen.

5. References

[1] Baltch, Aldona, and Raymond Smith. Pseudomonas Aeruginosa: Infections and Treatment. 1st. 1997. Print.

[2] Qarah, Samer. "Pseudomonas aeruginosa Infections." 17 Mar 2008. eMedicine. 3 May 2009 <http://emedicine.medscape.com/article/226748-overview>.

[3] Todder, Kenneth. "Pseudomonas aeruginosa." Online Textbook of Bacteriology. 2008. Web.3 Dec 2008.<http://www.textbookofbacteriology.net/pseudomonas.html>

Mar 10th - Aug 18th Batches

Sample	Ag/AgX	Ag% w/w	Aminoacid (AA)	Thiol compound (SH)	Ratio Ag:AA:SH (mol)	pH	E. coli log reduction	P. aeruginosa log reduction
1-10 March	Ag	0.23	-	Thiomalic acid	1:-:1		2.53	0.26
2-10 March	Ag	0.23	-	Thiomalic acid	1:-:3		2.06	0.27
3-10 March	Ag	0.23	-	Thiomalic acid (neutralized)	1:-:3		1.5	0.45
1-26 May	Ag	0.5	Aspartic acid	Thiomalic acid	1:5:0.10	~5.5	0.03	0.24
2-26 May	Ag	0.5	Aspartic acid	Thiomalic acid	1:5:0.25	~5.5	0.13	0.28
3-26 May	Ag	0.5	Aspartic acid	Thioglycine	1:5:0.10	~5.5	0	0.06
4-26 May	Ag	0.5	Aspartic acid	Thioglycine	1:5:0.25	~5.5	0.08	0.12
5-26 May	Ag	0.5	Aspartic acid	Thiooctic acid	1:5:0.10	~5.5	0	0.07
6-26 May	Ag	0.5	Aspartic acid	Thiooctic acid	1:5:0.25	~5.5	0	0.03
7-26 May	AgBr	0.5	Aspartic acid	Thiomalic acid	1:5:0.10	~4	2.06	1.75
8-26 May	AgBr	0.5	Aspartic acid	Thiomalic acid	1:5:0.25	~4	4.13	3.68
9-26 May	AgCl	0.5	Aspartic acid	Thiomalic acid	1:5:0.10	~4	0.72	0.4
10-26 May	AgCl	0.5	Aspartic acid	Thiomalic acid	1:5:0.25	~4	1.35	1.09
4-03 July	Ag	0.1	Leucine	Thiomalic acid	1:1:0.10		0.5	0.93
5-03 July	Ag	0.1	Leucine	Thioglycine	1:1:0.10		0.65	0.86
6-03 July	Ag	0.1	Leucine	Thiolactic acid	1:1:0.10		0.82	1.17
1-21 July	Ag	0.5	Aspartic acid	Thiomalic acid	1:5:0.25		0	0
2-21 July	AgBr	0.5	Aspartic acid	Thiomalic acid	1:1:0.10		3.48	1.06
3-21 July	AgBr	0.5	Aspartic acid	Thiomalic acid	1:1:0.25		2.99	1.45
4-21 July	AgBr	0.5	Aspartic acid	Thiomalic acid	1:1:0.50		4.14	3.09
5-21 July	AgCl	0.5	Aspartic acid	Thiomalic acid	1:1:0.50		1.69	0.01

3-21 July (2X concentration)	AgBr	0.5	Aspartic acid	Thiomalic acid	1:1:0.25		3.8	1.04
8-26 May (retest)	AgBr	0.5	Aspartic acid	Thiomalic acid	1:5:0.25	~4	3.02	0.67

1-29 July	AgBr	0.5	Aspartic acid	Thioglycine	1:1:0.50		4.55	3.15
2-29 July	AgBr	0.5	Aspartic acid	Thiooctic acid	1:1:0.50		1.56	2.23
3-29 July	AgBr	0.5	Aspartic acid	Thiolactic acid	1:1:0.50		4.25	3.07
4-29 July	AgCl	0.5	Aspartic acid	Thioglycine	1:1:0.50		2.8	3.59
5-29 July	AgCl	0.5	Aspartic acid	Thiooctic acid	1:1:0.50		1.51	1.32
6-29 July	AgCl	0.5	Aspartic acid	Thiolactic acid	1:1:0.50		1.29	0.94

4-06 August	Ag	0.25	Lysine	Thiomalic acid	1:0.75:0.25		0.39	0.07

1-18 August	AgBr	0.5	Polyvinylpyrolidone	Thiomalic acid	1:2.5:0.10		3.42	0.8
2-18 August	AgBr	0.5	Polyvinylpyrolidone	Thioglycine	1:2.5:0.10		3.8	1.18
3-18 August	AgBr	0.5	Polyvinylpyrolidone	Thioglycerol	1:2.5:0.10		3.21	0.8
4-18 August	AgCl	0.5	Polyvinylpyrolidone	Thioglycerol	1:2.5:0.10		1.28	0.07
5-18 August	AgBr	0.5	Polyvinylpyrolidone	-	1:2.5:-		0.92	0.28