SCREENING FOR HETERO-MULTIVALENT BINDING OF LECTIN: OPTIMIZATION OF A TURBIDITY-BASED EMULSION AGGLUTINATION ASSAY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Screening for Hetero-Multivalent Binding of Lectin: Optimization of a Turbidity-Based Emulsion Agglutination Assay

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A thorough understanding of the process bacterial pathogens use to infect host cells is an important factor in developing an antimicrobial that will provide efficient and effective defense against such infection. The first step of this infection process is initiated when adhesin, a protein on the surface of the bacteria, binds to ligands on the surface of the host cell. This binding, when comprehensively analyzed, can provide insight that improves design of special drug delivery techniques. We optimized a turbidity-based emulsion agglutination assay for high-throughput analysis of this binding mechanism between protein and ligands for applications in screening of specific bacterial characteristics. By successfully developing a protocol using simple, highly available instruments, including an UV spectrometer and tip sonicator, to prepare emulsions of model membranes and test binding within, our goal is to develop a method that allows us to screen large molecular libraries in order to identify the combinations of receptors that exhibit hetero-multivalent binding characteristics and apply this knowledge to improve drug delivery for specific bacterial infections.

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CHAPTER I

INTRODUCTION

A cell is contained from its extracellular environment by a complex and fluidic bilayer membrane. This membrane regulates a cell's interaction with its environment. Pathogens must pass this barrier during the process of infection. Lectins, which are proteins that are often components of pathogens, must bind to specific glycolipid receptors on the cellular membrane for pathogens to initiate infection [1,2]. Our understanding of these interactions that occur between glycolipid receptors and lectin has recently been altered by the discovery of hetero-multivalent binding. Rather than the more common belief that a single type of glycolipid receptor binding to a single type of lectin, this newly proposed mechanism suggests that when a cell interacts with a lectin, there can be several types of glycolipid receptors that bind simultaneously to the lectin [3,4]. This provides evidence for the significant efficacy of pathogen infection rates where, even among extremely low affinity receptors, pathogen proteins can still effectively bind to host cells initiating infection [5].

Proposition

Because hetero-multivalent binding is a newly identified mechanism, it is largely unknown what glycolipid receptors interact with various lectin in the mechanism's proposed fashion. An efficient assay must be developed in order to effectively screen potential ligands for this hetero-multivalent binding characteristic. In order to effectively screen ligands for hetero-multivalent binding, the model membrane must maintain the same fluidity of a natural cellular membrane, as this is an essential element of the proposed mechanism [3]. It is also beneficial to use an assay that is highly efficient, allowing for screening of large molecular libraries, and that requires a

very simple protocol that uses widely available materials and instruments so that the assay can be easily utilized in many different disciplines of study.

Assays

Traditional assays for binding characterization

Carbohydrate microarray technologies, which are the most commonly used assays for testing binding, all share commonalities in protocol and concept. In these assays, the receptors are fixed to a surface, allowing for a relatively high local concentration of receptors, making the assays high throughput [5]. The surface with receptors is then exposed to proteins that are marked, often with fluorescent markers, and the resulting binding can be quantified based on the amount of protein that interacts with the fixed receptors. But because the receptors are fixed to a surface, fluidity of these receptors is not possible. The mechanism of hetero-multivalent binding is completely dependent on the concept that membrane components and, therefore, receptors can move relatively freely within the membrane [3]. Because this fluidity is not possible in microarrays, these assays are not viable options for hetero-multivalent binding screening.

Nanocube assay

The nanocube assay, which was used during the discovery of the hetero-multivalent binding phenomenon, was first proposed as an assay that provided label-free quantitative analysis of protein-lipid interactions [4,6]. In this assay, silica-coated silver nanocubes are prepared such that a fluid bilayer membrane surrounds the nanocube. Glycolipid receptors can be added during the membrane preparation and then, upon addition of lectin, binding characteristics can be monitored by observing the localized surface plasmon resonance using a UV-Vis spectrophotometer [3,4]. While this is an effective method for analysis, a few drawbacks can impose limitations to the wide scale applicability of this assay. This assay calls for specialized

materials, which are not always available in labs that study these microbiological concepts, and the assay has a long, difficult protocol that can make it relatively difficult to replicate.

Turbidity-based emulsion agglutination assay

In order to address all of the components we desire in an assay, we propose using a turbidity-based emulsion agglutination assay. This assay has many parallels to a hemagglutination assay used for blood typing and virus titers, where cells or particles in a suspension are bound together by added proteins and receptors on the particles and the particles aggregate, allowing for various properties of the particles to be analyzed [7]. For this application, we use oil droplets as the particles and we add lipids and glycolipid receptors to act as the barrier between the particle and the surrounding aqueous environment. We can then add proteins and monitor binding between the receptors on the particles and the proteins by measuring the absorbance and increasing turbidity of the emulsion as the particles bind together to form larger particles. This assay maintains the fluidity of the glycolipid receptors on the oil particles, allowing for the hetero-multivalent binding mechanism to be executed. The emulsion can be prepared using simple instruments and the turbidity can be easily monitored using a common spectrometer. But because this assay depends on measures of absorbance for analysis, the particle size must stay within a certain range in order for us to effectively monitor the turbidity, as the assay is largely dependent on the linearity of the Rayleigh scattering theory [8]. Once a particle exceeds the size of the wavelength of light that it is scattering, the Rayleigh scattering theory no longer applies and the turbidity no longer increases linearly with particle size, making it nearly impossible to determine how turbidity is related to particle size [7]. In order for this assay to be a viable option for screening of hetero-multivalent binding, we must optimize certain components of the assay in order to maximize the linear relation of emulsion absorbance and particle size.

CHAPTER II

OBJECTIVE

In order for the turbidity-based emulsion agglutination assay to be a viable option for screening for hetero-multivalent binding, there are several aspects that must be optimized in order to maximize the region of linearity between absorbance and particle size.

Scattering efficiency factor, K, is directly related to turbidity [7]. K is dependent on three main components: particle size, wavelength of light being scattered, and relative refractive index of the particle to its surroundings. Worstell et al. computationally demonstrated K as a function of size parameter, α, and the relative refractive index, m, according to two different light scattering theories. Figure 1 (a) shows the Rayleigh scattering model and Figure 1 (b) shows the Mie scattering model that was obtained from the calculations.

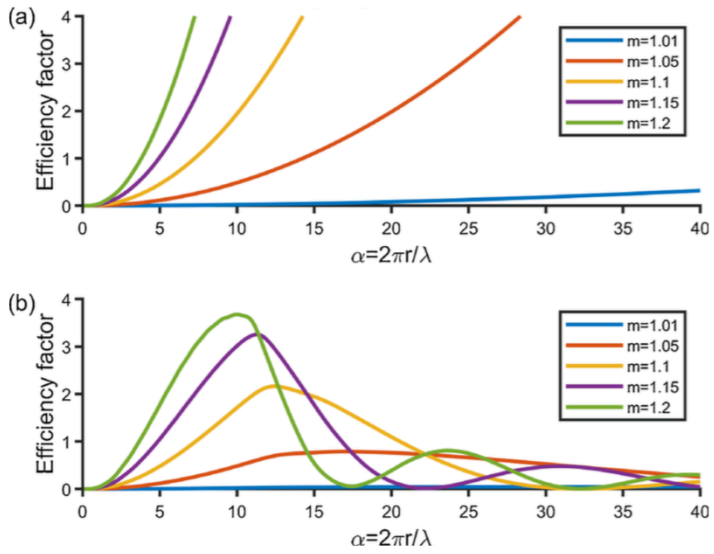

Figure 1. Relationship of scattering efficiency factor to size parameter. (a) According to the Rayleigh scattering theory. (b) According to the Mie scattering theory. [7]

According to the more widely applicable Mie scattering theory, the linear relationship between K and α only exists in the initial region where the α ranges from 0 to 10. When the emulsion is

under these conditions, we are able to easily predict the size of the particle simply by measuring K. This is the region that we must stay within while measuring absorbance and, consequently, turbidity in order to effectively analyze the emulsion and binding within.

By carefully optimizing the aspects of the assay that contribute to the particle size, wavelength of light being scattered, and relative refractive index of the particle to its surroundings, we will be able to maximize the efficiency of the assay by ensuring the particles remain in the linear Rayleigh scattering region for as long as possible.

Oil type and refractive index

By carefully selecting the type of oil that is used in the assay, we can minimize large fluctuations in the absorbance reading of the oil droplets. We first want to consider what refractive index, m, provides a larger range of linear relationship between K and α. Lower m values apparently result in this wider range of linearity (Figure 1). However, if m is too close to 1, then an absorbance reading will be too low to detect. Thus, we want to use oil and an aqueous buffer solution that give a refractive index of approximately 1.05.

Wavelength analysis

As observed in Figure 1, the relationship of the size parameter to the size of the particle and the wavelength of light that the particle is scattering is:

$$\alpha = \frac{2\pi r}{\lambda} \qquad (1)$$

Note that r is the particle radius and λ is the wavelength of the light being used to measure absorbance. In order to remain in the linear region for as long as possible, we want α to be small so we should use a relatively large wavelength of light when measuring absorbance in order to keep the size parameter small. However, we cannot use the largest wavelength available because of the absorbance characteristics of the oil droplet particles. At high wavelengths, absorbance of

an oil emulsion drops off sharply and approaches zero. Despite this, we do not want to use a very small wavelength, because this will make α large even when the particles are relatively small. Small wavelengths also have a higher energy that could possibly denature the proteins that we are using. Consequently, we must carefully select a wavelength that balances all of these considerations.

Particle size and emulsion composition

We must also consider particle size in order to further optimize the size parameter, α, which is directly related to particle radius according to Equation 1. By minimizing the diameter of particles, we can maximize the region of linearity between K and α. Many contributing factors affect the size of the particles, thus, much of the optimization for this assay can be accounted for in this regard. Particle size can be partially controlled by the relative lipid composition. More lipids allow for greater surface area of the particles and, therefore, a greater number of smaller particles. Beyond this, the particle size is largely dependent on protocol, which can be carefully adjusted to minimize particle diameter.

Lipid composition and cost

The concentration of lipids and proteins needed in order to effectively execute this assay is relatively high compared to the silver nanocube assay. Because the components are expensive, the composition should be carefully considered in order to maximize effectiveness while minimizing cost resulting from expensive materials such as lipids and proteins. Exploring substitutions for lipids, particularly surfactants, may allow us to decrease the concentration of lipids needed for this assay and may help lower the cost of experiment, ultimately increasing the viability of this assay.

CHAPTER III

MATERIALS AND METHODS

Materials

10X tris-buffered saline (TBS), silicone oil, olive oil, Streptavidin (STP) in a powder form, sodium dodecyl sulfate (SDS), and TWEEN® 20 were purchased from Sigma Aldrich in St. Louis, MO. Calcium chloride ($CaCl_2$) was purchased from BDH VWR Analytical in Radnor, PA. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased in a powder form from Avanti Polar Lipids in Alabaster, AL and mixed to prepare a chloroform solution per manufacturer's recommendations. Biotin was purchased from Matreya, LLC. in State College, PA.

Emulsion preparation

Emulsions were prepared in 1.5 mL Eppendorf tubes. 475 μL 1X TBS with 100 μM $CaCl_2$, 5 μL of silicone oil, and 220 μL lipid solution, which contained a chosen concentration of lipids in 1X TBS, were placed accordingly into the Eppendorf tube such that the final emulsion contained 700 μL. If the lipids were stored in an organic solution, such as chloroform, the desired amount of the lipids was placed in a 25 mL round bottom flask and the organic solution was removed using a rotary evaporator (Heidolph Hei-VAP Value®). The lipids were then rehydrated with 1X TBS with 100 μM $CaCl_2$ and 220 μL of this lipid solution was added to the emulsion solution. The Eppendorf tube containing the 700 μL of emulsion contents was then suspended in an ice bath inside the sonicator sound enclosure and the tip of the sonicator (Qsonica Q125 tip sonicator) was lowered into the Eppendorf tube so that the tip was approximately in the center of the solution. The sonicator was then set to either one of two settings: 60 minutes of 10 second

pulses at 60% power or 40 minutes of 10 second pulses at 80% power. Upon completion, the Eppendorf tube was removed from the ice bath and placed at 4 °C.

Emulsion stability check

After emulsion preparation, we must ensure that the emulsions remain stable. If there is any instability, flocculation and aggregation may occur, affecting the consistency of particle density in the emulsion and have an unintended effect on the absorbance reading and thus lead to false reports of particle size. In order to monitor the stability, we used a UV-Vis spectrometer (FLUOstar Omega®, BMG-Labtech) to collect an absorbance reading of each emulsion by placing 20 μL of the emulsion in a well on a 96-well microplate (Costar® 3370) and diluting it with 80 μL of 1X TBS with 100 μM $CaCl_2$. Three replicates and three controls containing only 100 μL 1X TBS buffer with 100 μM $CaCl_2$ were prepared. Absorbance was then tested at 450 nm at several time points after emulsion preparation: 0 hours, 1 hour, 3 hours, 4 hours, 24 hours, and 30 days.

Emulsion binding measurement

Emulsions were prepared as described earlier in the Emulsion preparation section with 1 mol% of the lipids being replaced with biotin. To test rate and strength of binding between particles in the emulsion, we placed 80 μL of 1X TBS with 100 μM $CaCl_2$ and 20 μL of emulsion into a well on a 96-well microplate (Costar® 3370) and added 2 μL of buffer containing 1 μg of dissolved STP. Two replicates and two controls containing 80 μL of 1X TBS with 100 μM $CaCl_2$, 20 μL of emulsion, and 2 μL of 1X TBS were prepared. Immediately after the addition of STP solution, the well plate was placed in a UV-Vis spectrometer and absorbance readings at 450 nm were collected every 10 seconds for the first 10 minutes and then every 60 seconds for 40 minutes.

This binding experiment procedure was repeated at different times after the emulsion preparation to monitor consistency.

CHAPTER IV

RESULTS

Emulsion stability

Emulsion stability is largely dependent on three components: oil fraction, lipid fraction, and sonicator power. During the optimization process, we considered the components in the listed order.

Oil fraction

Because the relative refractive index between silicone oil, which has a refractive index of 1.403, and the 1X TBS, which has a refractive index of 1.338, is 1.048, silicone oil nearly provided the ideal relative refractive index of 1.05 [7]. When setting the appropriate oil fraction, we considered the relative density of particles that we wished to have in the emulsion. We tried 3 μL, 4 μL, and 5 μL of silicone oil in initial experiments and determined that 5 μL provided the desired density of particles in the emulsion and remained consistently stable.

Lipid fraction

The ratio of the oil fraction to the lipid fraction in the emulsion has important implications on this assay. Because we use UV-Vis spectroscopy for analysis, we rely heavily on the relative refractive index between the oil and the aqueous solution and the assumption that the oil is only present inside the particles created by the lipids in the solution. If additional oil is present outside of the particles or some of the particles are filled with the aqueous solution, the absorbance readings collected for particle stability and particle binding may be inaccurate. Despite this, when carefully regulated, a high relative lipid concentration can increase the effectiveness of the assay.

If we hold the oil fraction constant, we can increase the lipid fraction and, thus, increase the potential surface area between particles and buffer, increasing the number of particles and, as a result, decreasing the size of particles. Because of the high price of lipids, we substituted a surfactant, TWEEN® 20, for these lipid fraction optimization experiments. Holding the silicone oil amount constant at 5 µL, we tried varying the concentration of TWEEN® 20, including 3 g/L, 5 g/L, 10g/L, and 19.91 g/L. The stability tests for 5 g/L, 10 g/L, and 19.91 g/L is shown in Figure 2, Figure 3, and Figure 4, respectively.

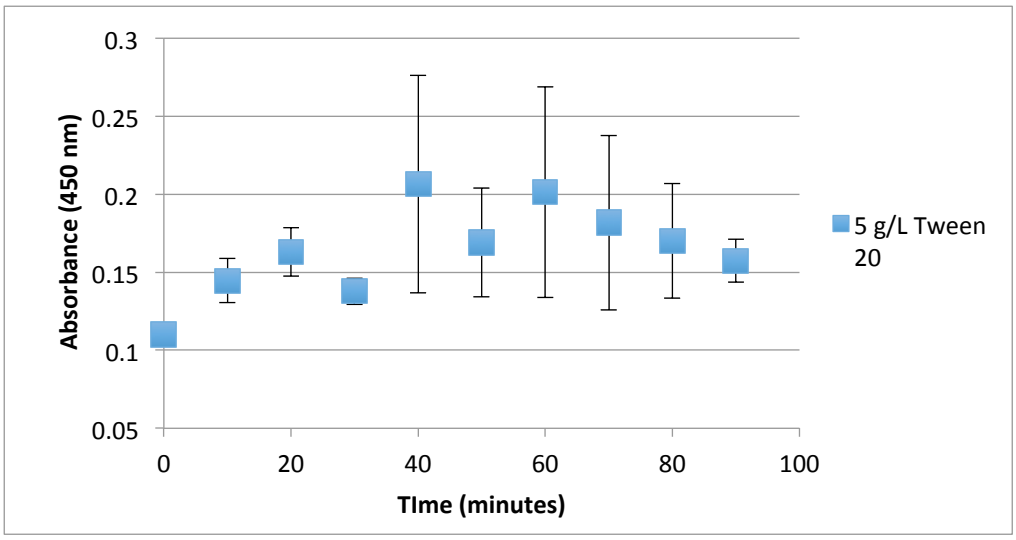

Figure 2. Stability test for emulsion with 5 g/L of lipids.

As apparent in Figure 2, there is no distinct trend in the absorbance of an emulsion containing 5 g/L of TWEEN® 20 as time progresses; absorbance fluctuates from 0.1 to 0.28 OD. While no flocculation or aggregation was visible to the eye, there is clearly some instability in the emulsion resulting from the ratio of lipids fraction to oil fraction being too low. In Figure 3, the absorbance does not change significantly with time for an emulsion with 10 g/L of TWEEN® 20; absorbance remains constant at approximately 0.09 OD. This consistency suggests that the emulsion is stable, and that particles are not aggregating or flocculating. In Figure 4, which displays the data collected on an emulsion prepared with 19.91 g/L of TWEEN® 20, we see

results also indicating stability, with a consistent absorbance of approximately 0.12 OD. This proves that a lipid concentration of 10 g/L or greater results in a stable emulsion. In further experiments, we used 10 g/L in order to minimize costs of expensive lipids.

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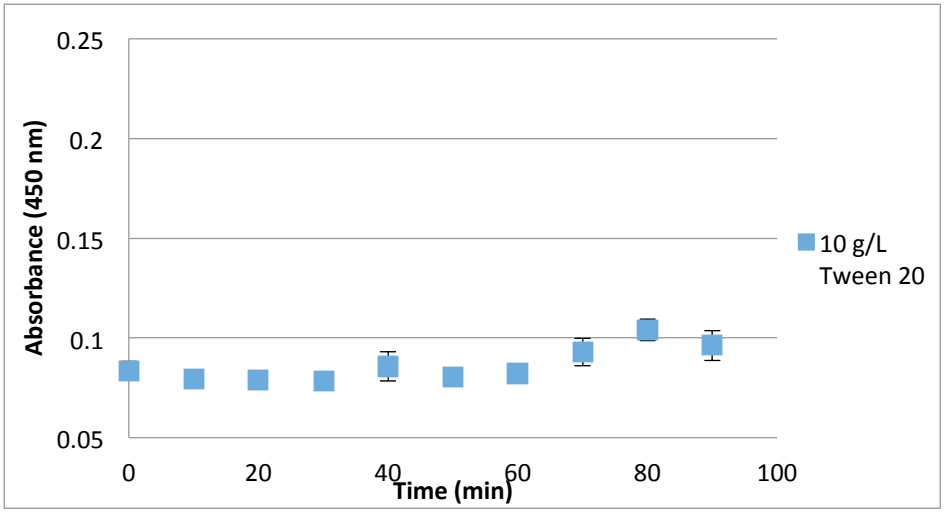

Figure 3. Stability test with 10 g/L of lipids.

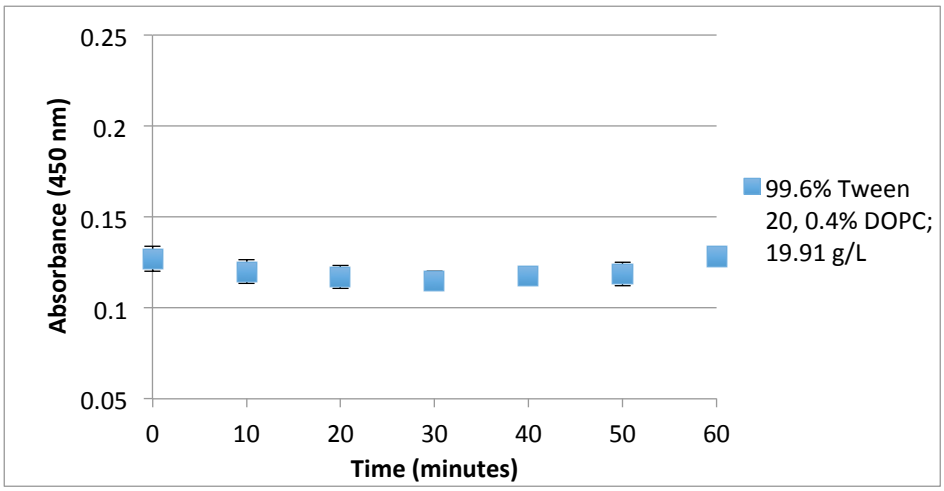

Figure 4. Stability test with 19.91 g/L of lipids.

We also determined that there was no significant difference in the results of the two stated sonicator settings and durations. We observed the same stability trends for the varied concentrations. Consequently, in further experiments, we chose the sonicator setting of 40 minutes of 10-second pulses at 80% power in order to reduce emulsion preparation time.

Protein addition and particle agglutination

In order to test binding occurring in this assay, we used biotin as the receptor added to the particles and streptavidin (STP) as the protein the particles bind to. Biotin and STP are commonly used in optimization of diagnostic assays because of the high affinity interactions. The immediate, irreversible binding that occurs between biotin and STP allows for the generation of a standardized calibration curve that can be used in quantification of biological systems [9]. STP is a homo-tetramer that has four binding sites for biotin. Crystal structures of STP obtained by Weber et al. show the binding sites are arranged approximately at each of the four corners of the protein's quaternary structure [9]. When STP is added to the emulsion, it will be able to bind to a biotin molecule on two different emulsion particles using binding sites diagonal to each other. This binding can occur between all of the protein added to the emulsion sample among different particles within the emulsion, resulting in the particles aggregating together. When absorbance is tested for this emulsion, the aggregates of particles will absorb the light as a large particle rather than an emulsion of the smaller particles. The difference in absorbance between unbound particles and particles that have aggregated as a result of added STP will provide data for quantification. Based on the results from the Emulsion stability experiments, we prepared emulsions with 5 μL of silicone oil and 10g/L of amphipathic molecules at a ratio of 99 mol% DOPC and 1 mol% biotin. The absorbance that was collected after the time that STP was added is shown in Figure 5. The control for this experiment was a sample of the emulsion to which no STP was added. While the experimental and control results only varied between 0.09 and 0.13 OD, the control and experimental values were significantly different indicating that upon the addition of STP, particles were binding together to increase in size and resulting absorbance. At time 0 seconds, there is an immediate jump in absorbance

between the experimental and control trials that results from the discussed high affinity between STP and biotin, where the association constant, K_a, is approximately 10^{15} mol^{-1} [10].

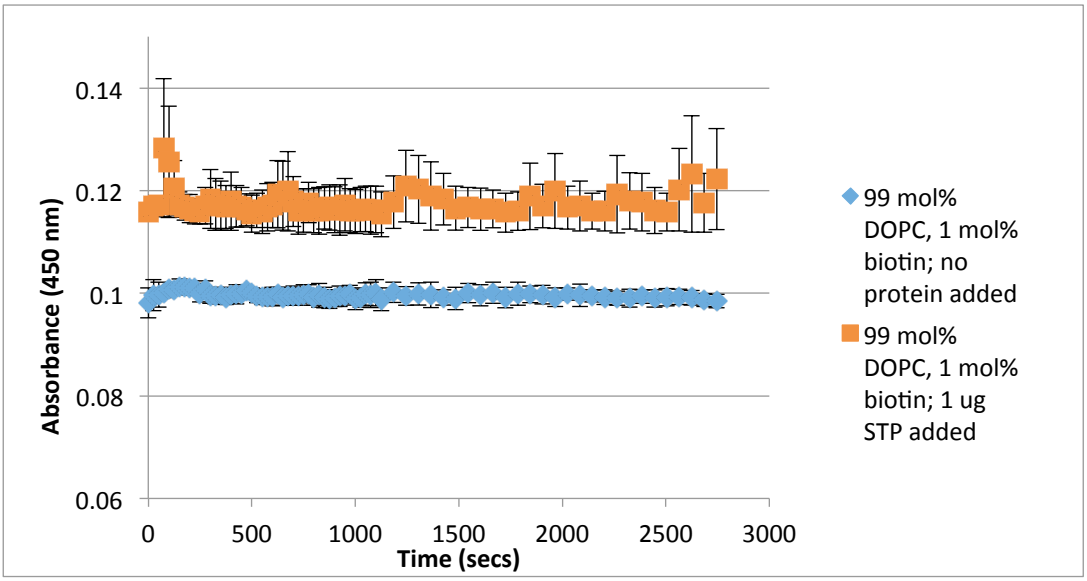

Figure 5. Pure lipid-silicone oil emulsion binding experimental results.

Protocol variations

Lipid substitutions

In order to reduce large costs of high concentrations of lipids, we considered substituting a portion of lipids with surfactants. We attempted to use both TWEEN® 20 and sodium dodecyl sulfate (SDS) as substitutions, preparing emulsions with silicone oil and 10 g/L of amphipathic molecules at a ratio of 49 mol% DOPC, 1 mol% biotin, and 50 mol% of the surfactant substitution. In Figure 6, the results for the trial using TWEEN® 20 as the surfactant are displayed. In Figure 7, the results for the trial using SDS as the surfactant are displayed. While resulting emulsions were stable with particles of desired size, we saw no increase in absorbance during binding experiments, indicating no binding is occurring. The controls for these experiments were samples of the emulsion to which no STP was added.

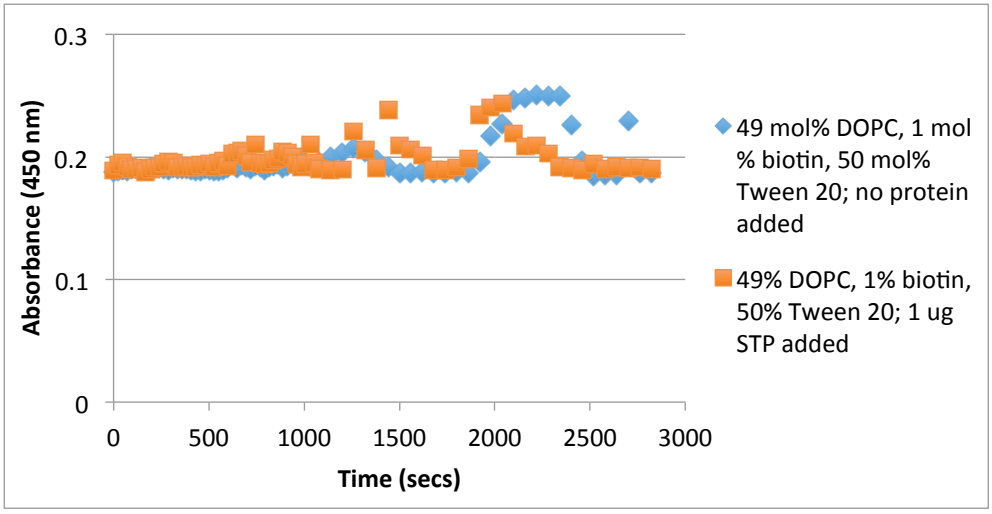

Figure 6. TWEEN® 20 surfactant and lipid-silicone oil emulsion binding experimental results.

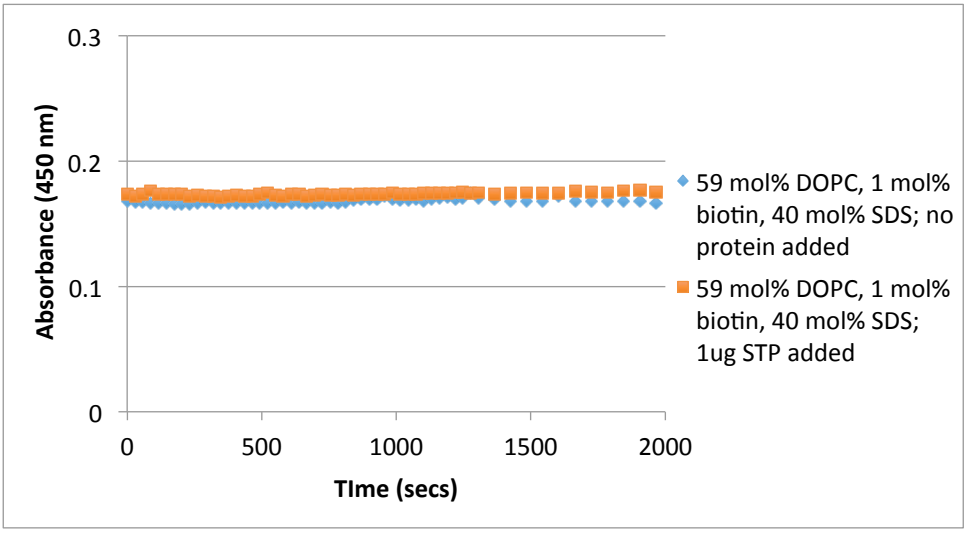

Figure 7. SDS surfactant and lipid-silicone oil emulsion binding experimental results.

No significant difference in absorbance was observed between the control trial and the trial in which protein was added for the emulsion with a TWEEN® 20 substitution or the emulsion with a SDS substitution. This indicates that no binding is occurring between the added STP and the emulsion particles. We predict that this is a result of the large, bulky head of TWEEN® 20 molecules and the charged head of SDS molecules. These molecular characteristics prevent the added STP from approaching the surface of the emulsion's particles, preventing interactions between the biotin receptors and the STP.

Olive oil

This assay also has potential to be used in other applications. In order to show variations that may be suitable for drug delivery systems, we optimized the system using olive oil, which is currently under review by the U.S. Food and Drug Administration for injection, according to the FDA database for Product-Specific Guidances for Generic Drug Development. Because olive oil has a refractive index of 1.47, the relative refractive index is approximately 1.09, which is nearly the desired relative refractive index of 1.05. Initially, we used 5 μL of olive oil in the emulsion preparation, which is the same amount of silicone oil used in earlier experiments. After stability analysis, this oil fraction did not appear to be stable. We repeated emulsion preparation with 3 μL and 4 μL of olive oil. The stability results are shown in Figure 8.

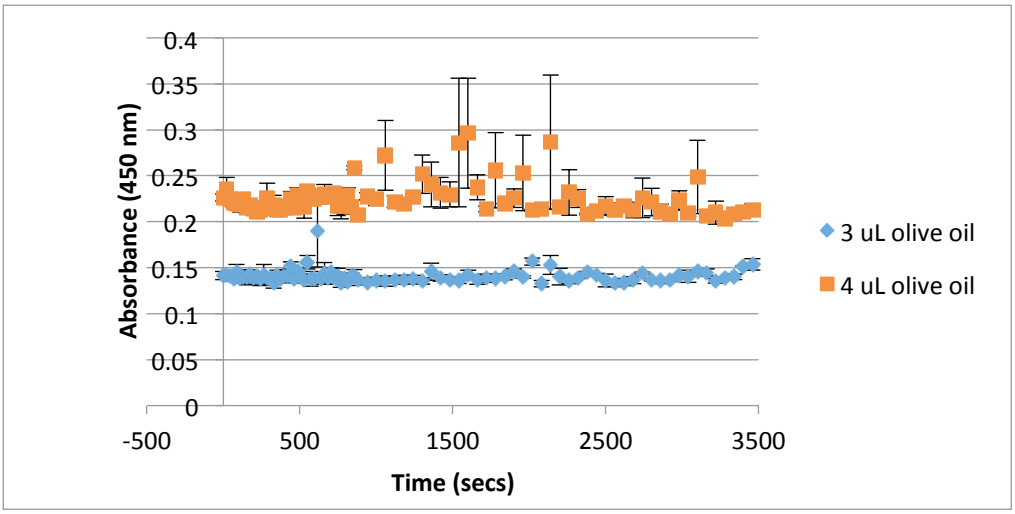

Figure 8. Stability test with 3 μL and 4 μL of olive oil.

The emulsion prepared with 4 μL of olive oil did not maintain a consistent absorbance over time. The absorbance ranged from 0.2 to 0.3 OD in the first hour after preparation. An emulsion prepared with 3 μL of olive oil appeared to be stable with absorbance remaining constant at approximately 0.15 OD for an hour after preparation. Based on this, we completed binding experiments using emulsions prepared with 3 μL or olive oil. We prepared emulsions with 3 μL

of silicone oil, 477 μL, and 220 μL of lipid solution, where the final concentration of lipid molecules is 10g/L, with a ratio of 99 mol% DOPC and 1 mol% biotin. The absorbance that was collected after the time that streptavidin (STP) was added to a sample of the emulsion is shown in Figure 9. The control for this experiment was a sample of the emulsion to which no STP was added.

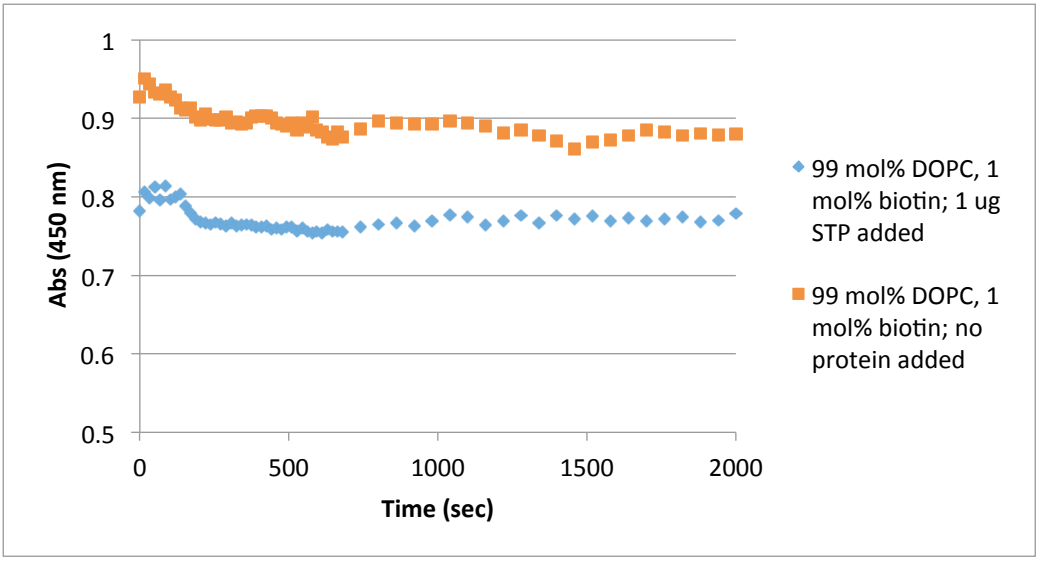

Figure 9. Pure lipid-olive oil emulsion binding experimental results.

The binding results for this emulsion prepared with olive oil are similar to the results we obtained for the emulsion prepared with silicone oil. The experimental results were constant at approximately 0.9 OD and the control results were lower and constant at approximately 0.77 OD. This significant different between the experimental and control trials indicates that, upon the addition of STP, particles were binding together to increase in size and resulting absorbance and proves that variations to the type of oil used in the emulsion can be optimized for the assay.

CHAPTER V

CONCLUSION

Conclusion

This study investigated the components of a turbidity-based emulsion agglutination assay that can be optimized so that the assay can be used to screen for hetero-multivalent binding. By carefully considering the oil type and fraction, lipid type and fraction, the sonicator settings, and the wavelength used to test absorbance, we can ensure that the emulsion particles remain in the Rayleigh scattering region for initial binding analysis, where a linear relationship between absorbance and particle size can be inferred and used to quantify binding.

Certain variations of the assay can be employed. For example, as discussed in the Protocol variations section, silicone oil can be replaced with olive oil. Only the oil fraction must be adjusted to account for this difference. However, as discussed in the same section, some variations may affect binding effectiveness. Lipids may not be substituted with surfactant in this particular assay. While stable emulsions were prepared, no binding was detected with the absorbance collections of these substituted emulsions.

Further work

We did not prepare emulsions with ranges of biotin fractions or ranges of STP amount additions. Rather, we only ran trials with one concentration, using 1 mol% biotin and solution containing 1μg of STP. From this, we are only able to make semi-quantitative observations based on the difference in absorbance OD between the control emulsion and the emulsion with the added STP. Further trials can be completed with these ranges and a calibration curve can be obtained in order to quantitatively analyze the binding that occurs.

Applications

As thoroughly discussed, this assay was optimized for diagnostics and to screen for hetero-multivalent binding. Worstell et al. employed this application, proving the existence of hetero-multivalent interactions between receptors Globotriaosylceramide (GB3) and Lactosylceamide (LacCer) and adhesin protein of *Pseudomonas aeruginosa*, LecA [7]. Similarly, this could be used to quickly identify combinations of receptors from large molecular libraries that participate in this binding mechanism.

Variations, such as the use of olive oil or other oils such as sesame oil, which is approved by the FDA for injection, may allow for other applications in antimicrobial treatments. Further experiments are being completed to determine if the emulsion particles can be loaded with drug and prepared with identified receptors so that the drug is specifically targeted to pathogen causing infection.

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