Molecular Origins of Recombinant Factor VIII (rFVIII) Stabilization with Pluronic® F68

By

Katherine M. Tadehara

A PROJECT

Submitted to

Oregon State University

University Honors College

In partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering (Honors Scholar)

Presented February 28, 2011
Commencement March 2011

AN ABSTRACT OF THE THESIS OF

Katherine M. Tadehara for the degree of Honors Baccalaureate of Science in Chemical Engineering presented on February 28, 2011. Title: Molecular Origins of Recombinant Factor VIII (rFVIII) Stabilization with Pluronic® F68.

Abstract approved: _____

Joseph Maguire

The capability of Pluronic® F68 to stabilize human recombinant Factor VIII in solution was assessed using zeta potential measurements. Hemophilia A is a genetic disorder in which the body is missing Factor VIII (FVIII) from the blood coagulation cascade. The pharmaceutical industry developed recombinant Factor VIII (rFVIII), the largest cloned molecule, as a viable treatment option for the missing protein. The protein suffers instability in solution which leads to aggregation, precipitation, and adsorption. Current formulations of rFVIII include the surfactant Tween 80 as a stabilization aid. Pluronic® F68, another surfactant, has been shown to prevent sheer in mammalian cell cultures mainly by enhancing cell resistance to sheer stress. Pluronic® F68 may be able to increase the stability of rFVIII for prevention of aggregation and precipitation. The stabilization of rFVIII was monitored using a zeta potentiometer with silica as a model surface. Zeta potential was unable to register a significant change for the initial concentrations of rFVIII used. Investigations were performed by increasing the amount of rFVIII and testing the effect of Tween 80 on the silica microspheres. The zeta potentiometer was not sensitive enough to register any conclusive results as to the effectiveness of Pluronic® F68 due to interactions with Tween 80. Further research is required to determine the effectiveness of Pluronic® F68 for rFVIII stabilization.

Key words: rFVIII, zeta potential, Pluronic® F68, silica microspheres, adsorption

Corresponding e-mail address: tadehark@onid.orst.edu

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APPROVED:

Mentor, representing Bioengineering

Committee Member, representing Bioengineering

Committee Member, representing Bioengineering

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon state University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Katherine M. Tadehara, Author

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Thank you, Brynn Livesay, for all the research you conducted over the summer. Your help was indispensible in affirming the inability of the zeta potentiometer to provide viable results. I wish you all the best in your coming years of research and hope that you have more luck in ascertaining conclusive results than me.

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Introduction

Hemophilia A and Protein Injections

Hemophilia A and B are genetic disorders that lead to a deficiency of essential proteins in the blood coagulation cascade. The diseases affect over 149,000 people worldwide, with symptoms ranging from mild to severe (Stachnik , 2010). Mild cases create complications for serious injuries while severe cases may bleed spontaneously in joints and muscles. In severe cases, hemophilia is diagnosed within the first year of life and must be treated regularly. Hemophilia A is more common, occurring 1 in every 5,000 male births in the U.S. The cofactor Factor VIII (FVIII) is absent in patients with hemophilia A.

FVIII functions as an antihemophilic factor and remains in the blood stream. The coagulation cascade produces thrombin which activates FVIII. Activated FVIII works with Factor IX to activate Factor X which stimulates the production of more thrombin. The absence or reduced amount of FVIII in the blood stream hinders the production of thrombin and causes a reduction in clotting rate.

The most common treatment for hemophilia A is the injection of the missing protein, FVIII, during a bleeding episode. Originally, FVIII was derived from human plasma despite concerns over pathogen transmission and supply limitation. These restraints led to the development of the largest successfully cloned molecule, recombinant Factor VIII.

Stability Issues of rFVIII

Recombinant Factor VIII (rFVIII) is now produced by several pharmaceutical companies. The recombinant protein is unstable in solution due rFVIII's large size (280 kDa). The protein denatures by one of several mechanisms including aggregation, precipitation, and adsorption. Adsorption occurs due to the hydrophobic regions of the protein, which are electroneutral. The protein will rearrange when in the presence of an interface, such as air-water or solid-water is present. The protein will refold at the interface to minimize the amount of hydrophobic regions in contact with water as shown in Figure 1. The deformity is thermodynamically driven and results in the loss of activity (Gray et al, 2008).

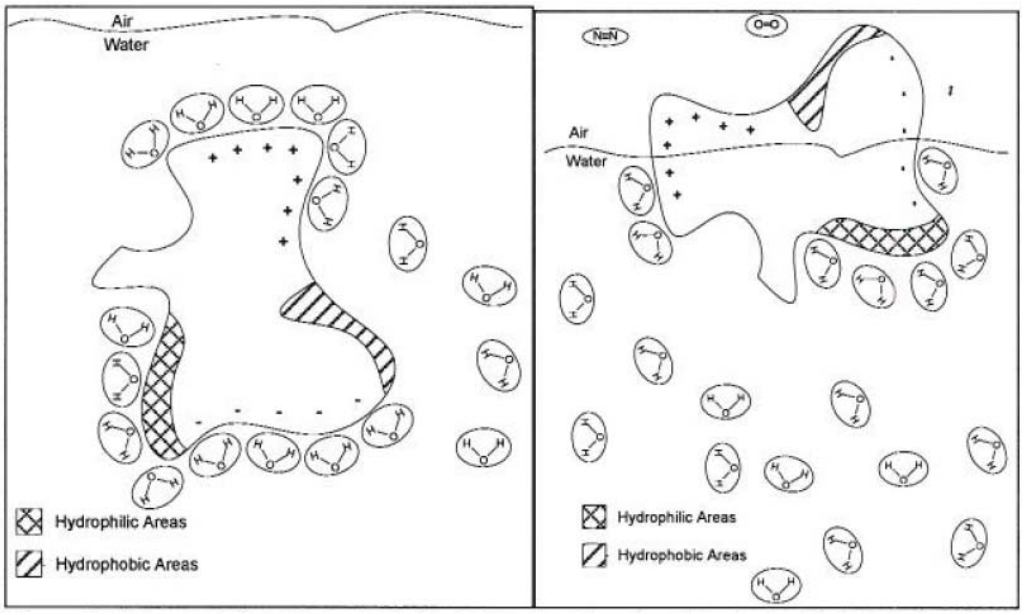

Figure 1. *The attractive and repellant forces that act on large proteins in solution. The dashed area represents the hydrophobic areas, while the cross-hatched area shows the hydrophobic areas. The charges that can arise on proteins due to these regions are shown as plus and minus signs. Note that the protein moves to the air-water interface to remove the hydrophobic region from contact with the water which is energetically more sound. (Figure courtesy of: Gray W, Thompson T. 2008)*

Aggregation is another problem as the protein will group with other proteins to reduce the amount of hydrophobic regions exposed to the water. Both aggregation and adsorption act to lower the overall energy of the protein, denaturing and inactivating the

protein. Pharmaceutical companies, like Bayer HealthCare, lyophilize rFVIII in order to maintain the protein's activity level. The rFVIII powder is then brought up in KG-2 buffer and injected into the patient at the required dosage. The protein is only stable for a short time once resolubilized which complicates administration of appropriate dosages.

Tween 80 Stabilization

Studies have shown that it is possible to use the surfactant Tween 80 to reduce interfacial folding of rFVIII. Tween 80 is a small molecule that can diffuse to and coat the interfaces quickly forming a physical barrier. This prevention is more effective at hydrophobic solid-water interfaces than at hydrophilic solid-water interfaces (Joshi et al, 2008). The hydrophilic surfaces were still coated with rFVIII despite the Tween 80, however, there was no significant change in the activity level of the proteins (Joshi et al, 2008). Tween 80's effectiveness at stabilizing rFVIII has led Bayer to include Tween 80 in its formulation of rFVIII to increase the stability time frame. Tween 80 is still unable to prevent the aggregation or precipitation of the rFVIII which is key to long term stabilization.

Pluronic® F68

Another surfactant, Pluronic® F68 has shown promise for the prevention of aggregation of rFVIII. Pluronic® F68 is a triblock copolymer used for the prevention of sheering in mammalian cell cultures. The surfactant coats the outside of the cells, providing a steric buffer. As a surfactant, Pluronic® F68 has two hydrophilic poly(ethylene oxide) (PEO) chains and one hydrophobic poly(propylene oxide) (PPO) chain. The structures of Tween 80 and Pluronic® F68 are shown in Figure 2. Ideally, the hydrophobic head of the Pluronic® F68 would cover the hydrophobic regions of rFVIII, allowing the PEO chains to provide a physical barrier preventing aggregation.

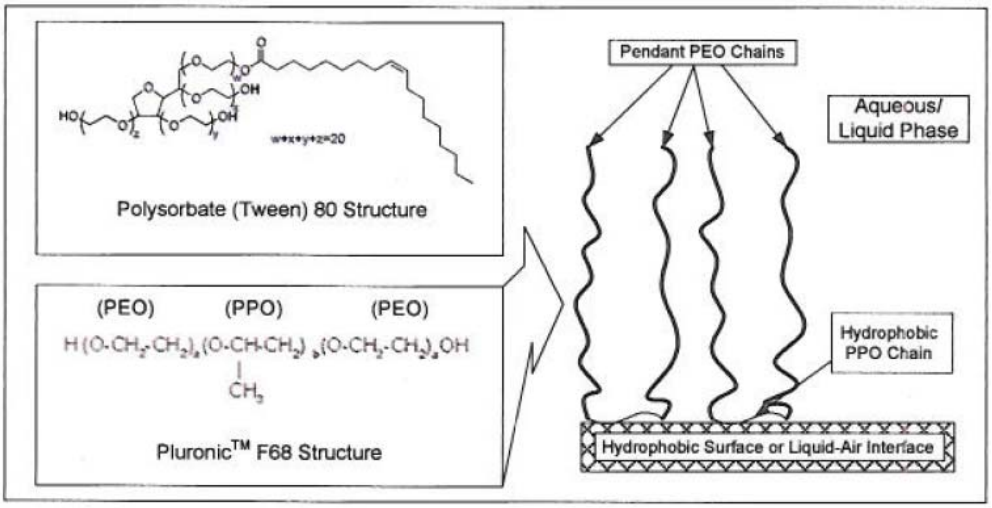

Figure 2. *The variable composition of the Tween 80 and triblock copolymer Pluronic® ® F68.Where Tween 80 only has one hydrophilic chain, Pluronic® F68 has two chains. Pluronic® F68 has been used for the prevention of sheering in mammalian cell cultures because its structure allows for it to coat the outside of cells and form a steric boundary.(Figure courtesy of: Gray W, Thompson T. 2008)*

Pluronic® F68 has behavior with rFVIII has been monitored at the air-water interface using tensiometer (Graham, 2009). The surfactant was shown to stabilize the rFVIII at 8 ppm which is lower than the Tween 80 amount of 18 ppm. The tensiometer was unable to indicate if this stabilization in surface tension was due to Pluronic® F68 coating the interface more effectively than the Tween 80 or if Pluronic® F68 is coating the protein's hydrophobic regions. A new testing medium was applied to see if Pluronic® F68 was forming the steric barrier: silica microspheres and zeta potential.

Zeta Potential

A zeta potentiometer examines surface migration phenomena by calculating the zeta potential of particles in solution. Zeta potential is a measure of surface charge in mV and it compares the electrical potential between the bulk solution and the slip plane charge about the particle. A higher magnitude zeta potential corresponds to a higher charge density on the particle surface. A zeta potentiometer has an anode on one side and a cathode on the other. It relies on the fact that particles of equal size experience equal drag consequently any change

in their velocity in the presence of an electric field can be attributed to a difference in surface charge as shown in Figure 3. Zeta potential also provides a measure of the degree of repulsion between particles. A lower magnitude value corresponds to less repulsion and higher likelihood of aggregation.

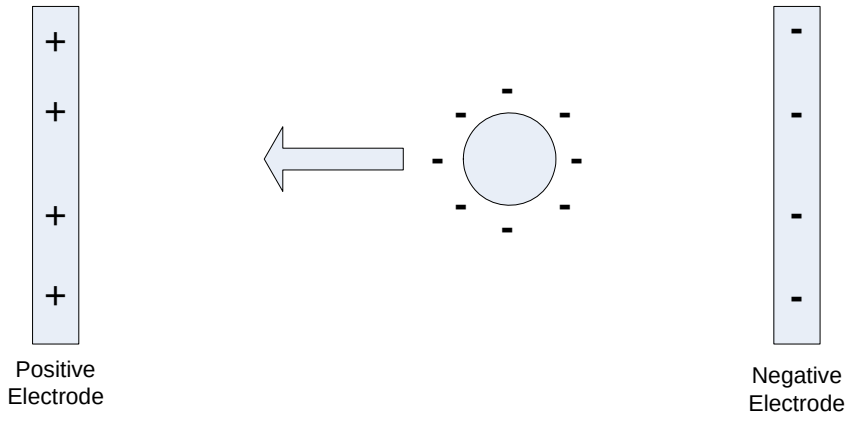

Figure 3. *The underlying operational concept of a zeta potentiometer. The negatively charged particle is driven from the anode to the cathode, migrating based on surface charge. The zeta potentiometer analyzes the movement of particles based on Stoke's Theorem.*

Silica microspheres were used as a testing platform due to their negatively charged surfaces and their size relative to the protein. The protein, rFVIII, has a higher affinity for negative surfaces than for itself consequently it will adsorb readily to the microspheres unless physically prevented. The silica microspheres will register a smaller negative charge when in the presence of rFVIII. The silica microspheres are also large enough that a microsphere coated in protein should register a drop in charge without significantly changing the size of the particle.

There are four basic experiments run on zeta potienometer to determine the effect of Pluronic® F68 on rFVIII stabilization shown in Figure 4. The first experiment will establish a baseline value for bare silica microspheres which has been shown to be around -90 mV (Ryder, 2010). The behavior of Pluronic® F68 in the presence of the spheres will be checked

to assure that it is not interacting significantly with the surface model. A non-significant drop in zeta potential would be less than 20 mV. The rFVIII would then be ran with the silica microspheres to show that the protein is adsorbing to the surface and remaining there throughout the wash procedures and sample preparations. The final experiment will establish the stabilization capabilities of Pluronic® F68 for rFVIII.

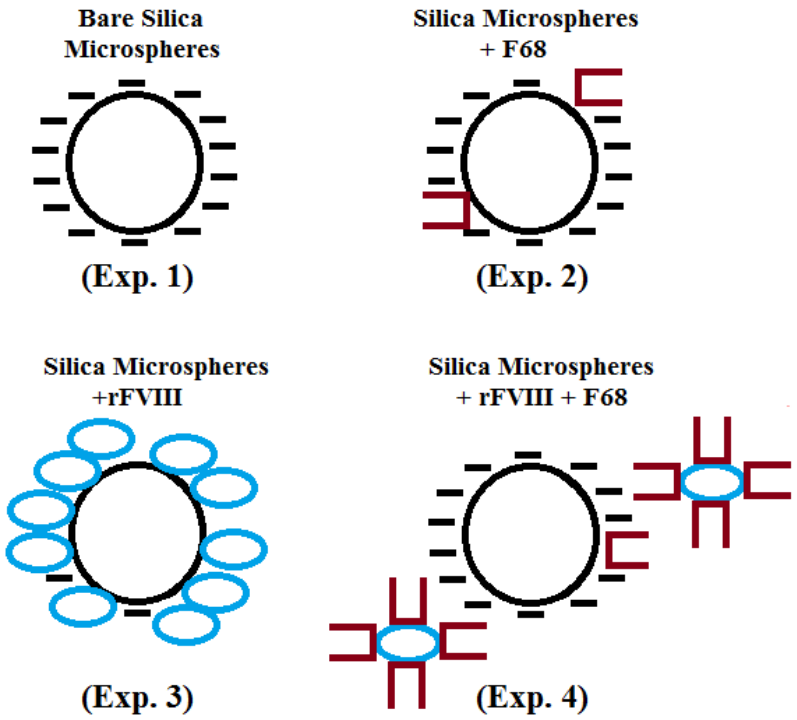

Figure 4. *Four zeta potential experimental conditions initially proposed to examine the anti-aggregation behavior of Pluronic® F68 on rFVIII. Experiment 1 produces a baseline for the incubation procedure on bare silica microspheres. Experiment 2 shows F68 behavior on microspheres. Experiment 3demonstrates the drop in zeta potential as rFVIII coats the silica microspheres. Experiment 4 is result hoped to prove F68 capable of stabilization.*

The efficacy of the zeta potentiometer depends on the change of the zeta potential between the bare silica microspheres and the subsequent runs. The rFVIII concentration will be kept very low, 10 IU/mL, to minimize the effect of Tween 80 already in formulation with the protein. There is the option of changing the sphere or protein concentration to provide for a larger drop in zeta potential. If the protein concentration is increased it will become

necessary to investigate Tween 80 with the silica microspheres as was proposed for Pluronic® F68 in experiment 2.

Materials and Methods

Protein and Buffers

Recombinant factor VIII (rFVIII) was donated by Bayer HealthCare (Berkeley, CA) as a freeze-dried powder. The concentration of the protein was 2000 IU. The rFVIII was reconstituted with 5 mL of DI water to a concentration of 400 IU/mL. The samples for the first half of the study were prepared to with a concentration of 10 IU/mL. The amount of rFVIII was increased for the second half of the study to 500 IU/mL by adding 4 mL of DI water to the lyophilized rFVIII.

The samples were prepared with a filtered (0.2 µm) KG-2 Buffer. The KG-2 buffer consisted of 30 mM NaCl, 2.5 mM $CaCl_2$, 22 g/L glycine, 3.1 g/L L-histidine and 10 g/L sucrose. The buffer was adjusted to pH 6.8 with 1 M HCl. The KG-2 buffer was stored at 4 ºC for up to four days, while the vials of rFVIII were stored at -20 ºC freezer. NaCl and $CaCl_2$ were purchased from Mallinckrodt AR, L-histidine from Fluka, and glycine and sucrose from Sigma Aldrich. The Pluronic® F68 (MW 8400) was purchased from BASF and dissolved into KG-2 buffer. F68 stock solutions were made at concentrations of 1000 and 10000 ppm. Tween 80 studies were conducted at a concentration of 100 ppm. Tween 80 was purchased from (?). The zeta potential samples were ran in 0.5 mM KCl solution. A 0.5 M KCl solution was filtered through 0.2 µm filter and then it was nano filtered through a 20 nm syringe depth filter.

Sample Preparation for Initial Experiments

Bare silica microspheres (1 µm AngstromSphere Monodispersed Silica Powder, Fiber Optic Center Inc.) were used as the testing surface for adsorption. Five grams of microspheres were cleaned with a piranha wash procedure to remove any particulates left

from processing. The microspheres were washed in a base solution of H_2O:NH_4OH:H_2O_2 in a 5:1:1 volume to volume ratio for 10 minutes in an 80ºC water bath with agitation. The solution was submerged in a room temperature bath for 5 minutes before being centrifuged at 3000 rpm. The supernatant was removed and the microspheres were washed with an acidic solution of H_2O:HCl:H_2O_2 in a 5:1:1 volume to volume ratio for 10 minutes in an 80ºC water bath with agitation. The microsphere suspension was then removed and centrifuged at 3000 rpm and the supernatant removed. The microspheres were rinsed three times. The microspheres were stored as a bulk solution in 1 M HCl. A brief study into the suspension of silica microspheres in the 1 M HCl solution was performed. The dry weights of three 1 mL samples were taken over three day period. The final sphere suspension was found to be 160 mg/mL. Aluminum trays were used and the samples were dried in a hood due to the HCl fumes.

Incubation of Samples

The microspheres were rinsed prior to incubation with filtered water and diluted to 16 mg/mL in KG-2 buffer. The solution was vortexed for 5 minutes to insure an even dispersion of microspheres. The contact incubations were performed with KG-2 buffer in 1.5 mL Eppendorf tubes. The concentration was 0.75 mg/mL, 0.145 mg/mL, and 10 mg/mL for microsphere suspension. The optimal amount of microsphere surface area for protein adsorption was calculated to be 10 mg/mL. The amount was decreased in later experiments to encourage a full layer of rFVIII to form on the microspheres and increase the change in zeta potential.

Samples were rotated for one hour and then centrifuged using a Eppendorf Centrifuge 5415 D at 3000 rpm for 3 minutes. The supernatant was removed and the pellet was

resuspended with KG-2 buffer and vortex. The centrifugation and resuspension was repeated two more times before the samples were reconstituted in WFI for zeta potential analysis.

Zeta Potential Measurements

The concentration of contacted microspheres used was 0.01 mg/mL. Nanofiltered 0.5 M KCl was added to the cuvette to bring the salt concentration to 1 mM. The remaining volume was made up of NanoPure water. The cuvettes for zeta potential required 3.5 mL of liquid. Each sample was tested individually in the zeta potentiometer (ZetaPALS, Brookhaven Instruments Corp.) and analyzed using a 10 run setting with 20 cycles per run. The electrodes were rinsed and cleaned using NanoPure water and a sonicator before each run. The cleaned electrodes were then rinsed with some of the sample prior to insertion into the cuvette. NanoPure water was used to reduce interference from trace ions, while BIC Particle Sizing Software was used to assure the blanks (NanoPure water only) were not contaminated prior to sample runs.

Experimental Design: Initial Runs

KG-2 buffer was used in all incubation runs including the bare silica microspheres (Run 1). The effect of KG-2 on the microspheres was tested in later experiments to see if excess calcium ions and charged amino acids alter zeta potential readings. The subsequent runs were performed as outlined in Table 1.

Table 1. *Experimental runs for zeta potential measurements on Brookhaven Instruments Corporation ZetaPals. Includes the initial runs with 0.073 mg/mL, 0.145 mg/mL, and 10 mg/mL of silica microspheres.*

Run	Silica Microspheres	F68	rFVIII (10 IU/mL)
1	X		
2	X	X	
3	X		X
4	X	X	X

Experiments requiring F68 and microspheres will be subjected to five different concentrations of the surfactant made up from stock solutions shown in Table 2 with the goal that one of the concentrations will produce similar zeta potentials for Runs 3 and 4.

Table 2. *Different concentrations of F68 to be used in Runs 4 and 6. Run 2 will only be tested using the highest concentration to test for unwanted interactions between F68 and the zeta potentiometer.*

	F68 Concentration (ppm)
A	1
B	10
C	100
D	1000
E	10000

Experimental Design: Increased rFVIII and Tween 80 Studies

The effect of Tween 80 on silica microspheres was evaluated at 100 ppm to allow differentiation between the effect of increased rFVIII, 500 IU/mL, and of the Tween 80 present in the solution. The experiments were ran with varying concentrations of Pluronic®

F68 shown in Table 3. The silica microsphere concentration was also decreased to 0.073 mg/mL for this study.

Table 3. *Experimental runs for zeta potential measurements on Brookhaven Instruments Corporation ZetaPals with increased amount of rFVIII (500 IU/mL) and decreased amount of microspheres (0.75 mg/mL).*

Run	Silica Microspheres	F68	rFVIII (500 IU/mL)	Tween 80 (100 ppm)
1	X			
2	X	X		
3	X		X	
4	X	X	X	
5	X			X

The concentrations of Pluronic® F68 used in these experiments were varied from 10 ppm to 10000 ppm in order to locate a point where the surfactant began to have an effect on rFVIII shown in Table 4.

Table 4. *Different concentrations of F68 to be used in Runs 4. Run 2 will only be tested using the highest concentration to test for unwanted interactions between F68 and the zeta potentiometer.*

	F68 Concentration (ppm)
A	10
B	100
C	1000
D	10000

Results and Discussion

Initial Runs: 10 mg/mL of Silica Microspheres for Incubation

The initial runs were performed with 10 mg/mL silica microsphere suspension for incubation. The zeta potential of the bare spheres was not as high as expected based upon previous research (-90 mV). The runs with the microspheres and the varying concentrations of Pluronic® F68 showed no consistent trend. The microspheres incubated with 100 ppm Pluronic® F68 showed an increase in magnitude, while 1000 ppm showed a decrease. This may be due to the formation of Pluronic® F68 mycelles that may have survived the post-incubation washes shown in Figure 5.

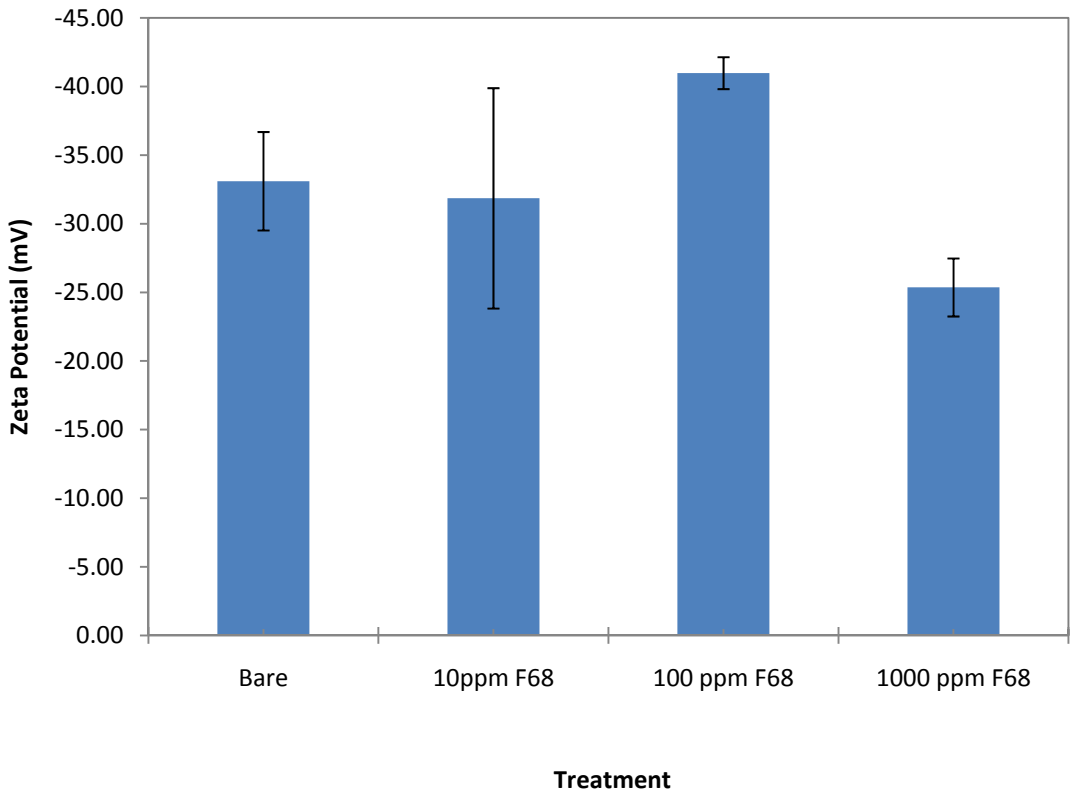

Figure 5. *Initial run of zeta potential with varying concentrations of Pluronic® F68 along with the 10 mg/mL microspheres. Samples were prepared in triplicates, each run was comprised of 10 cycles with 20 runs in each, which is 30 accessible data points for each sample. There is a no significant trend between the amount of Pluronic® F68 on the silica microspheres.*

Figure 6 shows the effect of rFVIII on the microspheres as well as the addition of the of F68 with rFVIII. The amount of protein used is 10 IU/mL in order to reduce the amount of interaction between the Tween 80 in the Bayer formulation and microspheres. The microspheres, on average, had a zeta potential of -33.1 ± 3.6 mV while the microspheres treated with rFVII showed -24.5 ± 4.3 mV. The rFVIII did reduce the negative charge on the silica microspheres, however, the change is not significant relative to the large standard deviations within the data sets. The 10 ppm F68 had the smallest standard deviation, ±5.7 mV, and differed from the bare microspheres by 1.97 mV. The increase to 100 ppm F68 decreased the difference to 1.33 mV, and 1000 ppm was even closer 0.62 mV difference. The standard deviation increased to ±9.0 mV and ±4.7 mV respectively, showing that the data is not consistent enough to prove that F68 is stopping interactions between rFVIII and the microspheres.

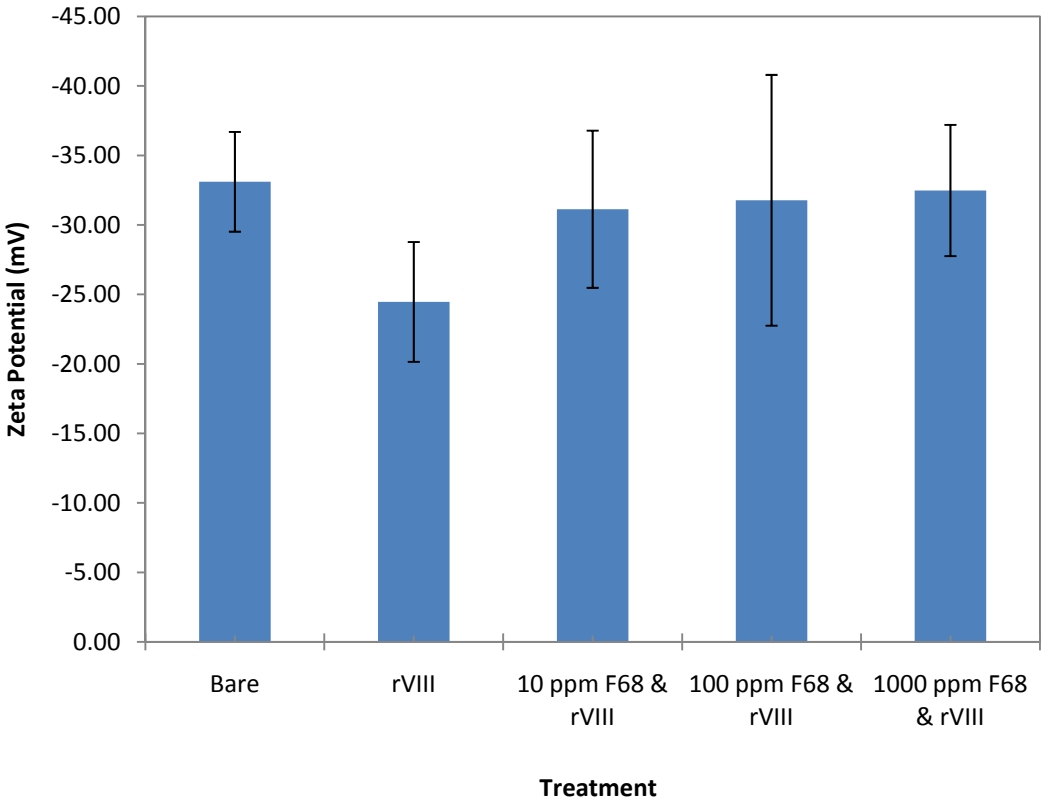

Figure 6. *The zeta potential of the samples with just silica microspheres, rFVIII, and varying amounts of F68 with rFVIII. The addition of F68 appeared to stabilize the rFVIII, however, rFVIII did not appear to decrease the zeta potential enough to indicate complete coverage.*

The small change between the bare silica microspheres and the rFVIII treated microspheres led to a retesting shown in Figure 7. The results were similar to the initial run with microsphere zeta potential of -32.9 ± 10.3 mV and rFVIII with -20.1 ± 9.5 mV. The large variation within the sample sets still indicate that there is not enough protein contacting the silica microsphere surface.

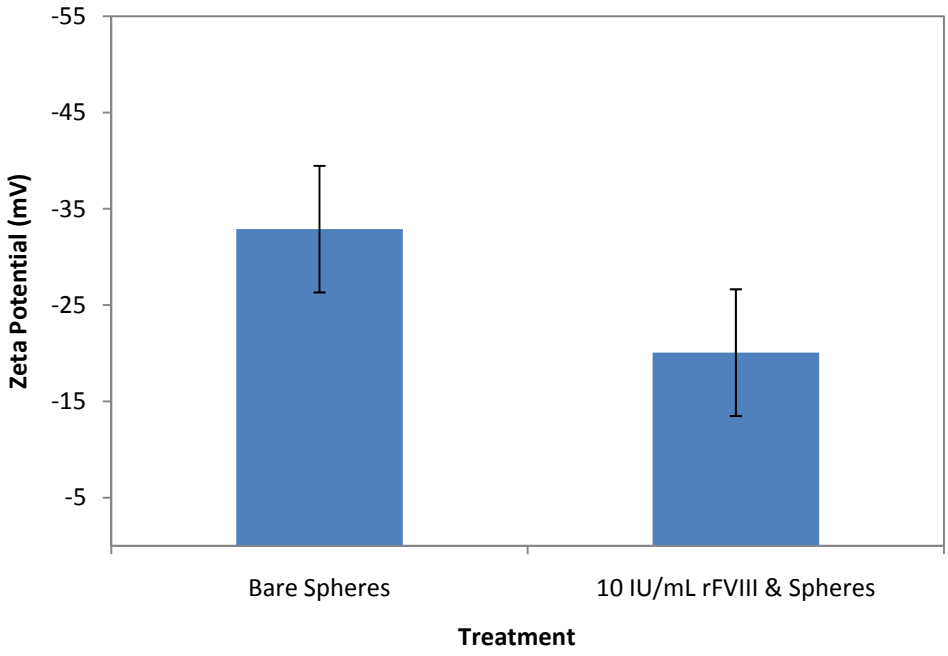

Figure 7. *Comparison to see if the rFVIII was fully coating microspheres. Given that the zeta potentiometer has an error of ±20 mV. The difference between the bare microspheres and the microspheres incubated with rFVIII is not significant enough, which means they may not be coated fully.*

Reduction of Microspheres: 0.145 mg/mL and 0.073 mg/mL

The amounts of the microspheres incubated were reduced so that the protein concentration was much greater than what is needed to coat the surface of spheres. The microsphere concentration was decreased instead of increasing rFVIII concentration to avoid increasing the concentration of Tween 80 unnecessarily. Figure 8 shows the results of the experiments with the 0.145 mg/mL of silica microspheres. The Pluronic® F68 appears to have an adverse affect on the zeta potential when combined with the rFVIII. There is a downward trend in the zeta potential, which shows that the surfactant may be interfering with the zeta potential by adhering to previously bond rFVIII on the microsphere surface.

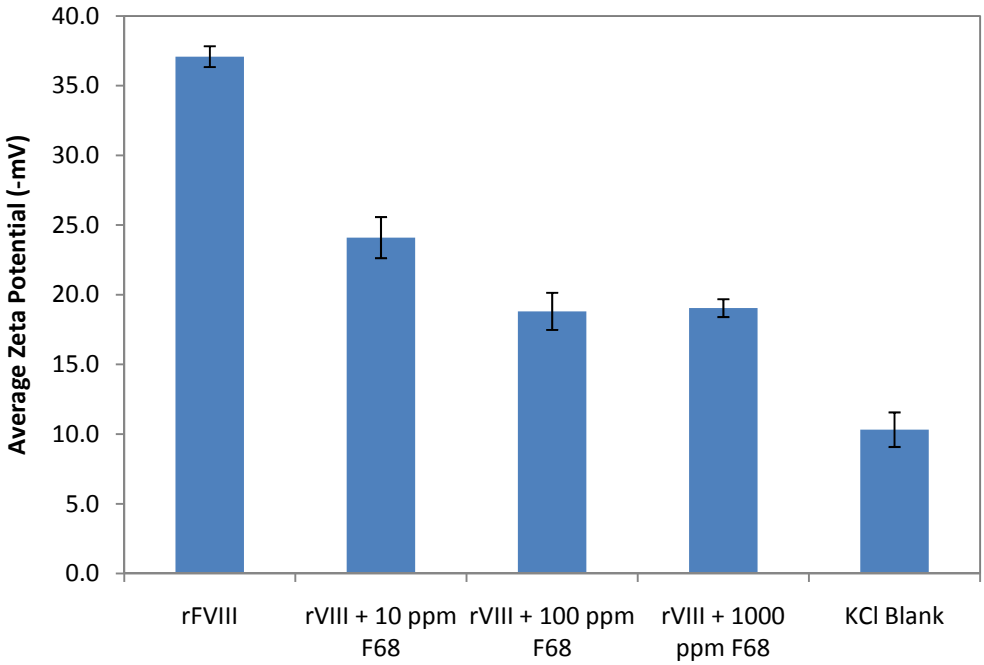

Figure 8. *The runs involved samples that were incubated with only 0.145 mg/mL of silica microspheres. The decrease in the microsphere concentration was to alter the ratio to favor rFVIII to fully coat the microspheres. The downward trend hints that Pluronic® F68 may not be stabilizing the rFVIII from aggregation, but by coating the microspheres instead.*

The same concentrations were ran again on the zeta potienometer to see if the trend persisted for a higher concentration of Pluronic® F68 in the presence of rFVIII. These subsequent runs had even higher variations than the previous set, especially for concentrations of 100 and 1000 ppm of Pluronic® F68. The higher standard deviations on these runs caused concern as to the effectiveness of the zeta potentiometer for measuring the stabilization of rFVIII. The variations within the runs of samples affected the accuracy of the data collected. The results from the earlier 10 mg/mL microsphere incubations with only the surfactant showed that the Pluronic® F68 to have a minimal affect on the charge of the microspheres. However, the presence of rFVIII in the incubations may have caused both the protein and the surfactant to adsorb to the microspheres creating the abnormalities seen in Figure 9.

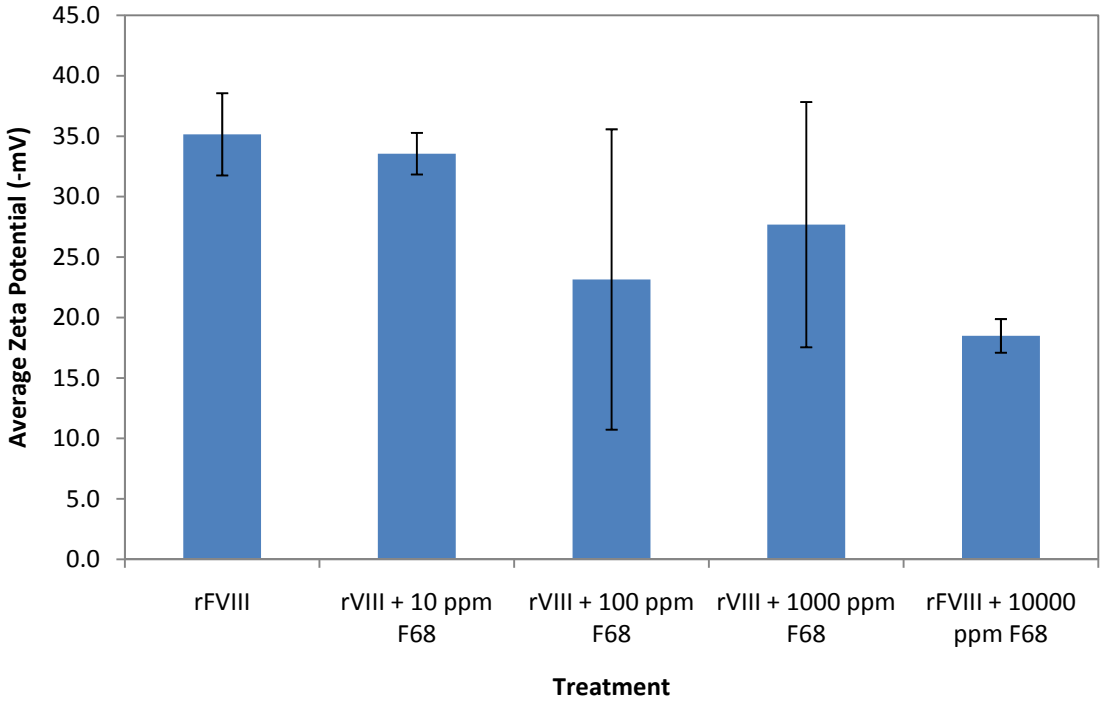

Figure 9. *Zeta potential for 0.145 mg/mL incubated spheres with 10 IU/mL. The trend seen in previous experiments was not replicated here, which hints that either the Pluronic® F68 is adsorbing to the microspheres or the rFVIII is not adsorbing. The change between the measurements is still not significant enough for any generalizations to be made especially with regards to the large standard deviations exhibited by the sample sets.*

A smaller amount of silica microspheres, 0.073 mg/mL, was used in incubations to see if the protein could coat the smaller area better as shown in Figure 10. The resulting average came to be -21.2 ± 7.2 mV which is similar to previous experiments showing that the reduction in microspheres was not helping to increase the difference in zeta potential between the bare silica microspheres and rFVIII coated microspheres.

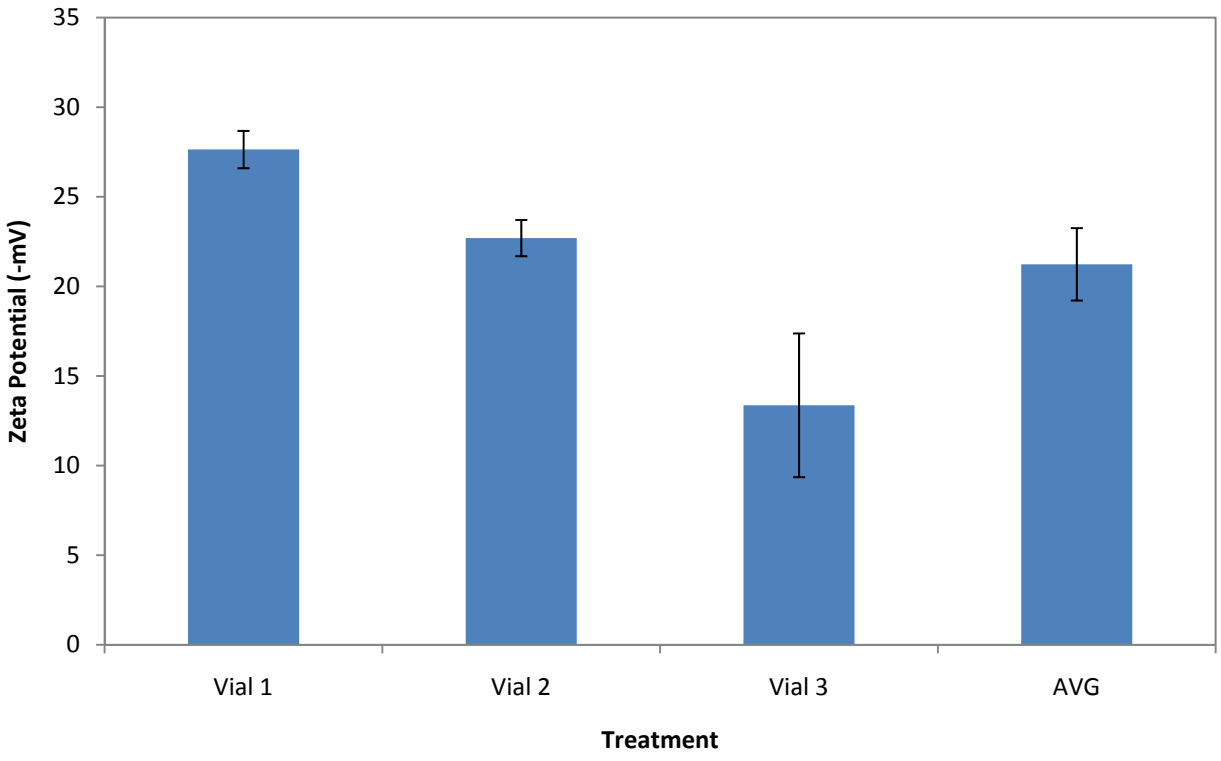

Figure 10. *Zeta Potential for 0.073 mg/mL incubated spheres with 10 IU/mL rFVIII. There is no difference in the average of the zeta potential of rFVIII, which means the amount of protein in the incubation needs to be increased to show any significant results on the zeta potentiometer.*

Increased rFVIII and Tween 80 Interactions

The protein was increased in an effort to coat the silica microspheres which were kept at 0.075 mg/mL for the incubation experiments. The Tween 80 was incubated at a concentration of 100 ppm to test for interactions. Tween 80 appeared to be coating the silica microspheres, lowering the zeta potential shown in Figure 11. This interaction makes it more difficult to validate the effectiveness of Pluronic® F68 in stabilizing rFVIII because even if the Pluronic® F68 is working the Tween 80 will still lower the zeta potential.

The effect of KG-2 buffer on the bare silica microspheres was tested by incubating bare microspheres with filtered water instead of the buffer. The non-buffered microspheres showed a larger zeta potential shown in Figure 11. The KG-2 buffer may also be dampening

the effectiveness of the zeta potentiometer in measuring the effects of Pluronic® F68 on rFVIII stabilization.

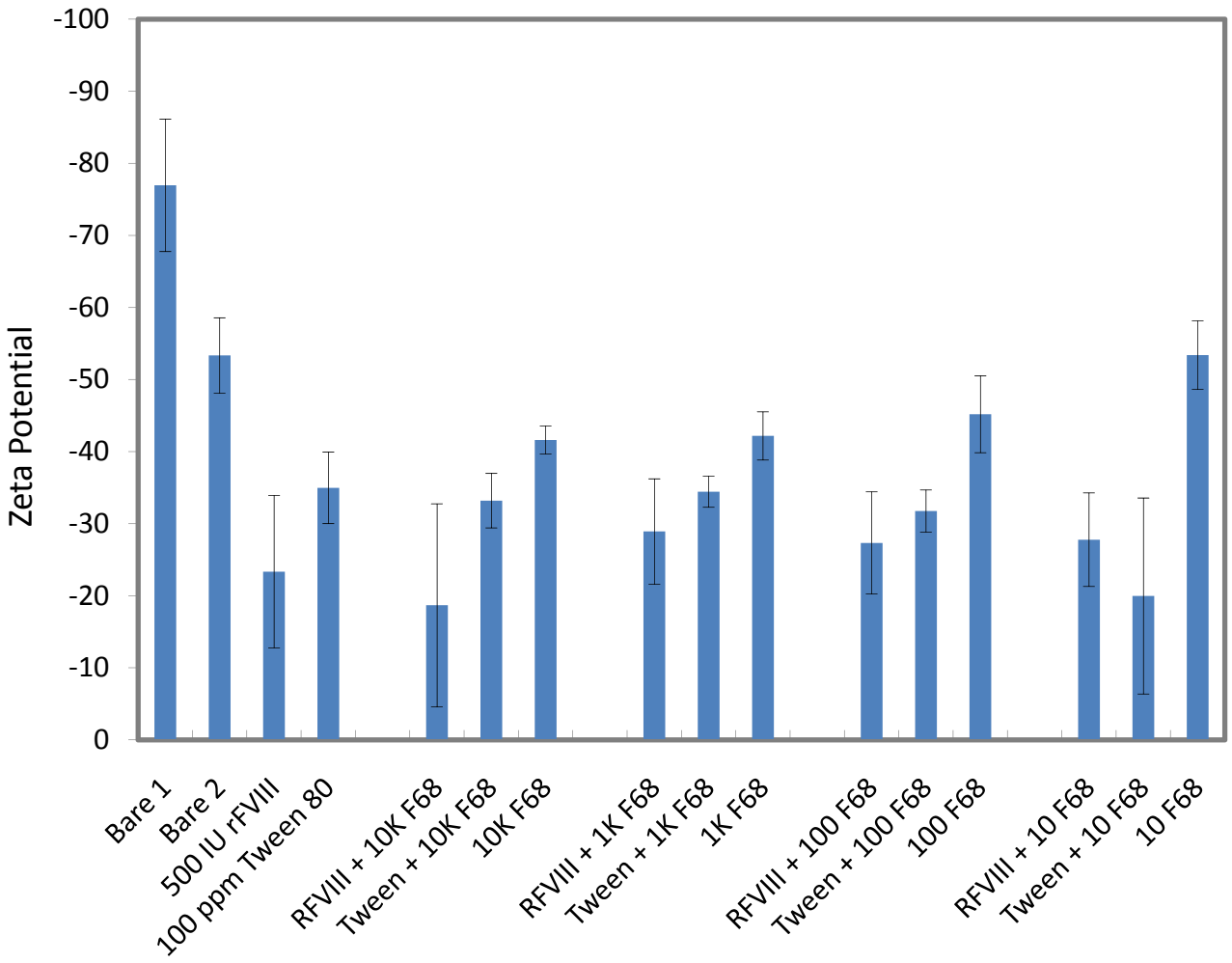

Figure 11. *Results from increased rFVIII concentration, 500 IU/mL, incubations and the Tween 80 study performed at 100 ppm. The amount of microspheres used in incubations was 0.75 mg/mL. The bare microspheres were incubated with filtered water unlike KG-2 buffer in the previous experiments. The results were still inconclusive as the effect of Tween 80 on microspheres was shown to decrease the negative surface charge.*

KG-2 buffer and Tween 80 were shown to have significant change on the zeta potential of the silica microspheres. As KG-2 buffer is essential to bringing up active rFVIII, it is indespenible for these experiments, despite the effect it has on the silica microspheres zeta potential. The Tween 80 also cannot be removed because it is part of the Bayer

formulation. The interactions created by the buffer and the Tween 80 severely limit the efficacy of using the zeta potentiometer. The results for the higher concentration of rFVIII are rendered inconclusive in light of these interactions. An alternative instrument should be used in substantiating the stabilization properties of Pluronic® F68 on rFVIII.

Conclusion

The zeta potential instrument gave only inconclusive results for the initial set of runs. There was never a steady trend for any of the experiments and it was concluded that there may not have been enough protein present to coat the microspheres. Pre-treatment of the samples before zeta potential included an hour of rotation at room temperature in 1.5 mL micro centrifuge tubes. The rotation was meant to provide the protein adequate time to bind to the surface of the microsphere, however, it is possible that the protein associated with the wetted surfaces of the centrifuge tubes.

A further investigation was conducted with a higher concentration of rFVIII with the microspheres. Experiments included the effect of the increased Tween 80 on silica microspheres and of KG-2 buffer on the microspheres. The zeta potential results were more promising with the higher amounts of protein (500 IU/mL), as the zeta potential exhibited a significant drop from bare microspheres to the microspheres and rFVIII. However, the amount of Tween 80 present also seemed to show a decrease in the charge of the microspheres, though not as drastic as rFVIII. The microspheres run with filtered water as a blank instead of the KG-2 buffer also showed a decrease in zeta potential. KG-2 buffer ions may also have been interfering with the silica microspheres dampening the changes in the zeta potential measurements. The presence of Pluronic® F68 did not change the zeta potential significantly enough to prove its capability in the stabilization of rFVIII.

Overall, the zeta potential was not sensitive enough to give conclusive results if Pluronic® F68 was able to stabilize rFVIII. Future work might consider application of direct surface spectroscopic analysis to the problem.

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