Improving the Delivery of Therapeutic Agents:
Creating Enhanced siRNA Carriers using Block Copolymers

A Major Qualifying Project submitted to the faculty of
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Degree of Bachelor of Science

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Abstract
Effective therapeutic delivery with carriers that easily complex, have low cellular toxicity, and have specified stability and disassembly mechanisms are ideal qualities in order to achieve ideal drug performance. A therapeutic that enables gene interference, short interfering RNA (siRNA), is currently being utilized in some clinical trials; however, limitations exist, such as their negative charge and short half-life in the body, with using siRNA. siRNA contains 21 base pairs and is negatively charged, resulting in a short half-life in the body making delivery carriers a necessity. Block copolymers have the ability to complex with siRNA and form micelles. These micelles have previously shown promise for drug delivery. The goal of this study was to assemble micelles of siRNA complexed with poly(ethylene glycol) (PEG) – poly-L-lysine (PLL) block copolymer and to investigate the disassembly and stability kinetics of the particles.

Anionic siRNA complexes with cationic PLL molecules through a self-assembly process. Micellization was confirmed using dynamic light scattering, which measured the particles to have a diameter of 70 nm. Two Alexa Flour molecules, Alexa Flour594 and Alexa Flour647, were included on the 5’ end of identical siRNA sequences in order to perform Förster Resonance Energy Transfer (FRET) assay, which is where excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon, to determine incorporation of siRNA within the micelles and the kinetics of the system when in a bulk buffer solution. FRET was achieved at charge neutral 1:1 molar ratio of siRNA:PEG-PLL. Micelle assembly measured over a period of time demonstrated their stability when in their bulk buffer condition. Since naked siRNA degrades in the body within minutes, micelle stability is essential to ensure effective siRNA delivery.
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Authorship

I certify that I am the primary author of all content in this report.
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1.0 Introduction and Literature Review

Short interfering ribonucleic acid (siRNA) was studied following Fire and Mello’s\textsuperscript{1} mechanistic discovery of RNA interference (RNAi).\textsuperscript{2} Since RNAi was discovered in 1998, siRNA has been used in over 30 early stage clinical trials with 21 drugs.\textsuperscript{2} siRNA’s ability to inhibit gene expression leading to diseases has the promise for advancing therapeutics within the clinic. For example, Kim et al. has studied polyelectrolyte complexes of dextran-siRNA in tumors of mice, resulting in better targeted delivery with the complexes opposed to naked siRNA.\textsuperscript{3} Similarly, another study utilized rat models with malignant gliomas.\textsuperscript{4} When treated with siRNA encapsulated in block copolymer delivery vehicles, there was observable tumor shrinkage in comparison to the untreated and naked siRNA.\textsuperscript{4} Another example is a drug involving siRNA in dendritic cells being investigated to target immunoportesome subunits in metastatic melanoma via transfection.\textsuperscript{2} The ability for complexed siRNA to achieve more effective delivery than naked siRNA is the groundwork for this current study.

siRNA contains at least 21 base pairs, is double stranded, and is negatively charged. Its hydrophobicity and negative charge makes naked siRNA non-ideal for transportation in the body.\textsuperscript{5} For example, the half-life of naked plasmid DNA (p-DNA) in the blood is only minutes.\textsuperscript{5,6} Additionally, the negative charge promotes siRNA to become degraded by nucleases found in serum and to have low cellular uptake.\textsuperscript{5,7} As a result, delivery agents such as viral vectors and block copolymers have been investigated to transport siRNA.\textsuperscript{5,7} The use of viral gene delivery, however, is limited due to safety factors for their use in clinical trials such as antigenicity and oncogenicity as well as quality control difficulties.\textsuperscript{7,9} Alternatively, block copolymers have shown efficient transfection, safety, and high productivity as delivery mechanisms for siRNA.\textsuperscript{5,7}
Block copolymers are considered one of the most promising candidates for non-viral gene delivery systems. They are beneficial for their low cellular toxicity and ease in complexing. For example, Oba studied a polyplex micelle formed of poly(ethylene glycol)-polycation (PEG-polycation) block copolymers containing pDNA and siRNA. This study utilized disulfide crosslink bonds within the micelle to demonstrate the stability of the micelle in the extracellular milieu (glutathione at 10 μM) and the release of pDNA/siRNA from the micelle in the intracellular milieu (simulated using glutathione at 10mM). In another investigation by Harada-Shiba et al. a block copolymer consisting of poly-L-lysine (PLL) and poly(ethylene glycol) (PEG) with pDNA were tested both in vitro and in vivo for their use as vectors for gene therapy. Results from this investigation showed that micelles with longer PLL chains (48 mer) have greater stability in the blood, which suggested that the binding between DNA and PLL is important for the overall micelle stability.

In this previous study conducted by Harada-Shiba et al., the importance of micelle stability prior to cellular uptake was a key factor while interacting with different components in the blood, such as IgM, fibrinogen, and fibronectin. The extracellular milieu plays a significant role during the delivery process of siRNA to a targeted site and is a critical interaction for delivery agents. Large NPs exceeding a diameter of 500 nm may not be successfully internalized by the cell. A recent review describes the challenges of delivering siRNA NPs through the blood even prior to reaching the targeted cell, such as clearance by the mononuclear phagocytic system. The ultimate goal for a carrying agent in the extracellular milieu is protection from degradation enzymes. An outer modification with PEG results in increased circulation time due to its hydrophilic and flexible characteristics. However, additional measures may be needed to then ensure complete and successful intracellular delivery through the endocytic compartments and
cytosol and release once reaching the nucleus. Block copolymers are ideal for such an application because of their controllability and modification abilities.

Once vehicles for delivery have been synthesized, the arrangement of siRNA within the complex is another crucial factor to consider for the most efficient delivery. Historically, studies have complexed siRNA within the core of the particle unit, where it can be protected by outer shell polymer units. However, a recent study demonstrated the effects of 10% fetal bovine serum (FBS) on siRNA chemisorbed to the surface of gold nanoparticles (NPs). FBS contains a plethora of molecules that easily degrade siRNA, including nucleases. This study suggested the rate of degradation of chemisorbed siRNA by serum nucleases was dependent primarily on siRNA sequence; however, particle degradation is influenced by the polymer, lipid, or other encapsulating agent when the siRNA resides at the core of the delivery complex (for examples see 13-14). The importance of circulation time in the blood demonstrates both the need for a high level of controllability and new methods to encapsulate siRNA and study its degradation properties.

In order to acquire a better quantitative understanding of the kinetic release of siRNA from its carrier, Alabi et al. developed siRNA-probes using Förster resonance energy transfer (FRET) analysis. FRET allows for a quantitative study of molecular assembly through a distance-dependent assay, where excitation is transferred from a donor molecule to an acceptor molecule. Utilizing these probes of siRNA containing Alexa Flour fluorescent units, Alabi et al. investigated fluorescently labelled siRNA encapsulated with branched polyethylene imine (b-PEI), Lipofectamine RNAiMax, and with lipidoid NPs. The sense and antisense strands were both labelled with Alexa Fluor 594 and 647 agents. When the siRNA duplexes were encapsulated within the NP, the FRET signal was “on,” showing a fluorescent signal, and when
the particle would disassemble, the FRET signal turned “off.” Microplate assays as well as incubation with HeLa cells demonstrated the use of these fluorescently labelled probes for quantifying the extent of siRNA NP disassembly.

In the current study, we utilized these FRET-labelled probes to encapsulate within a block copolymer. The block copolymer chosen to complex with the siRNA is a PEG-PLL polymer with a PLL length of 50 mer, which has been shown to be a stable delivery agent. PLL is a common cationic polymer used for delivering agents such as siRNA and nucleic acids to improve transfection efficiency and reduce toxicity. PEG is also often added to biomaterials to increase biocompatibility, improve solubility in NP complexes, and reduce cell toxicity. The goal of this work is to quantify the release kinetics of siRNA using FRET assays from a block copolymer which is suited as a delivery vehicle.

The first aim of this study was to form micelles complexing anionic siRNA with cationic PLL, leaving an outer shell of PEG. To ensure micelles were formed properly at charge neutralization, dynamic light scattering was performed, which measures the size of the particles. Once micelles were formed, the assembly of the siRNA within the block copolymer was measured using FRET assay and the system kinetics were investigated. Ultimately, the long term goal of this project is to be able to manipulate characteristics such as size and concentration of the micelles by altering properties of the block copolymer to achieve the most effective delivery. The assays and initial trials performed in this study set the groundwork for these future alterations.
2.0 Materials and Methods

2.1 Short Interfering Ribonucleic Acid (siRNA)

siRNA duplexes (HPLC purified) were purchased from Integrative DNA Technologies (IDT) (Coralville, IA). The 5’ end of the sense strand was labeled with either Alexa Fluor594 or Alexa Fluor647 dyes. The sequences are: (sense) 5’-Alexa594-
GAUUAUGUCCGGUUAUGUAUU-3’, (sense) 5’-Alexa647-
GAUUAUGUCCGGUUAUGUAUU-3’ and (antisense) 5’-
UACAUAACCGGACAUAAUCUU-3’. The molecular weight obtained for siRNA Alexa Flour594 (siAF594) and siRNA Alexa Flour (siAF647) were 13,747 Da and 13,882 Da, respectively. siRNA duplexes were stored in nuclease free duplex buffer purchased from IDT at concentrations of 7.7 nmoles/mL (equivalent to 107 μg/mL) at -20°C. The maximum amount of freeze-thaw cycles the duplexes can go through is 5, thus the stock concentration was aliquotted to reduce the number of freeze-thaw cycles. Once an aliquot was removed from the freezer, it was stored at 7°C, where the solution is stable for up to 14 months. The duplexes were stored separately. Precautions were taken to ensure photobleaching did not occur, and the siRNA molecules were stored in light impenetrable containers. Prior to micelle formation, siAF594 and siAF647 were mixed at a 1:1 molar ratio.

2.2 Poly(ethylene glycol) - Poly-L-lysine (PEG-PLL) Block Copolymer

PEG-PLL was purchased from Alamanda Polymers (Huntsville, AL) with a molecular weight of 28,000 Da. The molecular weight corresponds to 454 ethylene glycol repeat units, which have a molecular weight of 20,000 Da and 50 L-lysine hydrochloride repeat units, which have a molecular weight of 8,200 Da. The block copolymer was formed at a concentration of 1 mg/mL in HEPES buffer (20 mM, pH 7.4)\textsuperscript{10}, which was purchased from Sigma Aldrich.
2.3 Micelle Formation

Micelles were formed through achieving charge neutrality between the anionic siRNA and cationic PLL units. The basis to calculate this is determining the amount of negatively-charged phosphate groups in the siRNA concentration and then the amount of positively-charged nitrogen units needed by the PLL to create a charge neutrality.

Each base pair of siRNA contains one phosphate unit (P unit). Since siAF594 and siAF647 are duplexes, there are 21 base pairs in the sense strand and 21 base pairs in the antisense strand creating a phosphate total of 42 P units/nmoles. At a concentration of 7.7 nmol/mL, there are 323 P units/mL. For a volume of 5 μL of one siRNA duplex, the amount of P units would be 1.6. To form the micelles, a 1:1 molar ratio of siAF594 and siAF647 were mixed together. 5 μl of siAF594 is mixed in proportion with 5 μl of siAF594 to obtain a total of 3.2 P units within this solution.

To calculate the amount of PEG-PLL needed to micellize with this amount of siRNA to create charge neutrality, the amount of nitrogen units (N units) in 1 mg/mL of solution was first calculated. With a molecular weight of 28,000 g/mol and a 1 mg/mL concentration, there are 35.7 nmol/mL in the PEG-PLL solution. With 50 PLL units, there are 1,786 N units/mL. Knowing how many N units are present in 1 mL, and that the necessary amount of N units to achieve charge neutrality is 3.2 N units, a proportion can be set up to calculate the volumetric amount necessary for 3.2 N units. This amount was calculated to be 1.8 μl. Detailed calculations showing a step-by-step procedure can be seen in the Supplementary Materials 7.1 and 7.2.

Given these calculations, the final volume ratio required to achieve charge neutrality and for micelle formation to occur is 5 μl of siAF594, 5 μl of siAF647, and 1.8 μl of PEG-PLL. The siRNA duplexes are mixed together first by pipetting up and down. The polymer is then added to this solution and then pipetted up and down several times to ensure thorough mixing throughout.
This study did not require the utilization of a quencher molecule due to the siRNA encapsulation within the PEG-PLL complex.

Dynamic light scattering using a Malvern Instrument with Zetasizer software was used to ensure micelles were formed (see results, section 3.1). This technique has been utilized by previous studies for determining micelle size.17 A low-volume quartz batch cuvette was used to obtain measurements with minimal amount of micelle solution necessary. The low-volume quartz batch cuvette needed a volume of 0.40 μL of micelles in order to fill the window properly. 2-3 replicates were performed for each measurement.

2.4 Förster Resonance Energy Transfer (FRET) Analysis

FRET was performed using a SpectraMAX GeminiXS with SOFTmax Pro software. Black polystyrene 96 well optical bottom plates that were non-treated were purchased from Fisher Scientific. Experiments were performed using a 500 nM concentration of siRNA diluted in bulk HEPES buffer. The working volume contained in each well was 150 μL. For more details on plate dilution see Supplementary Material 7.3.

FRET is described as the energy transfer between a donor molecule to another acceptor molecule when the two are in close proximity, such as when they are in their micellized state. If not micellized, FRET is unlikely to take place due to the space between the donor and acceptor molecules (Fig 1a). In the current study, the siAF594 molecule is the donor as it is excited at a wavelength of 540 nm. If in close enough proximity, it will donate some of its emission energy to the siAF647 molecule, which will then also demonstrate emission (Fig 1b).
3.0 Results

3.1 Dynamic light scattering shows micelle formation

Dynamic light scattering was performed using a Malvern Instrument in order to ensure micelles were formed properly and at a size of interest for drug delivery studies. Size distribution by intensity showed a uniform peak at 100% intensity having a size of 55.15 radial nm and width of 35.63 r.nm (Fig. 2). Z-average results showed micelles formed with a diameter of 70 nm.
Figure 2: Dynamic light scattering size distribution by intensity
The size distribution intensity curve peaked at a radial size of 55.15 nm with 100.0% intensity and a radial width of 35.63 nm. Z-average results indicate a diameter size of 70 nm.

3.2 FRET observed with a stable mechanism

Control experiments were performed to first set a baseline to establish where the natural emission peaks for siAF594 and siAF647 were when alone in solution (Fig. 3a). When siAF594 was in a HEPES buffer environment, the emission peaked at a wavelength of 620 nm. When siAF647 was in a HEPES buffer environment, emission peaked at a wavelength of 670 nm. Further, to ensure the PEG-PLL solution had no auto fluorescence, an emission reading was taken at an excitation of 540 nm (Fig. 3b). No energy emission was observed, thus the polymer alone does not have fluorescent capabilities.

siAF594 and siAF647 were then mixed together at a charge-neutral 1:1 molar ratio; however, were not yet micellized and only in a HEPES buffer condition (Fig. 3c). The emission energy wavelength spectrum demonstrated a clear peak at 620 nm from the donor siAF594 molecule, and a minor insignificant shoulder at 670 nm. When compared to siAF594 and siAF647 micellized at a non-charge neutral 1:10 molar ratio of siRNA:PEG-PLL, the results are similar, and almost identical, with one major peak at 620 nm (Fig 3d).
Figure 3: Microplate readings at a wavelength emission range of 575 - 725 nm
Solutions were excited at a 540 nm wavelength for each trial. (a) Pure siRNA control with siAF594 (blue) and siAF647 (purple). Each siRNA probe was run separately from the other to obtain their natural emission peak when excited at 540 nm. siAF594 emission peaked at 620 nm and siAF649 peaked at a wavelength of 670 nm. (b) PEG-PLL with no siRNA present. (c) siAF594 and siAF647 together in HEPES solution not micellized with PEG-PLL. (d) 1:10 molar ratio complexation of siRNA:PEG-PLL.

When micelles were formed at a 1:1 molar ratio of siRNA:PEG-PLL, creating charge neutrality within the complex, and exposed with the HEPES buffer condition, FRET was achieved (Fig. 4). An emission peak at 620 nm is observed as well as a distinct shoulder peak at 670 nm, indicating a donor-acceptor relationship occurring.
3.2.1 Intensity Ratios

Intensity ratios were calculated by dividing the relative fluorescent unit (RFU) at a wavelength of 670 nm by the RFU at 620 nm (Table 1). When siAF594 was in a HEPES buffer condition, the intensity ratio was the lowest, at 0.25, and when siAF647 was in a HEPES buffer condition, the intensity ratio was the highest, at 36.8. These are the boundary conditions set by the two fluorescent molecules acting alone. When siAF594 and siAF647 were together in solution but not micellized, the intensity ratio was calculated to be 0.37, which is similar to the 0.4 intensity ratio with a 1:10 molar ratio of siRNA:PEG-PLL. The 1:1 molar ratio of
siRNA:PEG-PLL was calculated to be 0.61, which is higher than the non-micellized due to the donation of energy emission to the siAF647, resulting in a peak at a 670 nm wavelength.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>I670/I620</th>
</tr>
</thead>
<tbody>
<tr>
<td>siAF594 in HEPES</td>
<td>0.25</td>
</tr>
<tr>
<td>siAF647 in HEPES</td>
<td>36.8</td>
</tr>
<tr>
<td>siAF594 and siAF647 in HEPES no PEG-PLL</td>
<td>0.37</td>
</tr>
<tr>
<td>1:10 siRNA:PEG-PLL</td>
<td>0.4</td>
</tr>
<tr>
<td>1:1 siRNA:PEG-PLL</td>
<td>0.61</td>
</tr>
</tbody>
</table>

3.2.2 Kinetics

FRET for the 1:1 molar ratio of siRNA:PEG-PLL was measured over a time period of approximately 2 hours with measurements at five different time points (Fig. 5). These measurements allowed for the determination of siRNA release from the micelle or the stability of the micelle over a period of time. The intensity ratio was plotted versus time, which demonstrated that over a length of time the intensity ratio was unchanged. One replicate has been performed thus far.
Figure 5: Kinetic Results for 1:1 molar ratio of siRNA:PEG-PLL micelles
The intensity ratio $I_{670}/I_{620}$ was plotted for each time point measured. The kinetics of the system remained constant over the course of ~ 2 hours of measurements.

4.0 Discussion

4.1 Micelles formed drug delivery vehicles
Micelles have high colloidal stability due to the hydrophilic relationship between the PEG and PLL polymers.\textsuperscript{18} When the siRNA is encapsulated and complexed with the PLL unit, studies have proposed the possibility of a more toroidal shape forming opposed to a spherical shape due to the nature of the double stranded RNA complex.\textsuperscript{18-19} This shape is important to note when thinking about their delivery mechanisms. It has been shown that there is a dependence on the chain length of the hydrophobic unit on the size of the micelle complex. Studies have suggested that the size of the micelle is more strongly affected by the nonionic hydrophilic component of the polymer.\textsuperscript{18, 20} Thus, both the PEG at a MW of 20,000 g/mol and the 50 unit PLL influences the size at which micelles form.
Micelle size was measured using DLS to complex with a size of 70 nm in diameter with a distinct and uniform peak. Previous studies have investigated similar PEG-PLL complexations with DNA therapeutics, achieving similar sizes. Kataoka et al. synthesized polyion complex (PIC) micelles with a mixture of plasmid DNA (pGL3-Control) and PEG-PLL (48 mer) block copolymer. At a 1:1 ratio of DNA:PLL, the diameter of the PIC micelles formed at 121.0 nm. Another study utilizing a PEG-PLL block copolymer and both plasmid DNA and siRNA formed micelles at ~100 nm in diameter. It was noted that this size was good for blood circulation and had effective accumulation in solid tumors. Thus, the micelles formed in this study are also within the size of interest for drug delivery applications.

Additionally, Kataoka et al. investigated different ratios of DNA and PLL, ranging from 1:2 to 1:5, resulting in decreasing micelle diameters from 99.5 nm to 85.0 nm, respectively. Similarly, the long term focus of this study is to alter the block copolymers either by changing the concentration and ratio as done in this Kataoka study or by altering the structure of the block copolymer (see section 6.0).

4.2 FRET shows micelle assembly characteristics

FRET allowed for a comprehensive understanding of the assembly of the micelles with siRNA complexed with a PEG-PLL block copolymer. The natural emission peaks of the Alexa Fluor labelled siRNA molecules when measured were at 620 nm and 670 nm, respectively for siAF594 and siAF647. Alabi et al. conducted two studies utilizing the same siRNA probes, and found the natural emission peaks to be at 620 nm and 690 nm. This is a similar wavelength emission spectrum, and one slight difference could be attributed to difference in measurement reading devices.
The trial utilizing siRNA probes without micelle complexation demonstrated a distinct peak at 620 nm, and an insignificant shoulder at 670 nm. As depicted in the Fig. 1a schematic, the siAF594 and siAF647 were too separated for a donor-acceptor relationship to occur, and thus no FRET. The intensity ratio is relatively small, and close to the control siAF620 due to this separation and no micelle formation. Similarly, when micelles were not formed at the correct charge neutrality, a 1:10 molar ratio of siRNA:PEG-PLL instead of a 1:1 ratio, results were almost identical to those of the siRNA probes that were not micellized. This demonstrates the importance of the charge relationship when forming micelle complexes.

Micelles formed at charge neutrality with a 1:1 molar ratio of siRNA:PEG-PLL demonstrated a FRET interaction where, as depicted in the Fig 1b schematic, the siAF594 donated some of its energy to the siAF647 due to the close proximity within the micelle. The wavelength emission scan demonstrated both an emission peak at 620 nm and a distinct shoulder at 670 nm. This wavelength spectrum is similar to the one obtained by Alabi et al. using the siRNA probes with a b-PEI nanocomplex. This demonstrates the assembly of the micelles, ability to monitor their characteristics using FRET assay, and the repeatability between previous studies.

4.2.1 Kinetics demonstrate micelle stability

One limitation with using naked siRNA is the short half-life within the body. By using block copolymers and carrier vehicles, enhanced stability can be achieved. A focus on block copolymer use for this purpose has been seen in previous studies and for example, Zheng et al. has recently studied the long-term kinetics of DNA and oligonucleotides complexed with PLL. By studying these micelle’s over a length of time, an assessment could be determined on their stability or disassembly in a bulk buffer environment.
In the current study, over an approximate 2 hour time period, the intensity ratio of 0.61 remained unchanged in the HEPES bulk condition. Similarly, Alabi et al. performed a kinetic study on the siRNA probes utilized in this study with a lipidoid nanoparticle complexation.\textsuperscript{14} When in a bulk buffer condition, the intensity ratio of approximately 0.5-0.6 also remained unchanged over a period of approximately 1 hour.\textsuperscript{14} This study also promoted the release of the siRNA probes from the lipidoid NP by altering the bulk buffer condition to an environment that would dissemble the lipidoid particle.\textsuperscript{14} When this occurred, the intensity ratio started at a higher value at approximately 2.8 and decreased over the course of approximately 1 hour.\textsuperscript{14}

Now that we have developed micelles that have assembled, show a FRET response, and are stable in a bulk buffer environment, the future work of this study is to promote the release of siRNA from their micelle encapsulation similar. In the current work, this can be achieved by either manipulating the environmental condition or by altering or adding to the block copolymer structure, with the latter being more universally applicable since it would not rely on a bulk media for release.

5.0 Conclusions

Micelles were formed at charge neutrality with a 1:1 molar ratio of siRNA:PEG-PLL. The charge neutrality was determined based on the number of phosphate groups contained within the siRNA and the number of nitrogen groups with the PLL. This was balanced in order to gain a net neutral charge to promote ideal micellization between the anionic siRNA and cationic PLL. The nonionic PEG unit, with a MW of 20,000 g/mol, was suggested to have the most influence on the size of the molecules and formed a biocompatible outer shell. Micelle formation was confirmed with Dynamic light scattering, which yielded a distinct peak and a size measurement of 70 nm in diameter. This is a size of interest for particles in drug delivery studies.
FRET was achieved with the micelles when formed in their charge neutral state. The wavelength spectrum demonstrated the siAF594 was able to donate some of its energy emission to the siAF647 to emit due to their close proximity within the micelle complex. When measured over a time period of ~ 2 hours in a kinetic assay, the micelles proved to be stable with no change in the intensity ratio in their bulk HEPES buffer environment condition.

Micelle stability is an important characteristic for an ideal drug carrier. Naked siRNA has a short half-life in the body and degrades within minutes. By utilizing block copolymers, the assembly was stable for over two hours’ time. Future studies will work to control the release of the siRNA while maintaining necessary stability. This balance between controlled release and stability is crucial for achieving ideal transfer of siRNA therapeutics.

6.0 Future Work
Going forward, siRNA release from the micelle should be promoted with the kinetics of this system studied. There are two methods that could be used to achieve this. The first would be to alter the bulk environment of the system in order for the media to promote the release. With this method, the promotion would be reliant on the surrounding conditions, such as pH, temperature, and concentration.

Another approach that is possible with using block copolymers is adding to the PEG-PLL structure. This would also allow for a size dependent study to be conducted. For example, by adding a different block copolymer, such as a PEG-polypropylene glycol (PPG) as an additive, the micelle size would be altered. This would alter the size of the hydrophilic unit. Additionally, this study could also change the size of the hydrophobic part which would also influence both the concentration at which the micelle forms as well as the size. If the hydrophobic part increases in size, then the concentration at which the micelle forms decreases. By investigated these size and
concentration dependent relationships within the micelle complex, a mechanism for micelle assembly and disassembly would be obtained.
References

7.0 Supplementary Material

7.1 Micelle Preparation

Table 2: Supplementary Material Micelle Formation

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial amount received</th>
<th>Molecular Weight (g/mol)</th>
<th>Initial Mass</th>
<th>Buffer (diluting agent)</th>
<th>Component Amount</th>
<th>Buffer Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>siAF594</td>
<td>11.5 nmol</td>
<td>13,747</td>
<td>158 ug</td>
<td>Nuclease free duplex buffer</td>
<td>158 ug</td>
<td>1.5 mL</td>
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<tr>
<td>siAF647</td>
<td>7.7 nmol</td>
<td>13,882</td>
<td>107 ug</td>
<td>Nuclease free duplex buffer</td>
<td>107 ug</td>
<td>1 mL</td>
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<tr>
<td>PEG-PLL</td>
<td>100 mg</td>
<td>28,000 (PEG 20,000)</td>
<td>100 mg</td>
<td>HEPES buffer (20 mM)</td>
<td>10 mg</td>
<td>10 mL</td>
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Table 3: Supplementary Material Micelle Formation (continued)

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Molar Concentration</th>
<th>Individual N/P units</th>
<th>Total N/P units</th>
<th>Desired N/P units for 1:1 ratio</th>
<th>Volume for 1:1 molar ratio</th>
<th>N/P ratio for volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>siAF594</td>
<td>7.7 nmol/mL</td>
<td>42 (siRNA duplex)</td>
<td>323 P units/mL</td>
<td>323 P units/mL</td>
<td>5 ul</td>
<td>1.6 P units</td>
</tr>
<tr>
<td>siAF647</td>
<td>7.7 nmol/mL</td>
<td>42 (siRNA duplex)</td>
<td>323 P units/mL</td>
<td>323 P units/mL</td>
<td>5 ul</td>
<td>1.6 P units</td>
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<tr>
<td>PEG-PLL</td>
<td>35.7 nmol/mL</td>
<td>50 (PLL)</td>
<td>1786 N units/mL</td>
<td>646 N units/mL</td>
<td>1.8 ul</td>
<td>3.2 N units</td>
</tr>
</tbody>
</table>
7.2 Micelle Formation Sample Calculations

**siRNA stock**

Alexa594: 11.5 nmol -> 7.7 nmol:

\[
\frac{11.5 \text{nmol siRNA}}{1.5 \text{ mL buffer}} = \frac{7.7 \text{ nmol}}{\text{mL}}
\]

Alexa647: 7.7 nmol

\[
\frac{11.5 \text{nmol siRNA}}{1 \text{ mL buffer}} = \frac{7.7 \text{ nmol}}{\text{mL}}
\]

**P units per duplex**

Alexa594:

\[
\frac{7.7 \text{ nmol}}{\text{mL}} \times 42 \frac{\text{P units}}{\text{nmol}} = 323 \frac{\text{P units}}{\text{mL}}
\]

In 5 ul:

\[
\frac{323 \text{ P units}}{1 \text{ mL}} = \frac{x \text{ P units}}{0.005 \text{ mL}}
\]

\[P \text{ units in 5 ul} = 1.6\]

Alexa647:

\[
\frac{7.7 \text{ nmol}}{\text{mL}} \times 42 \frac{\text{P units}}{\text{nmol}} = 323 \frac{\text{P units}}{\text{mL}}
\]

In 5 ul:

\[
\frac{323 \text{ P units}}{1 \text{ mL}} = \frac{x \text{ P units}}{0.005 \text{ mL}}
\]

\[P \text{ units in 5 ul} = 1.6\]

Total siRNA P units: 646/ 2 mL

Total siRNA P units for 5 ul = 3.2
**PEG-PLL stock**

Amount to use: 10 mg

Molecular weight: 28,000 g/mol

\[
\frac{10 \text{ mg}}{28,000,000 \text{ mg/mol}} = 3.57 \times 10^{-7} \text{ mol/mL} = 357 \text{ nmol/mL}
\]

10 mg/10 mL buffer: 35.7 nmol/mL

**N units**

50 units PLL

\[
50 \text{ PLL units} \times 35.7 \text{ nmol/mL} = 1786 \text{ N units/mL}
\]

Desired N units for a 1:1 Molar ratio: 3.2 N units

\[
\frac{1 \text{ mL}}{1786 \text{ N units}} = \frac{x \text{ mL}}{3.2 \text{ N units}}
\]

\[
x \text{ mL} = 0.0018 = 1.8 \text{ ul}
\]

Final Volume Ratio for a 1:1 molar N/P ratio

5 ul siRNA Alexa594

5 ul siRNA Alexa647

1.8 ul PEG-PLL
7.3 Plate Dilution for FRET Assay

Table 4: Supplementary Material Preparation in HEPES for FRET

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Needed siRNA concentration</th>
<th>siRNA volume per well (uL)</th>
<th>PEG-PLL volume per well (uL)</th>
<th>Total volume per well (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelle</td>
<td>7.7 nmol/mL = 7700 nM siRNA</td>
<td>500 nM</td>
<td>10</td>
<td>1.8</td>
<td>11.8</td>
</tr>
<tr>
<td>HEPES</td>
<td>20 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>138.2</td>
</tr>
</tbody>
</table>