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Development of Silk Microparticles Capable of Bioluminescence

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Development of Silk Microparticles Capable of Bioluminescence

By

Monique Mariah Berg

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

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Monique Mariah Berg

May 19, 2023
Development of Silk Microparticles Capable of Bioluminescence

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
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Spring 2023
Abstract

The use of silk microparticles (µPs) as drug delivery devices has gained attention due to slow degradation properties, mild preparation conditions, and advantageous biocompatibility. However, little research has been done on where these particles go once injected. To expand these studies, the goal for this work is to create bioluminescent silk µPs that can be tracked in vivo. Here several methods are demonstrated for preparing bioluminescent silk µPs containing Nanoluciferase (NLuc) and/or its substrate furimazine (FZ). In this study, silk µPs were formed using a salting-out procedure.

The first method involved non-specific adsorption of NLuc and non-specific adsorption of FZ to separate silk µPs. Light output produced by introducing these two silk µPs sample types to each other was monitored over time in buffer as well as in the presence of other proteins. This study found that stable light emission could be achieved in all scenarios tested including when NLuc was absorbed to silk µPs and then exposed to FZ in solution, when FZ was absorbed to silk µPs and then exposed to NLuc in solution, and when silk µPs containing NLuc and silk µPs containing FZ were mixed in buffer. Both types of silk µPs were also found to stabilize light emission in the presence of other proteins.

For the next method, tyrosine residues of silk µPs were chemically modified to contain an azide functional group. The µPs were then soaked in dibenzocyclooctyne (DBCO)-linked NLuc solution. This resulted in the covalent linkage of silk µPs to NLuc via strain-promoted alkyne-azide cycloaddition. Light output produced by these luminescent silk µPs in the presence of FZ was monitored over extended periods of time. These two methods were compared to determine if
covalently attaching the NLuc enzyme to silk μPs results in a more stable light output. This study found that both modified and unmodified silk μPs that had been soaked in NLuc-DBCO produced a stable light output, indicating that covalent attachment of the NLuc enzyme to silk μPs was not necessary. The future role these BL silk μPs could play in drug delivery and cancer imaging is also discussed.
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Chapter 1. Introduction

1.1 Silk Fibroin Characteristics. Cocoons from the *Bombyx mori* silkworm are primarily composed of a fascinating and versatile protein called silk fibroin (SF). This protein has desirable traits such as biocompatibility, a slow degradation profile, low immunogenicity, mild aqueous processing conditions, and high mechanical strength. For these reasons, SF is studied as a biomaterial. SF is a large protein containing a heavy (391 kDa) and a light (26 kDa) chain that are covalently bound via a single disulfide bond at the C-terminus. The heavy chain of SF has a block copolymer-like assembly of 12 hydrophobic block regions linked by a total of 11 short hydrophilic regions. (Figure 1.) The hydrophobic regions of SF contain amino acid repeats of glycine-alanine-glycine-alanine-glycine-serine (GAGAGS) that self-assemble into highly crystalline anti-parallel $\beta$-sheets (Figure 2). These domains are held together by hydrogen bonding and van der Waals forces. The hydrophilic domains contain non-repetitive amino acids that form a random coil conformation. The content of the $\beta$-sheet structure plays a significant role in the crystallinity, hydrophobicity, mechanical strength, and degradation of SF. The versatility of SF allows silk solution to be reprocessed into a variety of structures. These structures include solutions, films, hydrogels, 3D scaffolds for tissue engineering, flexible electronic devices, and microparticles (µPs) for controlled drug delivery.
Figure 1. Schematic of the arrangement of silk fibroin heavy and light chains. The 12 hydrophobic regions (blue arrows) are separated by the 11 amorphous linker regions (adapted from reference 3).

Figure 2. Depiction of the silk fibroin β-sheet composition. GAGAGS domains self-assemble into anti-parallel β-sheets, strengthened by hydrogen bonding.

1.2 Chemical Modifications of Silk Fibroin. Although nearly 75% of the amino acid content of SF is aliphatic, SF contains reactive amino acids that are found primarily in the hydrophilic regions. The availability of these reactive residues allows for a variety of chemical modifications to take
place. The most prominent of these amino acids are serine (12%) and tyrosine (5.3%). Others include threonine (0.9%), glutamate (0.6%), and aspartate (0.5%). Increasing the functionality of SF, while maintaining its desirable characteristics, can expand the biomedical applications of this already versatile protein. Chemical modifications that are performed on silk include coupling reactions such as isocyanate coupling, carbodiimide coupling, and diazonium coupling.

Isocyanates are a family of highly reactive, low molecular weight chemicals that react with amino, carboxyl, and phenolic hydroxyl side groups. This means that roughly 3.5 mol% of SF amino acids could participate in modification with isocyanates. The reaction of SF with isocyanates introduces vinyl groups capable of radical polymerization. This reaction, however, typically requires an organic solvent which is not ideal and should be avoided if possible when working with proteins.

A more common method of SF modification is carbodiimide coupling. Carbodiimide coupling reactions are a method of forming amide bonds by reacting primary amines with carboxylic acids. There are several possible side reactions of carbodiimide coupling that can yield unwanted products. Targets for attachment, like proteins and biomolecules, often contain multiple amino or carboxylic groups, allowing variation in products and potential crosslinking or inactivation. There are few carbodiimide-coupling precursors within silk (1.1 mol% carboxylic acids), and as a result, the range of functionalization is drastically limited. A synthetic route to further carboxylate SF has been studied, which proved to be successful in providing more carbodiimide coupling reactive sites.

As previously mentioned, SF has a relatively high amino acid content of tyrosine (5.3 mol%, 277 per silk protein), making it a target for chemical modification. Because tyrosine is found
throughout the protein sequence of SF, modifications can be evenly distributed.\textsuperscript{15} Methods to modify the tyrosine residue include cyanuric chloride-activated coupling,\textsuperscript{16} tyrosinase catalyzed grafting,\textsuperscript{4,17} and sulfation.\textsuperscript{4} These modifications are limited by reaction yield and variation of incorporated molecules. In response to this, a method to modify silk using diazonium coupling chemistry was developed, which tripled the amount of functional group incorporation compared to carbodiimide coupling methods.\textsuperscript{12} Today, diazonium coupling is the most highly used tyrosine modification of SF.\textsuperscript{11} During diazonium coupling reactions, an electrophilic aromatic substitution reaction occurs between a diazonium salt and the tyrosine side chain. This substitution forms an azobenzene derivative.

Chemical modifications of silk in this study utilized a strategy published by Hausken et al.\textsuperscript{11} which began with azo modification of the tyrosine residues. The azo group was reduced with sodium dithionite, which yields a primary amine ortho to the hydroxyl group on the benzene ring (o-aminophenol, herein referred to as amino-tyrosine). The installation of a primary amine is necessary to be able to react the modified silk with N-hydroxysuccinimide (NHS) esters that contain desired functional groups.\textsuperscript{11} This study utilized an NHS ester containing an azide functional group. The purpose of these modifications will be further explained in the research aims.

\textbf{1.3 Silk as a Drug Delivery Device.} Ideal drug delivery materials should be non-toxic, have controllable release kinetics, and should stabilize loaded drugs. Synthetic polymers are typically used for drug delivery, as they have desirable pharmacokinetics and controllable degradation.\textsuperscript{1} However, synthetic polymers have restricted use in areas of sustained drug delivery due to acidic
degradation products and the need to be processed in organic solvents. Because of this, protein-based nano/micro-particle drug delivery systems are of increasing interest. In the last decade, SF has gained attention for use as a drug delivery material due to its higher biocompatibility and lower immunogenic potential compared to degradable polymers.\textsuperscript{18} Furthermore, the ability to functionalize SF side chains allows for bio-functionalization with numerous ligands or biomolecules, which could be applied to targeted drug delivery.\textsuperscript{1} Silk µPs as a drug delivery system have been studied and used in several anticancer therapy applications that have shown stability, non-toxicity, and controlled release.\textsuperscript{19–21}

There are several known techniques used to produce SF nano/micro-particles. Template assisted techniques utilize emulsion droplets to define size and shape, but are usually complicated to perform.\textsuperscript{22} Phase separation techniques, however, are relatively simple. One popular fabrication technique is the salting-out method.\textsuperscript{22} This method is inexpensive, simple to perform, and it avoids the use of toxic solvents. As the salting-out agent (potassium phosphate) is added to aqueous SF solution, protein-protein interactions increase, and SF eventually precipitates from solution. This simple method produces SF nano/micro-particles with controllable sizes within the range of 500 nm to 2 µm.\textsuperscript{8}

1.4 Tracking Silk \textit{in vivo}. Currently, little is known about where silk µPs travel once introduced \textit{in vivo}. Current techniques for tracking silk \textit{in vivo} include the use of quantum dots (QDs) or Lanthanide-doped upconversion nanoparticles (Ln-UCNPs). QDs are nanocrystals composed of semiconducting materials. Their use has gained popularity due to their high quantum yield, size-tunable absorption and emission, photostability, and broad excitation wavelength range.\textsuperscript{23}
However, the excitation wavelength of QDs may result in auto fluorescence background, tissue photodamage, and a low penetration depth.\textsuperscript{24} The major downfall of QDs, however, is that many are cytotoxic. Ln-UCNPs are an emerging type of fluorophore containing lanthanide ions featuring electronic transitions in the $4f$ electron shell. These nanoparticles have the ability to combine two or more lower-energy photons to generate a single, high-energy photon.\textsuperscript{25} These fluorophores are also biocompatible and exhibit low cytotoxicity, but their major downfall is that luminescence is strongly quenched in aqueous solvents.

The imaging techniques already described require a visible light excitation source, which can cause autofluorescence issues. One potential to expand the studies done on tracking silk \textit{in vivo} is to incorporate a bioluminescent component. Bioluminescence (BL) is a chemical process that involves emission of light by living organisms. Naturally occurring bioluminescent organisms emit light via oxidation of a small molecule substrate (e.g. luciferin for fireflies), catalyzed by a luciferase enzyme.\textsuperscript{26} Past research has utilized bioluminescent systems based on firefly and \textit{Renilla} (sea pansy) luciferases. However, applications of these systems are somewhat limited due to luciferase stability, size, and luminescence efficiency.\textsuperscript{27} These limitations constrict the scope of biomedical applications of such systems. Seeing the need for a brighter, more efficient luciferase, the Promega company engineered Nanoluciferase (NLuc), derived from a deep-sea shrimp, \textit{Oplophorus} luciferase (Scheme 1).\textsuperscript{28}
Scheme 1. Nanoluciferase enzyme catalyzes the oxidation reaction of optimized substrate furimazine. This chemical process yields furimamide and light.

Advantages of NLuc include small protein size (19.1 kDa), enhanced stability, and luminescence activity ~150-fold greater than both firefly and Renilla luciferases. This enzyme pairs with furimazine (FZ), a brighter, more stable substrate than the natural substrate, coelenterazine. The NLuc system has been employed successfully for several applications. These applications include investigating protein – protein and protein – ligand interactions, exploring gene regulation/cell signaling, monitoring protein stability, use as BRET-based biosensors, and BL imaging. Today, one of the most effective methods in imaging biological processes involves analyzing bioluminescent light emission. The relative non-toxicity of luciferin makes BL a promising imaging system regarding tracking silk μPs in vivo.
Research Aim

As mentioned earlier, the use of silk μPs as drug delivery devices has gained attention, but where these μPs go once introduced in vivo has not been extensively explored. Most in vivo imaging studies that utilize BL involve engineering cells to produce a bioluminescent enzyme, followed by the injection of its substrate into the bloodstream of the animal. While immensely useful for in vitro studies, cellular engineering could not be applied in human studies or used for practical therapeutic applications. In addition, many BL substrates can be toxic at the high doses often employed in animal studies. Therefore, a different approach was taken here to embed both the enzyme and substrate within silk μPs to avoid the use of mutated cells as well as minimize toxicity of the substrate.

The first goal was to determine methods to non-specifically incorporate the bioluminescent enzyme NLuc and its substrate within silk μPs. This was done by evaluating bioluminescence of silk μPs soaked in NLuc/FZ for varying amounts of time. These methods were used to create self-activated bioluminescent silk μPs, produced by introducing silk μPs that had been soaked in FZ to silk μPs that had been soaked in NLuc. The proximity of NLuc and FZ allows for the μPs to emit light, giving way to a new silk tracking technique that has not yet been explored.

The next goal of this study was to determine if it is advantageous to covalently bind NLuc to silk μPs. This was done by comparing light output of plain silk μPs soaked in NLuc to light output of azide-silk μPs covalently linked to NLuc-DBCO.

Lastly, initial studies to determine how these bioluminescent silk μPs perform in the presence of different biomolecules were carried out.
Chapter 2. Materials and Methods

2.1 Instrumentation. Proton nuclear magnetic resonance (\(^1\)H NMR) spectra of silk solutions and microparticles were recorded on a Bruker Avance III 500 MHz spectrometer. A VWR Clinical 50 Centrifuge was used to isolate silk microparticles upon formation. SEM images of the silk microparticles were obtained on a Tescan Vega 3 Tungsten Scanning Electron Microscope. Mass data for the Nanoluciferase protein was obtained with an Agilent 6545XT AdvanceBio LC/Q-TOF. Lastly, light output of luminescent silk microparticles was measured using a Biotek Synergy H1 Multimode Reader.

2.2 Silk Solution Preparation. First, silk fibers were isolated and purified via “degumming” to remove the sericin protein from the silk. In a 1.5 L solution of 0.02 M Na\(_2\)CO\(_3\), 12 *Bombyx mori* silkworm cocoons were cut up into 8 equal pieces and rapidly boiled for 1 h while stirring. The silk fiber strands were then removed from the Na\(_2\)CO\(_3\) and transferred to 1.5 L of boiling DI water for 10 min. Any remaining sericin was then removed by rinsing the silk fibers for 10 min in 2 L of DI water at rt; this rinse was performed 3x. Upon rinsing, the fibers were spread out to dry overnight.

The next day, the fibers were weighed and then covered in a 9.3 M LiBr solution (5 mL of LiBr per gram of silk). The fibers were then placed in a 60 °C oven for 1 h to fully dissolve into solution. Next, this solution was transferred into hydrated dialysis tubing (Fisher Scientific, 3.5K MWCO, 22mm) and dialyzed against 3 L of DI water. The dialysis water was changed after 1 h, then again after 3 h, and was then left to dialyze overnight. Over the next 12 h, the dialysis tubing was changed 3 more times and then removed. The final concentration of the silk fibroin aqueous
solution was determined by weighing the residual solid of a known volume of solution upon
drying it at 60 °C.

2.3 Silk Microparticle Formation. Silk microparticles were formed through induction of a phase
separation from the silk solution by addition to 1 L of 1.5 M potassium phosphate buffer (pH 9.5).
The buffer and silk solution were refrigerated overnight prior to use. The silk solution used to
form silk microparticles was 0.3% (w/v) in water. Upon cooling, the silk solution was mixed with
the potassium phosphate buffer in a volumetric ratio of 1:10. The silk solution was added
dropwise to the buffer solution, while stirring no faster than 160 rpm. After the entire volume of
silk solution was delivered, the bottle was capped, inverted, and placed back on the stir plate for
an additional 20 min. The mixture was then left to refrigerate overnight.

The next day, the particles were gravity filtered by passing the solution through filter paper (VWR
Scientific, 9 cm, 28310-048). The particles were then rinsed from the filter paper using nanopure
water into a 15 mL centrifuge tube and spun down at 4000 rpm in a centrifuge. The supernatant
was removed and replaced with ~10 mL of nanopure water to wash the particles. This
centrifuge/wash process was performed a total of 5 times. Upon completion of washing, the
centrifuge tube was filled with ~5 mL of nanopure water and placed in the freezer. Once frozen,
the sample was lyophilized which resulted in dried silk microparticles.

2.4 Chemical Modification of Silk Microparticles. Multiple chemical modifications were carried
out on the surface of silk microparticles. The first of these was the azo modification. First, 10 mg
of dry silk microparticles were suspended in 850 µL of borate buffer (100 mM, pH 9.5). For
future UV analysis, 50 µL was removed from the tube and the remaining 800 µL was used for the
following steps. The silk microparticles were placed on ice to cool. In a separate tube, 3.4 mg (0.020 mmol) of 4-aminobenzenesulfonic acid and 15.2 mg (0.089 mmol) of $p$-toluenesulfonic acid ($p$TSA) were added, along with 200 µL of nanopure water. This mixture was dissolved by vortexing and sonicating. Upon dissolving, the solution was placed on ice for 10 min. After 10 min, 6.8 µL of NaNO$_2$ (4 M, 0.027 mmol) were added to the solution and the tube was inverted and quickly returned to ice for 15 min. The resulting diazonium salt solution was pipetted into the tube containing the silk microparticles, inverted to mix, and quickly returned to ice. The particles were left to react for 40 min, while vortexing them periodically. After the reaction was complete, the microparticles were centrifuged for 1 min in a mini table-top centrifuge and the supernatant containing reactants was removed. Next, the microparticles were rinsed with 1 mL of nanopure water, vortexed, and centrifuged again. This wash process was repeated at least 3 times. After rinsing, the microparticles were resuspended in 1050 µL of borate buffer, again reserving 50 µL for future UV analysis.

The next modification was a reduction reaction of the azo bond to an amine group. After resuspending the azo-modified silk microparticles, 10 mg (0.057 mmol) of sodium dithionite was added to the tube and dissolved by vortexing. The silk was left to react for 30 min, while vortexing periodically. The same wash method described for the azo-modified microparticles was used. After the last rinse, the amino tyrosine (amtyr)-modified silk microparticles were resuspended in 1050 µL of phosphate buffered saline (PBS; 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2). Again, 50 µL was removed and kept for UV analysis.
The final chemical modification was the addition of an azide group. The suspension in PBS prepared above was used. The azido-dPEG₈-NHS ester (Quanta Biodesign, PN 10503) was removed from the freezer and brought to rt. To the suspension in PBS, 11 mg (0.020 mmol, ~3 equivalents relative to the number of tyrosine residues) of the azido-dPEG₈-NHS ester was added and vortexed to dissolve. This reacted for 3 h, while vortexing periodically during the reaction time. The same rinse method was used for these modified microparticles as the previously described method. After the last rinse, the azide-modified microparticles were resuspended in PBS. Lastly, 50 μL of the microparticle suspension was reserved for UV analysis. The remaining azide silk was saved for future reaction with NLuc-DBCO.

2.5 Preparation of Silk Microparticles for UV-Vis/NMR. First, 9.3 M LiBr was prepared in D₂O. Tubes containing particles to be analyzed were spun down in a table-top centrifuge for 1 min, and all liquid was removed with a pipette. Then, the samples were rinsed with 250 μL of D₂O, vortexed, spun down, and D₂O was removed. This wash was repeated. Next, 800 μL of the LiBr-D₂O solution was added to each sample tube and vortexed. If silk μPs were not dissolved within 15 min, samples were placed in a 60 °C oven and checked every 10 min. Once dissolved, 50 μL of each sample was transferred to a clear 96-well plate for UV analysis along with 50 μL PBS; absorption spectra were obtained (230-700 nm, 1 nm intervals). The remainder of each sample (750 μL) was transferred to an NMR tube and ¹H NMR spectra were obtained.

2.6 Expression of Nanoluciferase (carried out by the Amacher Group at WWU). Glycerol stock from the transformation of expression plasmid (pCold-His6-NLuc(C164S)-C) into BL21(DE3) cells was used for this step. A small amount of cells were used to initiate growth in 10 mL LB media
and 10 µL of ampicillin. The solution was then placed in a shaking incubator at 37 °C and 210 rpm and left overnight.

To a 3 L flask, 1 mL of ampicillin and 1 L of LB media were added. To this flask, 10 mL of the solution that had been incubating overnight was also added. The flask was incubated at 37 °C until OD = 0.6-0.8. Next, the solution was induced with 1 mL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG), and the protein was expressed at 37 °C for 4 h. Following this step, the sample was spun down to produce a pellet which was then lysed using 30 mL of lysis buffer (40.5 mM disodium phosphate, 9.5 mM monosodium phosphate, pH 7.5) and a Complete EDTA-free protease inhibitor tablet. The sample was placed on ice and sonicated. Next, the sample was split between small centrifuge tubes and a cell debris program was run in a prechilled centrifuge for 30 min. The protein was then purified using a 5 mL HisTrap column (Cytiva). The supernatant was loaded onto the Ni column and the flow through was collected and refrigerated. The column was then placed on an FPLC machine, and a wash was collected followed by the product fractions using wash buffer (40.5 mM disodium phosphate, 9.5 mM monosodium phosphate, 20 mM imidazole, pH 7.5) and elution buffer (40.5 mM disodium phosphate, 9.5 mM monosodium phosphate, 250 mM imidazole, pH 7.5). The flowthrough, wash, and desired fractions were analyzed using a 15% SDS-PAGE gel. The selected fractions were then dialyzed into 3 L running buffer (40.5 mM disodium phosphate, 9.5 mM monosodium phosphate, pH 7.5) to remove the imidazole.

Lastly, about 30 mL of running buffer was added to a 15 mL Amicon 10K centrifugal concentrator and centrifuged for 4 mins at 4000 rpm to wet the filter. The buffer was poured out and replaced
with selected protein fractions. The fractions were then centrifuged in 5 min intervals, interspersed with resuspension of protein. Next, the volume was reduced to the top portion of the filter, and protein was continually added to the upper reservoir and concentrated to 838 µM using the protein absorbance at 280 nm and extinction coefficient of 25440 M⁻¹ cm⁻¹.

Figure 3. The mutant Nanoluciferase protein sequence used with a total of 188 amino acids, and a calculated molecular weight of 21207.29 Da.

2.7 DBCO Linkage to Nanoluciferase. First, sulfo-DBCO-PEG₄-maleimide (Click Chemistry Tools, 1231-10) and NLuc were removed from the freezer and tris(2-carboxyethyl)phosphine (TCEP) was removed from the fridge. Once to rt, 1.0 mg (0.0035 mmol) of TCEP was dissolved in 1 mL of PBS giving final concentration of 3.5 mM. In a separate tube, 1.0 mg of sulfo-DBCO-PEG₄-maleimide (0.0012 mmol) was dissolved in 1 mL of PBS giving final concentration of 1.2 mM. In an Eppendorf tube, 66 µL of NLuc (100 µM), 22 µL of TCEP, and 267 µL of PBS was added giving final concentrations of 18.6 µM and 0.216 mM of NLuc and TCEP, respectively. This mixture was vortexed and left to react for 30 min. Next, 145 µL of the prepared DBCO stock solution in PBS was added to the tube, vortexed, and reacted at rt for 1 h. The NLuc-DBCO was then purified.
using a NAP-5 column that was equilibrated with PBS, giving a final NLuc concentration of 13.2 µM. The modification was verified using LCMS. HRMS (ESI/Q-TOF) m/z: [M + H+] calcd for NLuc-DBCO 22033.15; found 22033.3819. The NLuc-DBCO stock was stored in the freezer for future use.

2.8 Click Reaction of Azide Silk Microparticles with NLuc-DBCO. First, 2 mg of dry azide-modified silk μPs were placed in an Eppendorf tube. As a control, 2 mg of dry silk μPs were placed in a separate tube. To hydrate the particles, 1 mL of PBS was added to each tube and vortexed; particles soaked for 30 min. While soaking, 300 µL of a 10 µM solution of NLuc-DBCO was prepared. The 13.2 µM NLuc-DBCO stock was removed from the freezer. Once at rt, 227.3 µL of the stock was added to 72.7 µL PBS, giving a final concentration of 10 µM. Then, the hydrated silk particles were spun down in a tabletop centrifuge for 1 min, and buffer was removed from both tubes. To both the plain and azide-silk μPs, 100 µL of the 10 µM NLuc-DBCO stock was added and the samples were vortexed periodically. Silk μPs soaked in the NLuc-DBCO for varied lengths of time, depending on the experiment (2-24 h). After the soak time was completed, the μPs were spun down, and the supernatant of each was saved for BL analysis. Then, μPs were washed with 100 µL of phosphate buffer and again spun down, saving the supernatant for analysis. This rinse process was repeated 3 more times, giving a total of 5 washes to be analyzed. Tubes containing plain and azide-silk μPs were stored under refrigeration for future analysis.

2.9 Non-Specific Adsorption of Nanoluciferase and Furimazine to Silk. FZ silk μPs were prepared by soaking 2 mg plain silk μPs in 50 µL of either 0.5, 0.25, or 0.05 mM FZ solution. NLuc silk μPs were prepared by soaking 2 mg plain silk μPs in 50 µL of either 0.5, 0.1, or 0.05 µM NLuc solution.
Silk particle samples were tested in triplicates for all BL assays. Varying concentrations were used to determine optimal concentrations for stable light emission.

The enzyme and substrate dilutions were prepared as follows. FZ stock solution (NanoGlo Luciferase Assay Substrate, Promega, 5 mM), NanoGlo buffer (a proprietary buffer provided by Promega) and NLuc enzyme (838 µM) were removed from the freezer. Once at rt, the NLuc stock was diluted to 100 µM by mixing 24 µL of the stock with 176 µL of phosphate buffer (50 mM, pH 7.4), giving a total volume of 200 µL. This 100 µM NLuc solution was further diluted to 10 µM by adding 20 µL of the 100 µM stock to 180 µL of phosphate buffer giving a total volume of 200 µL. The same serial dilution was used to prepare a 1 µM NLuc stock, which was then used for the various desired concentrations. Unused 838 µM and 100 µM NLuc solutions were stored in the freezer for future use. The FZ stock was also diluted using phosphate buffer to the desired concentrations mentioned above.

Silk µPs were soaked in either FZ or NLuc solution (2 mg silk:50 µL FZ/NLuc solution) and vortexed periodically. Samples were left to soak overnight (about 20 h). The next day, samples were spun down in a tabletop centrifuge for 1 min. Supernatant was removed from the sample tubes and all samples were rinsed with 100 µL phosphate buffer, vortexed, and centrifuged. This rinse process was followed a total of 5 times for each sample. After the final rinse, FZ loaded µPs were resuspended in 50 µL phosphate buffer and NLuc loaded µPs were resuspended in 50 µL NanoGlo buffer. Bioluminescence was then ready to be evaluated by measuring relative light output (RLU) of the µPs in a plate reader.
2.10 BL Assay Parameters. BL assays were carried out using a BioTek Synergy H1 Multimode Reader. Samples to be measured were transferred to a Costar 96 black opaque plate. Full light emission was read with a normal read speed, a gain of 100, a delay of 100 msec, and a read height of 1 mm. Read time varied based on the experiment; all assays were read with an interval of 1 or 2 min, depending on the length of the read. Further specifications for each experiment type are discussed below. All silk particle assays were tested alongside standard controls of FZ/NLuc in solution. The series of dilutions varied depending on the concentrations that were being tested alongside them. Generally, the concentration of either FZ or NLuc was kept constant while the other was prepared in a series of dilutions to be used as a direct comparison to the silk µPs. Specific standards used for each experiment will be described in greater detail in the following assay parameter sections.

**BL Optimization.** Initial BL experiments involved determining optimal concentrations of FZ/NLuc in solution to use for future silk particle assays. FZ stock solution (5 mM) was serially diluted to various concentrations using phosphate buffer (50 mM, pH 7.4, no salt), and light output was measured in the presence of varying NLuc concentrations, prepared with either phosphate buffer or NanoGlo buffer. Through several trials, optimal concentrations for both FZ and NLuc were determined. Specifics on concentrations used and their effectiveness will be described in the results.

**NLuc-silk Particle Assays.** First, NLuc was adsorbed to silk µPs using the method described in section 2.9. Concentrations of 0.5, 0.1, and 0.05 µM were used, and each was tested in triplicates, soaking 6 mg of silk in 150 µL to keep the same ratio of 2 mg silk:50 µL NLuc solution. After the
NLuc silk μPs were rinsed and resuspended in NanoGlo buffer (150 μL), 50 μL of each particle suspension was transferred to 3 separate wells of a black 96-well plate. Enough of the NLuc dilutions were prepared so that a standard could be tested alongside the samples. For the standard control wells, 50 μL of each dilution was transferred to a separate well. FZ solution was prepared at a concentration of 0.1 mM by adding 12 μL of the 5 mM stock to 588 μL phosphate buffer. Upon setting up the plate reader with the settings listed above, 50 μL of the 0.1 mM FZ solution was transferred to each well containing sample or standard. Light output was measured immediately, reading every 1 min for 2 h.

FZ-silk Particle Assays. The FZ silk particle assays were performed in the same manner as the NLuc silk particle assays, again using the adsorption method described in section 2.9. Concentrations of 0.5, 0.25, and 0.05 mM FZ were used, and each was tested in triplicates. After the FZ silk μPs were rinsed and resuspended in 150 μL PBS, 50 μL of each particle suspension was transferred to 3 separate wells of a black 96-well plate. Enough of the FZ dilutions were prepared so that a standard could be tested alongside the samples. For the standard control wells, 50 μL of each dilution was transferred to a separate well. NLuc solution was prepared at a concentration of 0.005 μM by adding 30 μL of 0.1 μM NLuc (prepared via serial dilution of 100 μM stock with phosphate buffer) to 570 μL NanoGlo buffer. Upon setting up the plate reader, 50 μL of the 0.005 μM NLuc solution was transferred to each well containing sample or standard. Light output was measured immediately, reading every 1 min for 2 h.

NLuc-silk and FZ-silk Particle Assays. The next assay involved combining FZ-adsorbed silk μPs with NLuc-adsorbed silk μPs. NLuc μPs were prepared using 0.5, 0.1, and 0.05 μM NLuc solution, and
FZ µPs were prepared using only 0.25 mM FZ solution. Adsorption methods follow those previously described. Silk µPs (6 mg) were resuspended in 150 µL PBS (FZ µPs) and 150 µL NanoGlo (NLuc µPs), and 50 µL of each NLuc particle suspension was transferred to 3 separate wells of a black 96-well plate. To each of these wells, 50 µL of FZ particle suspension was added. A standard control well was also set up in the same manner as previous assays, testing all three NLuc concentrations in solution with 0.25 mM FZ in solution. Light output was measured immediately, reading every 2 min for 24 h.

**BL in the Presence of BSA and FBS.** BL of NLuc and FZ-adsorbed silk µPs were analyzed in the presence of other proteins/serums. Based on results from the NLuc/FZ-adsorbed silk µPs assays, concentrations of 0.1 µM NLuc and 0.25 mM FZ were used to prepare BL silk µPs. In two separate Eppendorf tubes, 24 mg of dry silk µPs were weighed out. In one tube, µPs were soaked in 600 µL of 0.25 mM FZ, and the other tube of µPs were soaked in 600 µL of 0.1 µM NLuc. Both substrate and enzyme solution were prepared using phosphate buffer, as described in section 2.9. Silk µPs soaked for 24 h, while vortexing periodically. The next day, samples were spun down and washed 5x as previously described. However, before the final rinse, both tube contents were separated into 4 equal volumes (150 µL, ~6 mg silk µPs). The 8 sample tubes (4 NLuc-µPs and 4 FZ-µPs) were spun down, and supernatant was replaced with 150 µL of each of the following 4 solutions: 1) 1:1 PBS and NanoGlo (normal conditions); 2) PBS alone; 3) 1% bovine serum albumin (BSA); 4) 10% fetal bovine serum (FBS). Next, 50 µL of each NLuc particle suspension was transferred to 3 separate wells of a black 96-well plate. To each of these wells, 50 µL of FZ particle suspension was added. Standard controls were prepared by diluting FZ and NLuc in the 4 conditions listed above. Light output was measured immediately, reading every 2 min for 24 h.
**Evaluation of Covalently Bound NLuc-silk Particles.** The last experiment was split into two parts which analyzed BL of NLuc-DBCO-azide silk particle supernatants and their suspensions. Silk particle preparation is described in section 2.8 for both plain- and azide-DBCO-NLuc silk µPs. Part 1 of the experiment analyzed supernatant and rinse RLU of both plain- and azide-silk samples. Due to the scale of this assay, a concentration of 0.1 mM, rather than 0.25 mM, was used for FZ solution. Briefly, this was prepared by adding 36 µL of FZ stock (5 mM) to 1764 µL of NanoGlo buffer. To an Eppendorf tube containing 990 µL of PBS (pH 7.2), 10 µL of the 10 µM NLuc-DBCO solution was added to make 0.1 µM NLuc-DBCO. The following standard control wells were prepared:

<table>
<thead>
<tr>
<th>Well</th>
<th>0.1 µM NLuc-DBCO</th>
<th>Phosphate Buffer</th>
<th>0.1 mM FZ/NanoGlo</th>
<th>Final [NLuc]</th>
<th>Final [FZ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 µL</td>
<td>0 µL</td>
<td>50 µL</td>
<td>0.05 µM</td>
<td>50 µM</td>
</tr>
<tr>
<td>2</td>
<td>30 µL</td>
<td>20 µL</td>
<td>50 µL</td>
<td>0.03 µM</td>
<td>50 µM</td>
</tr>
<tr>
<td>3</td>
<td>10 µL</td>
<td>40 µL</td>
<td>50 µL</td>
<td>0.01 µM</td>
<td>50 µM</td>
</tr>
<tr>
<td>4</td>
<td>5 µL</td>
<td>45 µL</td>
<td>50 µL</td>
<td>0.005 µM</td>
<td>50 µM</td>
</tr>
<tr>
<td>5</td>
<td>2.5 µL</td>
<td>47.5 µL</td>
<td>50 µL</td>
<td>0.002 µM</td>
<td>50 µM</td>
</tr>
</tbody>
</table>

To directly compare with supernatant/rinse samples, the same dilution procedure as the stock was used. In separate tubes, 10 µL of the supernatant/rinse samples were added to 990 µL PBS (pH 7.2) for both the plain and azide samples. Sample tubes were vortexed, and 50 µL of the diluted samples were added to 3 different wells in the plate. Then, 50 µL of the 0.1 mM FZ solution was added to each well. Light output was measured immediately, reading every 1 min for 1 h. Part 2 of this experiment analyzed BL of plain- and azide- particle suspensions. The particles from Part 1 above had all remaining buffer removed and were then resuspended in 1250
µL of PBS. The particles then soaked for 1 h, vortexing periodically – this gave the enzyme time to desorb from the silk µPs. The solution was then vortexed again and 50 µL of each suspension was quickly transferred to 3 different wells. The µPs were spun down and 50 µL of each supernatant was transferred to 3 different wells. Following this, 50 µL of the 0.1 mM FZ solution was added to each well. The control for this assay was prepared in the same way as Part 1. Light output was measured immediately, reading every 1 min for 1 h. To keep the buffer: µPs ratio the same, 150 µL of PBS (pH 7.2) was added to the silk sample tubes. These steps were repeated every ~24 h until light emission ceased.
Chapter 3. Results and Discussion

3.1 Silk Microparticle Formation. Silk µPs were formed using a salting-out procedure adapted from Lammel et al.\textsuperscript{22} where silk solution was mixed with potassium phosphate in volumetric ratios of 1:5. The process was optimized by varying the silk and phosphate concentrations, and SEM was used to check for relatively uniform size and shape.

Figure 4 (A and B) show the difference in size distribution when silk solution concentration was doubled. Based on random selection of 30 µPs, silk µPs made with 0.3% silk solution have an average diameter of 2.4 ± 1.1 µm. An average diameter for the 0.6% silk µPs was not calculated, as spherical µPs were not formed. After determining 0.3% silk solution successfully prepared silk µPs with the tested buffer parameters, buffer pH and concentration were manipulated for further optimization. First, the ratio of silk to buffer was changed to 1:10, keeping the concentration of buffer at 1.25 M, pH 7.84 (Figure 4C). Buffer properties were also manipulated. As discussed in Lammel et al., salting out efficiency should increase as pH and ionic strength increase, with maximum efficiency near 1.5 M, pH 9. Figure 4D shows silk µPs that have been prepared with 1.5 M, pH 9.3 buffer. The SEM results indicate that a 1:10 volumetric ratio gives a narrower size distribution. The downfall of this was an increase in buffer waste. The results also show that when concentration and pH of the buffer was increased, silk µPs had a smaller, more uniform size and shape. Using 1.25 M buffer with a pH ~7.8 (Figure 4C) produced silk µPs with an average diameter of 1.5 ± 0.4 µm. Using 1.5 M buffer with a pH ~9.3 (Figure 4D) produced silk µPs with a slightly smaller average diameter of 1.4 ± 0.4 µm. However, slight aggregation of particles was observed. Up to this point, the stir plate was set to ~400 rpm when silk solution was added dropwise into buffer. This time, the stir plate was set to 160 rpm, with more time in
between each dropwise addition. Figure 4E shows the results for silk µPs prepared using 0.3% silk added dropwise to 1.5 M (pH 9.5) buffer stirring at 160 rpm. Based on random selection of 30 µPs, these µPs have an average diameter of 1.5 ± 0.5 µm. Going forward, silk µPs were prepared for future modifications and BL assays using 0.3% silk solution and 1.5 M potassium phosphate buffer (pH ~9.5) using a 1:10 volumetric ratio and spin speed of 160 rpm.

Figure 4. Silk µPs made from varying conditions. (A) 0.3% silk solution and 1.25 M potassium phosphate buffer (pH 8.01); 1:5 volumetric ratio at 400 rpm. (B) 0.6% silk solution and 1.25 M potassium phosphate buffer (pH 8.01); 1:5 volumetric ratio at 400 rpm. (C) 0.3% silk solution and 1.25 M potassium phosphate buffer (pH 7.84); 1:10 volumetric ratio at 400 rpm. (D) 0.3% silk solution and 1.5 M potassium phosphate buffer (pH 9.36); 1:10 volumetric ratio at 400 rpm. (E) 0.3% silk solution and 1.5 M potassium phosphate buffer (pH 9.5); 1:10 volumetric ratio at 160 rpm.
3.2 Optimal FZ/NLuc Concentration Determination. Initial BL experiments involved determining which concentrations of FZ/NLuc produce a stable, long-lasting light output (no silk involved). First, it should be noted that Promega provides a proprietary ‘NanoGlo’ buffer to use in the NLuc/FZ BL assays. The ingredients of this buffer are unknown. However, we elected to use this NanoGlo buffer for most of the optimization experiments described here, as light output is more stable than in PBS alone. Later experiments described in section 3.3.4 compare light output with and without NanoGlo buffer. The Promega company also has recommended assay parameters for evaluating different NLuc concentrations, which always use a large excess of FZ. Using their recommended 0.1 mM FZ stock solution, NLuc concentrations in the range of 0.005-0.5 µM were found to produce stable light output for >1 h.

All published biological assays utilizing NLuc seek to evaluate the NLuc concentration; therefore, an excess of the FZ substrate is always employed. However, we also needed assay parameters to evaluate the effectiveness of encapsulating different concentrations of the FZ substrate. Therefore, BL assays were carried out using a range of FZ and NLuc concentrations. Here, it was observed that as NLuc concentration was lowered, light emission became more stable. The lowest NLuc concentration tested (0.005 µM) gave the most stable light output with a range of FZ concentrations (Figure 5, right). However, while the BL intensity scales linearly with NLuc concentration when an excess of FZ is used, varying the concentration of FZ with a fixed NLuc concentration does not scale linearly with light output. For example, 0.5 mM FZ did not produce light double the intensity of 0.25 mM FZ. In fact, multiple trials showed that 0.25 mM FZ produced light similar to that of 0.5 mM, implying a potential upper limit. Because of this, 0.25 mM FZ was used for future assays. Figure 5 shows a graph of time vs. relative light units (RLU) produced by
0.01 µM NLuc (left) 0.005 µM NLuc (right) mixed with 0.5, 0.25, 0.05, and 0.025 mM FZ in 1:1 NanoGlo/PBS. This assay was evaluated for 1 h, and light output remains steady during this time, most notably for 0.25 mM and 0.5 mM FZ.

![Graph](image)

**Figure 5.** BL measurements of 0.005 µM NLuc (left) and 0.01 µM NLuc (right) mixed with 0.5, 0.25, 0.05, and 0.025 mM FZ in solution (1:1 PBS to NanoGlo buffer).

### 3.3 Bioluminescence Assays with Silk Microparticles

BL studies with silk µPs had several goals. The first goal was to determine methods to prepare NLuc-adsorbed silk µPs and evaluate light output in the presence of FZ in solution (**Figure 6A**). The second goal was to determine methods to prepare FZ-adsorbed silk µPs and evaluate light output in the presence of NLuc in solution (**Figure 6B**). The third goal was to combine the results of goals 1 and 2 and evaluate light output of a mixture of NLuc-adsorbed silk µPs with FZ-adsorbed silk µPs (**Figure 6C**). These assays were carried out to determine if a more stable light emission is produced when both enzyme and substrate are adsorbed to silk µPs. This would aid in long-term light output for the future *in vivo*
use of BL silk μPs. The fourth goal was to covalently attach NLuc to silk μPs and compare stability and light output of these μPs to non-specifically NLuc-adsorbed μPs (Figure 6D vs A). These experiments were each performed multiple times for proof of reproducibility, and one representative data set per experiment is shown for each of the following sections. All samples were prepared so that triplicates could be tested to get average RLU values. For triplicates, 6 mg of silk μPs were soaked in 150 µL of each NLuc or FZ concentration to keep the ratio of 2 mg silk:50 µL solution. This allowed for 50 µL of particle suspensions to be transferred to a total of 3 wells to be analyzed and averaged.

Figure 6. Cartoon depiction of (A) NLuc-silk μPs BL assay, (B) FZ-silk μPs BL assay, (C) BL assay of NLuc-silk particles mixed with FZ-silk μPs, and (D) covalently bound NLuc-silk μPs mixed with FZ solution.

After these assays, a different type of BL assay was performed that evaluated the stability of BL silk μPs in the presence of other proteins/serums. For these tests, BL assays shown in Figure 6A and B were again performed, this time in PBS alone, 1% BSA, and 10% FBS, and compared to light
output of NLuc and FZ in the listed solutions without silk µPs. This allowed for a better understanding of how silk µPs would perform in a more biological setting.

3.3.1 BL of NLuc-Silk Microparticles + FZ Solution. BL studies first began with determining methods to encapsulate NLuc within silk µPs to prevent degradation and control the reaction rate of the bioluminescent process. Initially, silk µPs were soaked in 0.005 µM NLuc solution as that was determined above to give stable light output with a range of FZ concentrations. However, when adsorbed to µPs, this low concentration produced significantly lower RLU than when in solution. Because of this, 0.5, 0.1, and 0.05 µM NLuc solutions (150 µL) were used for soaking silk µPs (6 mg). After soaking overnight in NLuc solution, the silk µPs were washed extensively with phosphate buffer. This was done by replacing the supernatant with fresh phosphate buffer, vortexing, centrifuging, and repeating this process a total of 5 times. Samples were then resuspended in 150 µL NanoGlo buffer, and 50 µL of each suspension was transferred to 3 different wells of a 96-well plate. FZ solution at a concentration of 0.25 mM was initially used for these assays, but due to the high cost of FZ, tests were continued with a concentration of 0.1 mM to conserve material. To the wells containing NLuc-silk suspensions, 50 µL of 0.1 mM FZ was added and light output was immediately analyzed. RLU values of the triplicate samples were averaged to give the graph shown in Figure 7A. The light emission of the control wells containing the same three concentrations of NLuc in solution are shown in orange. For further comparison, light output at 1 h for each of the samples is plotted in Figure 7B. This experiment was repeated several times.
Silk μPs soaked in NLuc exhibit a steady light output for >2 h, similar to NLuc in solution. Notably, RLU is brighter for μPs that have been soaked in NLuc compared to the RLU of NLuc in solution at the same concentration. These encouraging results indicate that the NLuc enzyme is still active after non-covalent absorption to the silk particles. These results also imply that silk μPs may aid in stabilizing the enzyme as the emission is brighter from the particle-bound NLuc as compared to free NLuc in solution.

### 3.3.2 BL of FZ-Silk Microparticles + NLuc Solution

The next step was to see if BL stability was also observed for FZ-adsorbed silk μPs. Silk μPs (6 mg) were soaked overnight in FZ concentrations of 0.5, 0.25, and 0.05 mM FZ (150 μL). After soaking in FZ solution, the silk μPs were washed repeatedly as described above for the NLuc-silk μPs. After the final wash, samples were resuspended in 150 μL fresh PBS, and 50 μL of each suspension was transferred to 3 different wells of a 96-well plate. To each of these wells, 50 μL of 0.005 μM NLuc solution (in
NanoGlo buffer) was added, and light output was immediately analyzed. The emission intensity and stability of these FZ-silk µPs were compared to control wells containing the same concentration of FZ in solution. Figure 8A shows light emission for FZ-adsorbed silk µPs (blue) and control wells (orange) over time. A further comparison of the light output for each sample at 1 h (Figure 8B) and 2.5 h (Figure 8C) is plotted.

![Graph A](image1)

**Figure 8.** (A) BL measurements of 0.005 μM NLuc in NanoGlo and silk µPs soaked in 0.5, 0.25, and 0.05 mM FZ (blue); control wells containing solution only are shown in orange. RLU values for silk µPs are averages of triplicates. (B) Comparison of the light output for each sample at 1 h and (C) 2.5 h.
Results indicate that silk µPs slow the rate at which the reaction occurs. During the first ~20 min, there is a gradual increase in RLU for silk µPs, likely because the bound-FZ is gradually leaching out from the µPs. Alternatively, we see that RLU begins to drop immediately and at a quicker rate for FZ in solution. Notably, the light emission from the FZ- µPs is quite steady over the 2 h evaluation window. Similar to the solution optimization study discussed in Section 3.2, the light intensity does not linearly scale with FZ concentration. Here, particles soaked in 0.5 and 0.25 mM FZ gave similar light output.

As mentioned in the introduction, for current in vivo bioluminescence imaging studies, FZ must be infused into the animal at high concentrations that have been shown to be toxic. Our results are exciting in that we have demonstrated that the silk µPs can encapsulate the FZ and slow the rate of release to the NLuc enzyme, which will lower the total amount of FZ required and reduce the overall toxicity.

3.3.3 BL of FZ-Silk Microparticles + NLuc-Silk Microparticles. The successful silk-adsorption results from the previous sections led us to mix FZ- and NLuc-silk µPs for a potentially even more stable light output. For this study, silk-FZ µPs were prepared by soaking 18 mg of silk µPs overnight in 450 µL 0.25 mM FZ, as the results in Figure 8 show that higher concentrations do not result in increased light output. Silk-NLuc µPs were prepared by soaking 6 mg of silk µPs in 150 µL of 0.5, 0.1, and 0.05 µM NLuc overnight. After soaking, the samples were washed repeatedly the same way as described in the past sections. After the final wash, FZ samples were resuspended in 450 µL PBS and each NLuc sample was resuspended in 150 µL NanoGlo. To 3 separate wells of a 96-well plate, 50 µL of each NLuc particle suspension was added. To each of
these wells, 50 µL of FZ particle suspension was added and light output was immediately analyzed, monitoring for 24 h. The results are given in Figure 9.

**Figure 9.** (A) BL measurements of 0.25 mM FZ-silk µPs mixed with 0.5, 0.1, and 0.05 µM NLuc-silk µPs (blue); control wells containing solution only are shown in orange. RLU values for silk µPs are averages of triplicates. (B) Comparison of the light output for each sample at 2 h and (C) 12 h. The gradual initial increase in RLU is again observed for the silk µPs over the first 30 min, whereas the
control solutions start bright and diminish at a faster rate. When not bound to silk µPs, the enzyme and substrate react faster, creating a brighter light output. However, as time goes on, a brighter and more stable light is produced from the silk µPs.

These results are very promising for the future use of silk µPs for bioimaging. Not only can FZ- and NLuc-encapsulated silk µPs luminesce for 24+ h when mixed together, but the use of silk µPs stabilizes light emission when compared to NLuc and FZ in solution alone.

3.3.4 BL Stability in the Presence of Other Biomolecules. How RLU signal changes in the presence of other proteins or serums is important to note for the future in vivo use of BL silk µPs. As an initial test, we evaluated the stability of BL from NLuc-silk µPs and FZ-silk µPs in the presence of bovine serum albumin (BSA) and fetal bovine serum (FBS). BSA is a serum albumin protein derived from cows and is often used as a protein concentration standard. BSA is a major component of FBS, and both are common ingredients in cell cultures. Concentrations of 0.1 µM NLuc and 0.25 mM FZ were used to prepare the NLuc-silk µPs and FZ-silk µPs used in this study. The FZ µPs were prepared by soaking 24 mg silk µPs overnight in 600 µL of 0.25 mM FZ, and the NLuc µPs were prepared by soaking 24 mg silk µPs overnight in 600 µL of 0.1 µM NLuc. After soaking, samples were washed in the same way as the previous silk µP experiments. However, before the final rinse, both tube contents were separated into 4 equal volumes (150 µL, ~6 mg silk µPs). The sample tubes were spun down, washed 5x with phosphate buffer, and buffer was replaced with 150 µL of each of the following 4 solutions: 1) 1:1 PBS and NanoGlo (normal conditions); 2) PBS alone; 3) 1% BSA; 4) 10% FBS. Next, 50 µL of each NLuc particle suspension was transferred to 3 separate wells of a 96-well plate. To each of these wells, 50 µL of FZ particle suspension was
added. Standard controls were prepared by diluting FZ and NLuc solution in the 4 conditions listed above. Light output was immediately analyzed, monitoring for 24 h.

The BL results for the four tested conditions are shown in Figure 10. Normal conditions (1:1 PBS and NanoGlo) and PBS alone show similar results, but when BSA and FBS are introduced, relative light intensity for solution-only wells drastically drops to zero in less than 1 h. Silk μPs, on the other hand, maintain a stable light emission for 24+ h in both 1% BSA and 10% FBS. These are encouraging results for the future use of bioluminescent silk μPs in vivo.

Figure 10. BL of FZ-silk μPs (0.25 mM, light blue), NLuc-silk μPs (0.1 μM, dark blue), and FZ/NLuc solution only (orange, control) in the presence of 1) 1:1 PBS and NanoGlo (normal conditions, top left); 2) PBS alone (top right); 3) 1% BSA (bottom left); 4) 10% FBS (bottom right).
Figure 11. Comparison of relative light intensity of FZ-silk μPs, NLuc-silk μPs, and FZ/NLuc solution only in the presence of 1) 1:1 PBS and NanoGlo; 2) PBS alone; 3) 1% BSA; 4) 10% FBS at 2 h (top) and 12 h (bottom).

A further comparison of the light output for each sample at 2 h and 12 h is plotted in Figure 11. Here we clearly see the negative effect other proteins have on light production when silk μPs are not present. The orange columns, representing substrate and enzyme in solution, report a light intensity less than 1000 RLU after 2 h. By 12 h, solution light intensity is less than 100 RLU. Silk μPs, however, produce drastically brighter light and continue to do so for the entire 24 h.
By encapsulating NLuc and FZ into silk µPs, we can protect the BL components from interacting with biomolecules within the surrounding solution, thereby maintaining light intensity. These results are compelling in that we have demonstrated that silk µPs can play a role in stabilizing light emission of NLuc and FZ in the presence of other biomolecules. This will be a key requirement when employing these particles \textit{in vivo}.

3.4 Chemical Modifications of Silk Microparticles. After determining silk µPs successfully luminesce when soaked in NLuc/FZ and aid in stabilizing light emission, a different method was pursued for preparing BL silk µPs. The next goal of this study was to covalently attach NLuc to silk µPs and compare stability and light output of this method to the non-specific adsorption method. This involved 1) modifying silk µPs to contain an azide group, 2) modifying NLuc to contain a DBCO group, and 3) “clicking” the two pieces together and evaluating relative light intensity of the resulting NLuc-DBCO-azide silk µPs.

Tyrosine residues of plain silk µPs were first functionalized with nucleophilic amines prior to azide functionalization (Scheme 3). Diazonium coupling was performed on silk µPs, similar to the method reported by Hausken et al. which describes diazonium coupling of aqueous silk solution.\textsuperscript{11} This modification installs negatively charged sulfonic acid groups, which aid in increasing reactivity of the diazonium salt due to the electron-withdrawing nature of sulfonic acid. Likewise, the use of organic co-solvents can be avoided, as sulfanilic acid is water soluble.\textsuperscript{11} Upon reaction with the diazonium salt, the silk µPs change from white to a red-orange color. This color change is indicative of an increase in conjugation within the system, and therefore successful installation of azo bonds. Next, azo bonds were reduced using excess sodium dithionite.
under basic conditions, yielding primary amines ortho to the hydroxyl group on silk tyrosine residues.

**Scheme 2.** Diazonium coupling of silk tyrosine residue followed by reduction of the introduced azo bond yielding amine functionalized tyrosine residues.

Following amine functionalization of the tyrosine ring, further modification had to occur right away to avoid degradation as the amino-tyrosine ring is prone to oxidation. Amidation with an NHS-ester was used to install bio-orthogonal azide groups that can react selectively with alkynes, specifically, DBCO. Amtyr-silk µPs reacted with azido-dPEG₈-NHS ester for 3 h (Scheme 3). During this time, there were no visible changes to the silk µPs.

The azo- and amtyr-silk µPs were initially characterized using UV-Vis spectroscopy (Figure 12B). After each modification, 50 µL of particles suspension was reserved for analysis. These samples were first resuspended in PBS prior to UV-Vis characterization. Sample tubes were centrifuged for 1 min and all buffer was removed. To each tube, 100 µL of PBS was added and samples were vortexed to resuspend the µPs. The entire 100 µL of each suspension was transferred to a clear 96-well plate, and absorption spectra were obtained from 230-700 nm using 1 nm intervals. Since solid silk particles are being analyzed, the UV signals were broad and less distinct than previously reported for modified silks in solution. Azo-modified silk µPs have a broad absorbance near 330 nm, corresponding to the azo bond. This absorbance is greatly diminished after reduction.
However, there is still some background absorbance near 330 nm for the amtyr μPs, which aligns with the yellow color exhibited by the μPs. There is also a shift in tyrosine absorbance from 278 nm for the plain μPs to 290 nm for the amtyr μPs. This is expected, as the shift is consistent with the effect of a new amino group on the aromatic ring.

![Figure 12](image)

Figure 12. (A) Photographs of modified silk μPs, and (B) UV-Vis spectra of plain, azo-, and amtyr-silk μPs in PBS. Plain silk $\lambda_{\text{max}} = 278$ nm, azo-silk $\lambda_{\text{max}} = 330$ nm, amtyr-silk $\lambda_{\text{max}} = 290$ nm.

Modifications were further evaluated with $^1$H NMR (Figure 13). To obtain NMR spectra, we attempted to dissolve the μPs in 9.3 M LiBr-D$_2$O with varying success. The aromatic region of plain silk μPs was compared to that of modified silk μPs to determine the extent of functionalization of the tyrosine ring with a primary amine. The spectra of the μPs were further compared to previously published spectra of silk solutions modified with the same reactions.\textsuperscript{11} Obtaining clear $^1$H NMR spectra of modified silk μPs presented many challenges. We hypothesize that only the outer surface of silk μPs was being modified, and when μPs were dissolved in 9.3 M
LiBr-D$_2$O for NMR, the majority of sample was unmodified silk. Silk fibroin amino acids are 5.3 mol% (277 residues/protein) tyrosine, but some of these residues are likely buried when silk is in microparticle form, limiting the extent of reaction. In addition, after the amtyr reduction step, the particles are more challenging to dissolve so the sample may be enriched in the more soluble unmodified or azo-modified forms. This lack of modification can be seen in the aromatic region of the stacked $^1$H NMR, as proton peaks for plain silk $\mu$Ps appear to somewhat remain with the samples throughout modification. There are some visible changes to the aromatic region in the $^1$H NMR, but no distinct peaks to prove successful modification of the entire sample.

The azide-silk $\mu$Ps were also characterized using $^1$H NMR. For comparison, the $^1$H NMR spectrum of azide-silk solution is included in Figure 13, and peaks were assigned according to Hausken et al.$^{11}$ Labels A-G are used for the protons of the azide product and are displayed in Scheme 3 and Figure 13; label E is not shown in the $^1$H NMR azide particle spectrum as the solvent peak is overlapping it. Like previous modifications, obtaining a high-resolution spectrum that confirmed azide modification was challenging due to difficulty in re-dissolving the particles after modification. In addition, the spectra were taken in solutions containing LiBr, which shifts the peaks making comparisons to the spectra in D$_2$O tricky. However, there were changes observed that indicate at least partial modification occurred. Most notably, chemical shifts in the aromatic region were similar to those observed when the reactions are carried out on dissolved silk proteins, indicating at least partial modification. In addition, resonances likely corresponding to ethylene glycol appeared near 3.6 ppm. Also, a new resonance appeared around 2.8 ppm, which was assigned to the methylene protons adjacent to the amide carbonyl carbon. The methylene protons adjacent to the azide group should have a distinct signal at 3.4 ppm, and a new peak with
that chemical shift appeared. The azide-silk μPs exhibited several similarities to the azide-silk solution spectrum and were therefore used for the future reaction with NLuc-DBCO, assuming that the reaction would occur with whatever azide functional groups were present.

**Scheme 3.** NHS-ester readily reacts with the installed primary amine on the silk tyrosine residue and forms a stable amide bond. The azide product has protons labeled A-G, referenced in the $^1$H NMR in Figure 13.

![Scheme 3](image)

**Figure 13.** $^1$H NMR spectra of unmodified silk μPs (green), azo-silk μPs (dark blue), amtyr-silk μPs (maroon), and azide-silk μPs (light blue) dissolved in 9.3 M LiBr-D$_2$O. For comparison, azide silk solution is also shown (gold). Labels A-G from Scheme 3 are used here for the azide product.
3.5 **DBCO Linkage to Nanoluciferase.** Introduction of a DBCO group to the NLuc protein was necessary for future bio-orthogonal conjugation with azide-silk μPs. As shown in **Scheme 4**, the C-terminal cysteine of NLuc-C forms a disulfide with itself. Before reacting with a maleimide group, the disulfide had to first be reduced. **Figure 14A** shows the mass spectrum for NLuc-C, displaying a mass of 21208.0338 Da. Upon reaction with sulfo-DBCO-PEG₄-maleimide (825.89 Da), a mass peak at 22033.3819 Da was observed; the sum of NLuc-C and sulfo-DBCO-PEG₄-maleimide. Both spectra show trace NLuc dimers (~42,414 Da), and **Figure 14B** shows trace unreacted NLuc-C. Overall, this reaction proved to be highly selective, yielding NLuc-DBCO. With this proof, NLuc-DBCO was used for a click reaction with azide-silk μPs.

**Scheme 4.** (A) Reduction of NanoLuc disulfide to free the thiol groups which then (B) selectively react with sulfo-DBCO-PEG₄-maleimide to form NLuc-DBCO.
Figure 14. (A) NLuc-C HRMS (ESI/Q-TOF) m/z: [M + H⁺] found at 21207.7666 Da, with trace amounts of uncleaved NLuc dimer indicated by m/z: [M + H⁺] 42413.6208. (B) NLuc-DBCO HRMS (ESI/Q-TOF) m/z: [M + H⁺] found at 22033.3819 Da, with trace amounts of uncleaved NLuc dimer indicated by m/z: [M + H⁺] 42414.3246, and trace amounts of unreacted NLuc-C indicated by m/z: [M + H⁺] 21207.5379 Da.

3.6 BL of NLuc-DBCO-Azide Silk Microparticles. Stability and light output of non-specifically bound silk μPs to NLuc/FZ was then compared to silk μPs that had been covalently attached to NLuc. The goal was to see if bioluminescence was more stable over time for silk μPs with covalently bound enzyme. DBCO is a cycloalkyne that reacts with azide functional groups
(Scheme 5) via strain-promoted 1,3-dipolar cycloaddition.\textsuperscript{32} This reaction is highly specific, as azide groups react only with DBCO in the presence of -NH\textsubscript{2}, -SH, -COOH, and other protein functionalities. When these two groups come together, the result is the formation of a stable triazole. Due to the favorable reaction kinetics of DBCO and azide functional groups, it is assumed that bio-orthogonal conjugation of azide-silk µPs and DBCO reagent occurred with whatever azide functional groups were present. With this assumption in mind, light output was measured for NLuc-DBCO-azide silk µPs.

Scheme 5. Azide-functionalized silk reacting with a DBCO group covalently linked to NLuc enzyme to form a stable triazole bond between silk and enzyme.

Azide-silk µPs (2 mg) were soaked in 100 µL of 10 µM NLuc-DBCO solution for various lengths of time, depending on the experiment. As a control, this same procedure was performed with plain silk µPs also soaked in 10 µM NLuc-DBCO solution for direct comparison. After soaking, the µPs were spun down and the supernatant of each was saved for BL analysis to determine how much of the initial NLuc solution was absorbed to the particles. Then, µPs were washed with 100 µL of phosphate buffer and again spun down, saving the supernatant for analysis to see if any weakly bound NLuc was washed off. This wash process was repeated 3 more times, keeping all washes
in their own tube and saving the µPs for future analysis. For Part 1 of the experiment, RLU of supernatants and washes were analyzed to see how well the enzyme was retaining with azide µPs compared to plain µPs.

**Figure 15.** Overview of the procedure for Part 1 performed for both azide- and plain-silk µPs. Upon soaking silk µPs in NLuc-DBCO, samples are washed multiple times. Relative light intensity of the 5 washes is evaluated in 0.1 mM FZ.

As mentioned in section 2.10, control wells were prepared by first diluting the 10 µM NLuc-DBCO stock by a factor of 100. This was necessary as the 10 µM stock is too concentrated to achieve steady BL. Therefore, for a direct comparison of wash samples, the same dilution factor was used by diluting 10 µL of each wash sample into 990 µL PBS. In triplicates, 50 µL of each diluted sample was added to a 96-well plate. Finally, 50 µL of the 0.1 mM FZ solution was added to sample and control wells, and light output was evaluated.

Part 2 of this experiment was to analyze the light output of plain- and azide-silk particle suspensions to see if enzyme was still active when bound to the azide µPs. This was done using the azide and plain silk µPs that were reserved from Part 1 of the experiment. Suspensions of
these samples were transferred to a well plate to evaluate the activity of NLuc still bound to the particles. Supernatants of the particle samples were also added to the well plate to evaluate how much of the enzyme was desorbing from the µPs. As mentioned earlier, this 2-part experiment was performed several times, and the following data sets are representative of experiments of similar silk particle soak times.

The first experiment described was for µPs that were soaked in 10 µM NLuc-DBCO for 2 h. Results for Part 1 are shown in Figure 16. The supernatant of the azide-silk µPs had a lower light output than the plain-silk µPs supernatant. This was promising, as it indicates the enzyme was retained better by azide-modified µPs. Control wells of NLuc-DBCO standard dilutions were run alongside sample wells, using concentrations of 0.05, 0.03, 0.01, 0.005, and 0.002 µM. For the data shown on the right of Figure 16, RLU values of the standard dilutions at time = 30 min were used to obtain a calibration curve that relates enzyme concentration to RLU (Figure 17). Then, average RLU values for each supernatant/wash at t = 30 min was used in the linear regression to give a corresponding enzyme concentration. This concentration was compared to the maximum possible concentration of 10 µM to evaluate % NLuc-DBCO washed off. After the final phosphate buffer wash, the azide-silk µPs retained about 7.9 µM of the original 10 µM NLuc-DBCO. The plain silk µPs, however, retained only about 4.2 µM of the original 10 µM after the final wash.
Figure 16. BL of plain (blue) and azide (orange) μPs initial supernatants (left); graph of % NLuc-DBCO washed off plain (blue) and azide (orange) μPs for each wash based on RLU of standards (right).

Figure 17. Linear regression of NLuc-DBCO standard dilutions for 0.05, 0.03, 0.01, 0.005, and 0.002 μM in 0.1 mM FZ solution.

Next, light output of plain- and azide- silk particle suspensions was analyzed to evaluate enzyme activity when bound to plain or azide-silk μPs. This was done using the azide and plain silk μPs from Part 1 of this experiment. Both particle samples were soaked in 1250 μL PBS for 1 h, vortexing periodically, giving the enzyme time to desorb from the silk. Both samples were then
spun down and 50 µL of supernatant was transferred to 3 different wells. After vortexing again to suspend the µPs, 50 µL of each suspension was transferred to 3 different wells. To both suspension and supernatant wells, 50 µL of 0.1 mM FZ solution was added and light output was analyzed in 0.1 mM FZ solution (Figure 18, left). To each sample tube, 150 µL of PBS was added to maintain the silk to buffer ratio. The next day, silk particle suspensions and their corresponding supernatants were transferred to a well plate in the same manner and light output was again analyzed in 0.1 mM FZ solution (Figure 18, right).

**Figure 18.** Light output of plain- and azide-silk particle suspensions that were soaked in 10 µM NLuc-DBCO for 5 h, using 0.1 mM FZ. Suspensions on day 1 of experimentation are on the left, while suspensions on day 2 are shown on the right.

The next experiment described is for µPs that were soaked in 10 µM NLuc-DBCO for a longer time of 5 h. Results for Part 1 are shown in Figure 19. The supernatant of the azide-silk µPs had a lower light output than the plain-silk µPs supernatant, indicating the enzyme was retained slightly better by azide-modified µPs. However, the stability of light produced is the same; both plain and azide particle supernatant light outputs are decreasing at a similar rate. For the data shown on
the right of Figure 19, a calibration curve relating enzyme concentration to RLU was again used (Figure 20). Average RLU values for each wash at t = 30 min were used in the linear regression to give corresponding NLuc-DBCO concentrations. These values indicated the amount of NLuc-DBCO present in each wash. For this experiment, both plain and azide-silk µPs retained nearly equal amounts of NLuc-DBCO. Here, after the final wash, azide-silk µPs retained about 9.5 µM, whereas the plain µPs retained about 9.4 µM. From this experiment, and similar experiments, we saw that NLuc-DBCO adhered nearly equally well to both the plain and azide-silk µPs when soak time of µPs was increased.

![Particle Supernatants](image)

**Figure 19.** BL of plain (blue) and azide (orange) µPs initial supernatants (left); graph of % NLuc-DBCO washed off plain (blue) and azide (orange) µPs for each wash based on RLU of standards (right); supernatant is referred to as wash 1.
Figure 20. Linear regression of NLuc-DBCO standard dilutions for 0.05, 0.03, 0.01, 0.005, and 0.002 µM in 0.1 mM FZ solution.

Part 2 of this experiment was to analyze the light output of plain- and azide- silk particle suspensions. This was done using the azide and plain silk μPs from Part 1 of this experiment. The same procedure described for the previous experiment was followed. This time, however, light output was analyzed every 24 h for 7 d. The results are shown in Figure 19 and 20.

Figure 21. Light output of plain- and azide-silk particle suspensions that had been soaked in 10 µM NLuc-DBCO for 5 h, using 0.1 mM FZ. Suspensions on day 1 of experimentation or on the left, while suspensions on day 4 are shown on the right.
The goal of the covalent attachment study was to determine whether BL could be further stabilized using click chemistry. Here, we see there is also no visible increase in BL stability for azide-modified silk µPs. Light output observed for the plain particle suspensions is in fact slightly more stable than the azide particle suspensions based on results in Figure 21 (right), as there is a lower rate of decrease in RLU observed for plain particles. Relative light intensity comparisons in Figure 22 show azide particles suspensions initially outperforming plain particle suspensions in terms of brightness, but performance becomes equivalent by day 4. These initial results indicate that covalent attachment of the NLuc enzyme to azide-modified silk µPs is not necessary for a more stable BL light output, as adsorbed enzyme performs with equal stability.
Chapter 4. Conclusions

This study demonstrates advancement of the use of silk µPs for bioimaging. In this study, silk µPs capable of bioluminescence were prepared by encapsulating silk µPs with bioluminescent enzyme NLuc and/or the substrate FZ. Our results demonstrate that FZ-encapsulated silk µPs slow the rate of release to the NLuc enzyme. This results in a lower total amount of FZ required in comparison to the toxic levels required for current in vivo bioluminescence imaging studies. We also found that FZ- and NLuc-encapsulated silk µPs luminesce for 24+ h when mixed together, and that silk µPs stabilize light emission when compared to NLuc and FZ in solution alone.

This study also addresses how light intensity changes for BL silk µPs in the presence of other proteins. We found that by encapsulating NLuc and FZ into silk µPs, the BL components are more protected from biomolecules within the surrounding solution. The “shielding” of the silk allows the BL reaction to proceed with a stable light emission for 24+ h. These results are compelling for the future employment of BL silk µPs in vivo.

Covalent attachment of NLuc to silk µPs was also investigated. Diazonium coupling was performed on tyrosine residues of silk µPs to form azo bonds, followed by reduction using sodium dithionite to produce nucleophilic primary amines. Amines could then react with azide-functionalized NHS esters for the formation of stable amide bonds and silk µPs functionalized with azide groups. These µPs were then reacted with NLuc-DBCO to form silk µPs covalently attached to NLuc enzyme. $^1$H NMR results for the modified silk µPs did not show convicting evidence that successful modification had occurred. This made it challenging to prove that bio-orthogonal conjugation of azide-silk µPs to DBCO occurred, and one can assume that a click
reaction was occurring with the azide functional groups present. Plain- and azide-silk µPs performed equally well, however, indicating that covalent attachment of the NLuc enzyme to silk µPs is not necessary for a more efficient light output.

This study opened the door to a new, non-invasive way of tracking silk \textit{in vivo}. Here, we demonstrated the first account of utilizing bioluminescent systems as a way to image silk µPs which showed promising advancement in non-invasive imaging of silk µPs. By embedding both NLuc and FZ within silk µPs, the toxicity of the substrate was reduced, and light output of the BL system was stabilized. The impressive performance of BL-silk µPs in the presence of BSA and FBS points at a promising future of the employment of these particles \textit{in vivo}. 
References


