Project Title: Efficacy and Delivery of Novel FAST Agents for Coronaviruses

We proposed to test and develop advanced delivery for novel agents from our collaborators Facile Accelerated Specific Therapeutics (FAST) platform to reduce coronavirus replication. Sachi Bioworks Inc., Prof. Anushree Chatterjee, and Prof. Prashant Nagpal at the University of Colorado Boulder have developed a bioinformatics and synthesis pipeline to produce sequence specific theranostic agents (agents that can be therapies and/or diagnostics) that are inherently transported into the cytoplasm of mammalian host cells and sequence-specifically interfere in nucleic acid replication.

The agent comprises a small nanoparticle (2-5 nm) chosen for ideal cellular transport and/or imaging conjugated to a short, synthetic DNA analog oligomer designed for binding to one or more target viral sequences. The sequence specific binding of the FAST agent to its target prevents nucleic acid replication due to its high affinity binding. While the small nanoparticle facilitates delivery *in vitro*, we plan to package the FAST agents into a larger nanoparticle (80-300 nm) for future *in vivo* delivery applications. Our team at Sandia has expertise encapsulating biomolecules including protein, DNA, and RNA into solid lipid nanoparticles (LNP) and lipid coated mesoporous silica nanoparticles (LC-MSN) and shown successful delivery in mouse models to multiple tissues.

Our team focused on formulation parameters for FAST agents into lipid nanoparticles (LNP) and lipid coated mesoporous silica nanoparticles (LC-MSN) for enhanced delivery and/or efficacy and *in vivo* translation. We used lipid formulas that have been shown in literature to facility *in vitro* and more importantly, *in vivo* delivery. In our work discussed below, we successfully demonstrate loading and release of FAST agents on silica core and stable LC-MSN in a reasonable size range for *in vivo* testing.

Results

Our project collaborator demonstrated efficacy of the FAST agents against BSL3 SARS-CoV-2 *in vitro* early in our project which motivated our focus on the encapsulation portion of the project. Their successful inhibition of SARS-CoV-2 *in vitro* demonstrates the importance and potential impact of the nanoparticle formulations from this work. NL-63, the proposed BSL2 coronavirus for testing in this project, proved difficult to culture and we were not able to conduct viral inhibition studies as part of this project.

LNP formulation was attempted with an ionizable lipid (DLin-MC3-DMA). LNP formulation depends on electrostatic interactions between cargo and lipids. We were able to synthesize a stable, 80-90 nm LNP using microfluidic mixing however upon testing the encapsulation efficiency determined that the resultant LNP did not contained significant FAST agent. This can be explained by the different electrostatic properties between mRNA and FAST agents. Given this result, our collaborator synthesized a negatively charged FAST agent that was intended to more closely mimic the electrostatic interactions that mRNA has with the lipids during formation. Unfortunately, this did not produce better results and we were not able to synthesize a LNP with a high degree of encapsulated FAST agent.

To formulate LC-MSN, we first tested a range of loading ratios for FAST agents onto both bare (negatively charged) and amine (positively charged) cores (Figure 1). The amine cores showed strong

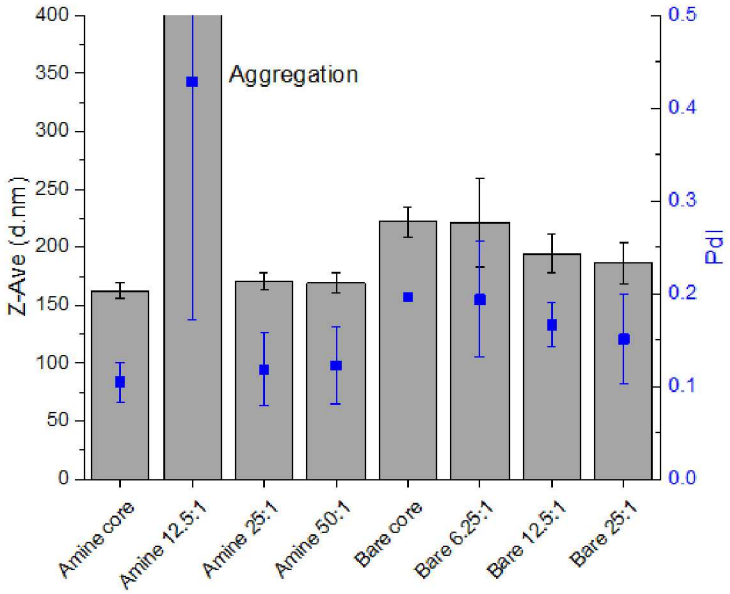

Figure 1. Silica core loading with FAST agents at various mass ratios. Z-ave is the hydrodynamic radius and PdI is the polydispersity index determined by DLS.

aggregation at loading ratios higher than 25:1 whereas bare cores did not show a strong change in physical characteristics at any loading ratio. The aggregation seen at 12.5:1 with the amine core indicated to us that we were likely saturating the surface and causing colloidal instability.

We next took these samples and pelleted the loaded cores with centrifugation to test the supernatant for remaining unloaded FAST agent. As controls, we also used FAST agent and core only controls and removed any background signal which was minimal. As shown in Figure 2, the amine cores successfully loaded the highest fraction of FAST agent at the 12.5:1 mass ratio. This curve indicated that the FASTmers are loading in a concentration dependent manner and that the surface is likely saturated at the 6.25:1 loading ratio. Interestingly, the raw signal of the 50:1, 25:1, and 12.5:1 samples were similar leading us to believe there were unique surface interactions with the FAST agents. We additionally assayed release from washed, loaded amine silica cores by adding a reducing agent (β-mercaptoethanol) and demonstrated reduction dependent FAST agent release from the core (data not shown).The bare silica cores showed similar loading at the highest mass ratios tested where the 6.25:1 ratio loaded ~80% of the added FAST agent and the 12.5:1 ratio loaded ~75% of added FAST agent. For lipid coating experiments, we proceeded with the highest loaded, stable silica core for bare and amine which corresponded to bare cores

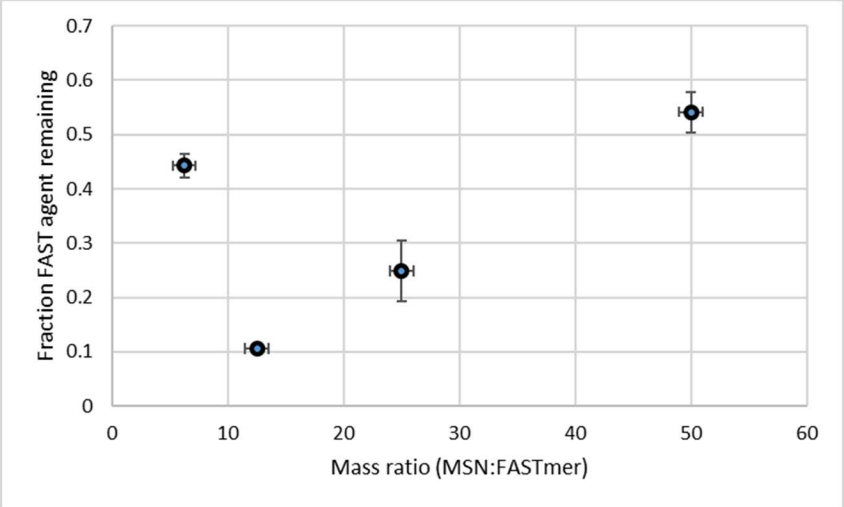

Figure 2. Fraction of FAST agent unloaded at varied mass loading ratios. Fraction is expressed as the amount remaining in the supernatant compared to amount added to loading incubation period.

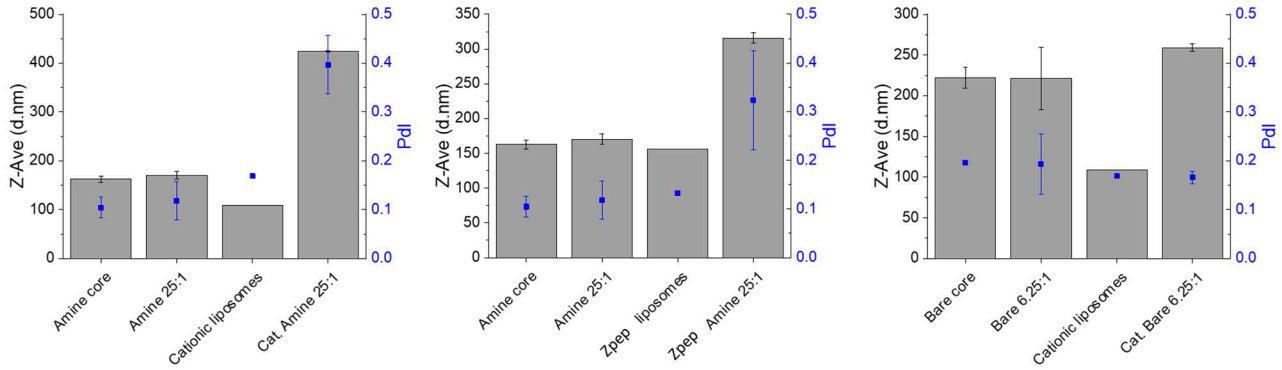

Figure 3. DLS data for three leading LC-MSN formulations of FASTmers. Amine cores loaded at the 25:1 ratio were successfully coated with the cationic DCD3330 liposomes (left panel) and the zwitterionic Zpep liposomes (middle panel). The bare cores loaded with 6:25:1 ratio were successfully coated with the cationic DCD3330 liposomes (right panel).

with 6.25:1 loading and amine cores with 25:1 loading.

We tested three liposome formulations for lipid coating of the FAST agent loaded silica. We tried one cationic lipid formula (DCD3330) and two zwitterionic formulations (Zpep and E100C) (see Methods). The three liposomes were stable and 100-150 nm in size after mechanical extrusion/sizing. The liposomes were added to loaded cores at 1:1 gram of silica core to µL of liposomes and sonicated to coat. The resultant LC-MSN were pelleted by centrifugation and resuspended in PBS. It is important to note that uncoated silica cores are *unstable* in PBS and the production of particles that are stable in PBS was used as an indicator of lipid coating.

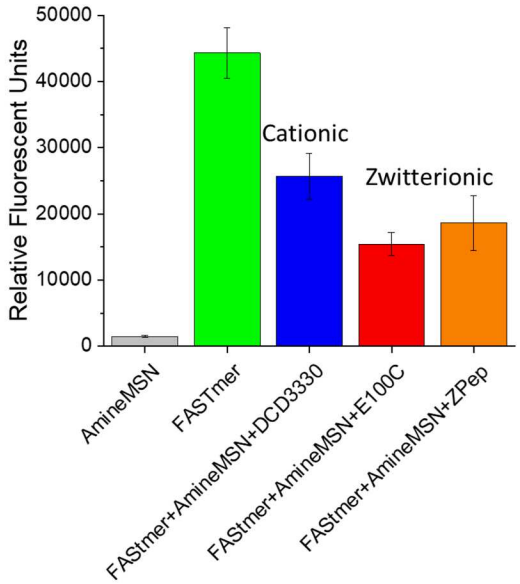

Figure 4. FASTmer release from amine core LC-MSN after lysis of lipid coating by heat. This study was performed with 25:1 core loading.

As shown in Figure 3, there were three formulations that successfully produced loaded LC-MSN that were stable in PBS. The amine cores were stable once coated with DCD3330 and Zpep. The DCD3330 liposome coating produced a LC-MSN that was slightly above what we would normally consider in a good size range but have included it here since parameter optimization could reasonably bring the size down. The bare cores with DCD3330 produced a stable LC-MSN around 250 nm which is in the range ideal for *in vivo* applications.

We further characterized these formulations by heating the LC-MSN to lyse the lipid and release the FAST agent from the silica core. Unfortunately, this heat crashed the bare silica core from the LC-MSN out of solution but we were able to perform this assay for the amine cores as shown in Figure 4. We measured significant FAST agent RiboGreen signal in the

supernatant of the lysed LC-MSN. Since these LC-MSN have been pelleted by centrifugation and washed multiple times during formulation, this signal is due to encapsulated FAST agent being released from the LC-MSN.

At the conclusion of the project, we successfully demonstrated three LC-MSN formulations of FAST agents which are ready for *in vitro* and *in vivo* translation. These final formulas were amine cores loaded with 25:1 mass ratio FAST agent and either DCD3330 or Zpep and bare cores loaded with 6.25:1 mas ratio FAST agent and DCD3330. The FAST platform is agile and readily adaptable to emerging threats like COVID-19 and others. The chemical properties of the FAST agent do not change with target allowing for a successful LC-MSN formulation to applied to a range of potential current and future applications. Immediately, these LC-MSN formulations are ready for testing in our collaborators BSL3 model of SARS-CoV-2 infection for viral inhibition.

Methods

Lipid Coated-Mesoporous Silica Nanoparticle (LC-MSN) Formulation. LC-MSN components were synthesized and LC-MSN's were formulated as outlined in Noureddine et al. 2020. For FASTmer loading, silica was incubated in water with FASTmers for 20 min at room temperature at a range of mass ratios of silica to FASTmer (6.25:1, 12.5:1, 25:1, and 50:1). Lipid formulas were as follows where all percentages are mole percent: DCD3330 (33% DOTAP, 30% Cholesterol, 33% DOPE, 4% DSPE-PEG(2000)), Zpep (77.5% DSPC, 20% cholesterol, 2.5% DSPE-PEG2000), and E100C (62% DOPE, 35% cholesterol, 3% DSPE-PEG2000).

Lipid Nanoparticle (LNP) Formulation. LNPs were synthesized using the NanoAssemblr bench top with DLin-MC3-DMA as the main lipid following formula in Aretea et al. 2018. Given the FASTmer properties that differ from mRNA, agents were suspended in water for formulation.

Nanoparticle Characterization. Immediately after synthesis, nanoparticles were characterized using dynamic light scattering (DLS) on a Malvern Zetasizer to determine the hydrodynamic radius and polydispersity index.

FASTmer Encapsulation. After FASTmer loading onto silica cores, samples were pelleted using centrifugation and the supernatant was assayed by RiboGreen for FASTmer quantification. We used UV-Vis extinction coefficients for FASTmers to generate RiboGreen calibration curves that were correlated to FAST agent concentration. RiboGreen was used according to manufacturer's instructions (with the exception that water was used in place of TE buffer) and signal was assayed on a Tecan microplate reader at 485/535 nm.

FASTmer Release. For release studies, LC-MSN were heated to 96C for 10 min to lyse lipid coat. The samples were then pelleted to remote silica and the supernatant was assayed for FASTmer quantification using RiboGreen method mentioned in FASTmer Encapsulation.