Optimization of silk fibroin nanoparticles for encapsulation of piscine hemoglobin: A novel oxygen delivery system

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Statement of Purpose: Many conditions and injuries such as diabetes, anemia, stroke, traumatic injury, and cancer lead to the local or systemic lack of oxygen (O_2) within tissues [1,2]. Blood donations and human-derived blood products are common treatments for low O_2 loss due to trauma, however they are in limited supply, require extensive pathogen screening, and require cold-chain storage. Because of these limitations, many options including recombinant hemoglobin, bovine hemoglobin and synthetic perfluorocarbons have been investigated for their potential use as O_2 carriers or red blood cell replacements [3]. However, issues with toxicity, dose efficiency, and clearance rate have been major barriers for clinical success. Thus, there is still a need for a temperature stable, injectable, and pathogen-free solution to aid with tissue oxygenation over more than 5 days. The hemoglobin from teleost fish (salmon, tuna, etc.) is unique due to the root effect. The root effect is a phenomenon in which an acidic pH decreases the affinity of hemoglobin for O_2, leading to efficient delivery of hemoglobin bound O_2 to the hypoxic tissues during systemic circulation. Fish, such as salmon, leverage this phenomenon to regulate their buoyancy in the water, as well as the oxygenation of their tissues. We aim to leverage this evolved advantage to deliver O_2 efficiently to hypoxic regions during circulation in humans. To improve both shelf life and dose efficiency, the hemoglobin proteins can be stabilized in a biopolymer matrix. Silk fibroin, referred to as silk, is a protein isolated from *Bombyx mori* cocoons that is well established as a biocompatible and inert natural polymer that can be processed into multiple biomaterial formats, including nano/microparticles and degraded into simple amino acids [4, 5]. We have established a design space to control particle size and analyzed hemoglobin incorporation. We simulate O_2 release via a COMSOL® reaction-diffusion model to inform *in vitro* experiment design.

Methods: Silk particles were formed via phase separation with polyvinyl alcohol (PVA) as described previously [4]. The phase separation is induced via probe sonication. To test for shifts in particle size, we varied the sonication amplitude (0, 8, 12, 16, 25, 40%), silk concentration (20, 50, 70 mg/mL), silk to PVA ratio (1:1, 1:3, 1:4, 1:5), and silk degumming time (30, 60, 90 min, inversely proportional to silk molecular weight). Samples were assessed for particle size, stability, and morphology using light microscopy, dynamic light scattering (DLS), and scanning electron microscopy (SEM). Bovine hemoglobin was incorporated into the particles at concentrations of (0.25, 0.5, 1.0. and 1.5 mg/mL) by reconstituting substrate powder hemoglobin in ultrapure water and combining with the silk solution prior to sonication. Immunofluorescent imaging was performed to confirm colocalization of the hemoglobin in the silk particles.

Results: We found that all sonication amplitudes are statistically different from no sonication (***p<0.001), silk concentration is negatively correlated to particle size, and silk degumming time is positively correlated to size (Figure 1A-C). Silk to PVA ratio was not observed to have a critical effect on particle size, necessitating on-going investigation into the thermodynamic interactions of the polymers participating in the phase separation. Moderate levels of polydispersity were observed across all samples, prompting investigation into strategies to narrow polydispersity. While altering the sonication probe and probe to sample volume ratios can impact polydispersity, we are also investigating post-processing strategies, like filtration. Bovine hemoglobin was observed to be incorporated in particles (Figure 1D). No significant changes in particle size were observed as a function of the hemoglobin inclusion. These results inform the control of particle size and level of hemoglobin incorporation, which will be used to develop a 2D Reaction-Diffusion COMSOL® model to predict the O_2 dynamics of the system. Ongoing work aims to evaluate O_2 delivery profiles using an *in vitro* perfusion flow system equipped with Presens® O_2 sensors.

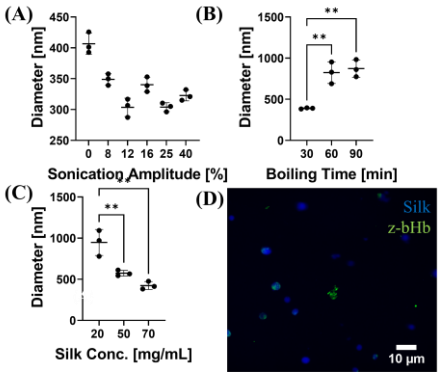

Figure 1. Particle size from DLS **(A)** Particles made at differing sonication amplitudes while holding silk concentration (5%), silk:PVA ratio (1:4), and degumming time (60 min) constant. **(B)** Particles made at different silk concentrations while holding sonication amplitude (25%), silk:PVA ratio (1:4), and degumming time (60 min) constant. **(C)** Particles made using different degumming times while holding sonication amplitude (25%), silk concentration (5%), silk:PVA ratio (1:1) constant. **(D)** Silk particles (blue) loaded with 1.5 mg/mL bovine hemoglobin were stained for the zeta subunit of bovine hemoglobin (green). All data expressed as mean ± 1SD (n=3). Analyzed with 1-Way ANOVA with Tukey's test post-hoc analysis. Statistical significance is reported as *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

References: 1. Boedtkjer E et al. Annu Rev Physiol. 2020;82:103-26. **2**. Catrina SB et al. Diabetologia. 2021;64(4):709-16. **3**.Rockwood DN et al. Nat Protoc. 2011;6(10):1612-31. **4**. Sen Gupta A. Shock. 2019;52(1S Suppl 1):70-83. **5**. Jameson JF, Pacheco MO et al. Front Bioeng Biotechnol (2021).