Final Technical Report

Period: March 1, 2017- February 28, 2019

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Grant Number: DE-FG02-03ER46099

Goals and Objectives: This focus of this grant is to study the interrelationships between proteins that form the high performance bioceramic nacre layer of the mollusc shell. In particular during this grant period we investigated (1) the interactivity between two framework nacre proteins, n16.3 and Pif97 of the Japanese pearl oyster, Pinctada fucata, and (2) the incorporation and subsequent material property modifications that accompany the intracrystalline incorporation of two nacre proteins, AP7 (H. rufescens) and n16.3 (P. fucata).

1 Intracrystalline incorporation of nacre protein hydrogels modifies the mechanical properties of calcite crystals: A microcompression study.

The fracture toughness of mollusk shell nacre has been attributed to many factors, one of which is the intracrystalline incorporation of nacre-specific proteins. Although mechanical force measurements have been made on the nacre layer and on individual calcium carbonate crystals containing occluded organic molecules and macromolecules, there are few if any studies which examine the impact of occluded proteins on the mechanical properties of calcium carbonate crystals. To remedy this, we performed microcompression studies of calcite crystals grown in the presence and absence of two recombinant nacre proteins, r-AP7 (H. rufescens, intracrystalline proteome) and r-n16.3 (P. fucata, framework proteome),

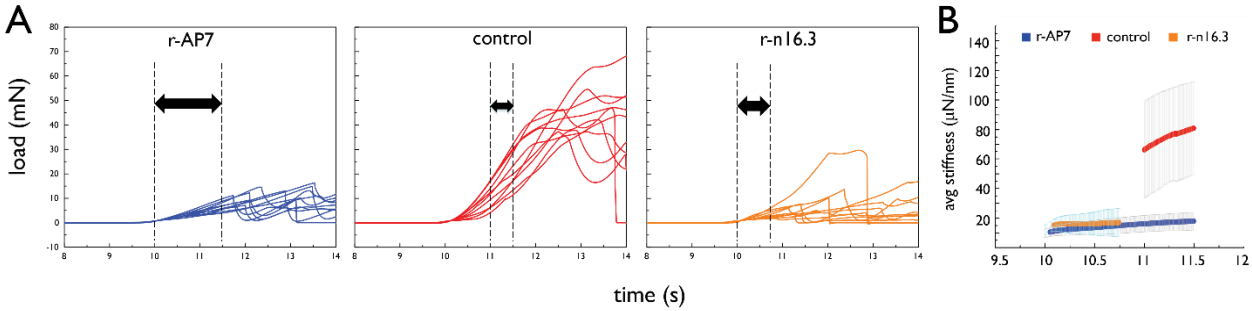

Figure 1. (A) Microcompression stress-strain curves obtained for control, r-AP7, and r-n16.3 calcite crystals (n = 10). (B) Histogram plots of the < average > critical stress and critical strain values (± S.D.) that cause crystal failure (n = 10).

both of which are known aggregators that form hydrogel nanoinclusions within *in vitro* calcite. We find that, relative to protein-free calcite, the intracrystalline inclusion of either r-AP7 or r-n16.3 nacre protein hydrogels within the calcite crystals leads to a reduction in strength. However, nacre protein – modified crystals were found to exhibit elastic deformation under force compared to control scenarios, with no discernable differences noted between intracrystalline or framework protein-modified crystals (Figure 1). We conclude from our in vitro microcompression studies that the intracrystalline incorporation of nacre proteins can contribute to fracture-resistance of the crystalline phase by significantly reducing both modulus AND critical strength.

2 Selective synergism created by interactive nacre framework-associated proteins possessing EGF and vWA motifs: Implications for mollusk shell formation.

In the nacre layer of the Pinctada fucata oyster shell there exists a multi-member proteome, known as the framework family, which regulates the formation of the aragonite mesoscale tablets and participates in the creation of an organic coating around each tablet. Several approaches have been developed to understand protein-associated mechanisms of nacre formation, yet we still lack insight into how protein ensembles or proteomes manage nucleation and crystal growth. To provide additional insights we have created a proportionally-defined combinatorial model consisting of two recombinant framework proteins, r-Pif97 (containing a von Willebrand Factor Type A domain) and r-n16.3 (containing an EGF-like domain) whose individual *in vitro* mineralization functionalities are distinct from one another. We find that at 1:1 molar ratios r-Pif97 and r-n16.3 exhibit little or no synergistic activity regarding modifying existing calcite crystals. However, during the early stages of nucleation in solution, we note synergistic effects on nucleation kinetics and ACC formation/stability (via dehydration) that are not observed for the individual proteins. This selective synergism is generated by Ca^{2+} - mediated protein – protein interactions (~ 4 molecules of r-n16.3 per 1 molecule of r-Pif97) which lead to the formation of nucleation-responsive hybrid hydrogel particles in solution (Figure 2). Interestingly, in the absence of Ca^{2+} there are no significant interactions occurring between the two proteins. This unique behavior of the framework-associated n16.3 and Pif97 proteins suggests that the Asp, Glu-containing regions of the vWA and EGF-like domains may play a role in both nacre matrix formation and mineralization.

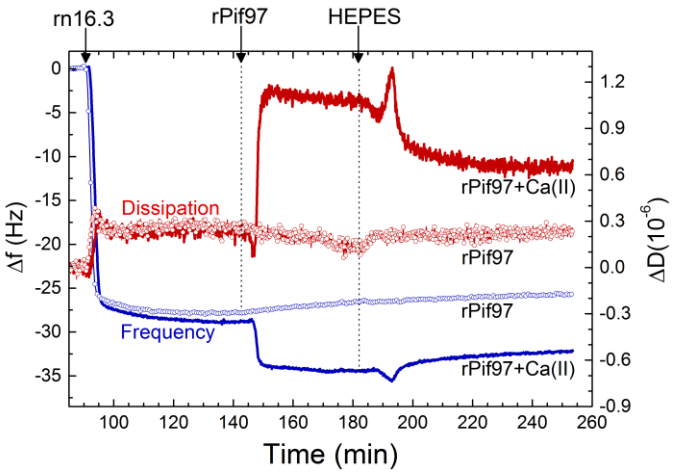

Figure 2. QCM-D experiments of immobilized r-n16.3 (2.5 µM in HEPES) exposed to 2.5 µM r-Pif97 under two different conditions: r-Pif97 in 10 mM HEPES (pH 8.0) or r-Pif97 in 10 mM HEPES / 10 mM $CaCl_2$ (pH 8.0). Plots show the fifth harmonic frequency (F5, blue) and dissipation (D5, red) observed under each scenario. The time-dependent introduction of r-Pif97 alone (circles) or r-Pif97+Ca^{2+} (lines) and HEPES washing solutions is noted on the plots by arrows and extended dashed lines. These experiments were duplicated (see Figure S3) and found to be reproducible.

Grant activities: The PI and post-docs funded by this grant presented their work at several scientific meetings during this period (MRS, GRC).

Publications:

Risan, J., Jain, G., Pendola, M., Evans, J.S (2018) Intracrystalline incorporation of nacre protein hydrogels modifies the mechanical properties of calcite crystals: A microcompression study. *Journal of Materials Chemistry B* **6**, 4191 – 4196.

Jain, G., Pendola, M., Huang, Y.C., Gebauer, D., Koutsoumpeli, E., Johnson, S., Evans, J.S. (2018) Selective synergism created by interactive nacre framework-associated proteins possessing EGF and vWA motifs. Implications for mollusk shell formation. *Biochemistry* **57**, 2657-2666.