

## Real-Time Observation of Ferritin Biomineralization Using Graphene Liquid Cells Electron Microscopy

Surya Narayanan<sup>1</sup>, Reza Shahbazian-Yassar<sup>2</sup> and Tolou Shokuhfar<sup>1\*</sup>

<sup>1</sup> University of Illinois at Chicago, Dept. of Bioengineering, Chicago IL, USA.

<sup>2</sup> University of Illinois at Chicago, Dept. of Industrial and Mechanical Engineering, Chicago IL, USA.

\*Corresponding author: snaray8@uic.edu

Multiple studies have speculated that iron accumulation in the brain can accelerate many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and epilepsy [1]. Excessive iron ions in the cellular cytosol are regulated by ferritin in a physiological condition, an iron storage protein. Ferritin is composed of an inner iron core and an outer protein shell that consists of heavy (H) chain and light (L) chain amino acid subunits. While H subunits help oxidize iron from Fe<sup>2+</sup> to Fe<sup>3+</sup> through a series of chemical reactions, L subunits are responsible for iron nucleation and mineral formation (Figure 1A). Regardless of these mechanisms, genetic factors such as mutations in L-subunits are known to affect the protein structure as well as influence the type of iron oxide minerals formed, especially in neuroferritinopathy [1]. Despite abundant knowledge obtained through convention spectroscopy and electron microscopy techniques, the causes of iron accumulation are not well understood. Further, iron nucleation pathways in physiological and pathological ferritins are yet to be discovered. Here, graphene liquid cells (GLC-TEM) are used to elucidate the biomineralization iron within ferritin in real-time and at the nanoscale (Figure 1B). Graphene's unique capability to encapsulate biological materials in liquid state, while providing high spatial resolution allows imaging of the 5nm to 7nm iron core in ferritin [2]. Further the ability to study the oxidation state of the iron core via electron energy loss spectroscopy (EELS) while protecting the protein's integrity allows investigation of the real-time assembly of iron minerals in ferritin [2]. With increasing number of iron ions over time, the iron oxides phases formed during different stages of biomineralization could be different. Likewise, observation of iron oxidation states at different stages can reflect the reaction kinetics of the proteins to oxidize iron.

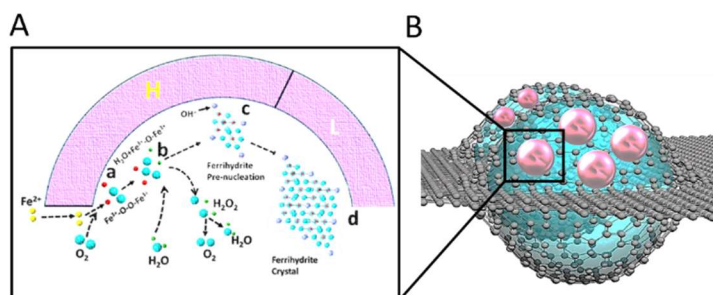
Here, biomineralization of iron in ferritin was first performed through *ex-situ* biochemical experiments. The commercially available known concentration of equine spleen apoferritin (protein without iron) was mixed with ferrous ammonium sulfate solution (iron source) and 3-(N-morpholino) propane sulfonic acid (MOPS) buffer [3]. The change in absorbance (310nm) as a result of iron internalization was measured via UV visible Spectroscopy. Further, 2μl of the solution mixture (apoferritin, iron solution, and MOPS buffer) with the same concentration was encapsulated in GLC to observe the process via *in situ* electron microscopy. The formation of iron core in the protein was monitored in real-time. The iron signal (Fe L<sub>23</sub> edge) from EELS, and the change in the contrast of the protein in the high angular annular dark field-scanning transmission electron microscopy (HAADF-STEM) image confirmed the iron biomineralization in ferritin.

The presence of liquid around ferritin, and the intactness of graphene during biomineralization was verified through the plasmon region of the low loss EELS. The three EELS peaks at 5eV, 9eV, and 33eV indicated the  $\pi \rightarrow \pi^*$  transition of the graphene layer, water exciton, and  $\pi + \sigma$  plasmon of graphene respectively [4]. Further, the iron L<sub>23</sub> edge obtained during different stages of biomineralization was analyzed with respect to electron energy near edge structure (ELNES) to understand the mechanism of nucleation of iron oxides (Figure 2). The ELNES of the iron edge obtained from ferritin was compared with the known iron oxide standards. It was observed that the iron oxide core formed after one hour of biomineralization in ferritin is

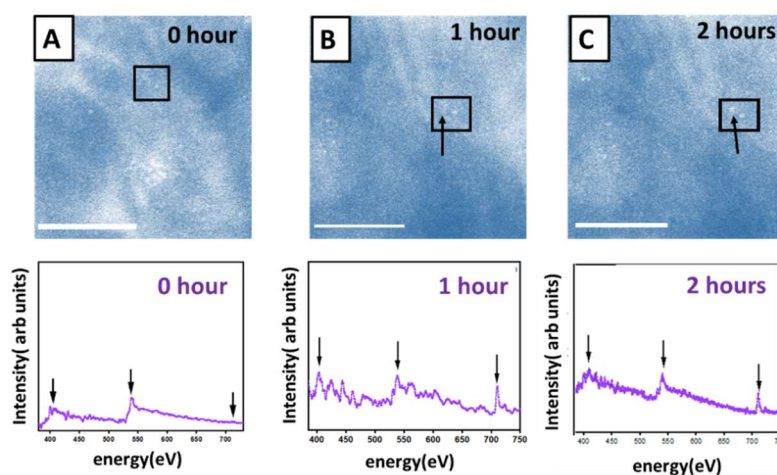
comparable with ferric oxyhydroxide ( $\text{FeO}(\text{OH})$ ). However, with time, the near edge structure of the ferritin's iron oxide core resembled both magnetite ( $\text{Fe}_3\text{O}_4$ ), and ferrihydrite ( $\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$ ) structure. The presence of magnetite also indicates the slow conversion of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  form during the stages of biomineralization. The knowledge gained from this study can be applied to understand the biomineralization process in different living organisms. Future studies will focus on comparison of iron biomineralization in physiological and pathological ferritin [5].

#### References:

- [1] Y Ke and ZM Qian, *The Lancet Neurology* **2** (2003), p. 246.  
 [2] C Wang et al., *Advanced Materials* **26** (2014), p. 3410.  
 [3] CL Snow, *Journal of Inorganic Biochemistry* **105** (2011), p. 972.  
 [4] T Eberlein et al., *Condens Matter Mater. Physics* **77** (2008).  
 [5] The authors acknowledge funding from the National Science Foundation- CAREER award- Grant No- DMR- 1564950. Dr Tolou Shokuhfar is acknowledged for her many useful discussions and contribution to this work. This work made use of instruments in the Electron Microscopy Service (Research Resources Center, UIC)



**Figure 1.** A schematic of biomineralization process in GLC-TEM. **A.** Shows the step-wise mechanism of iron core formation inside ferritin. **B.** GLC encapsulating ferritin proteins in the liquid pockets. GLC-TEM is utilized to visualize the iron biomineralization in ferritin.



**Figure 2.** GLC-TEM indicates the different stages of *in situ* biomineralization in ferritin acquired from same area. The indication of iron signal (via EELS at 702eV) and the contrast change (indicated by black arrow) in the protein observed at 1<sup>st</sup> and 2<sup>nd</sup> hour after the onset of biomineralization (via HAADF-STEM- Figure B and C) confirms the process. The white contrast in the images indicate the presence of liquid. The images are artificially colored to enhance the contrast.