Supporting Information

The Enzyme-mimic Activity of Ferric Nano-Core Residing in Ferritin and Its Biosensing Applications

Zhiwen Tang, Hong Wu, Youyu Zhang, Zhaohui Li, Yuehe Lin*

Pacific Northwest National Laboratory, Richland, WA 99352 (USA)

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich. Deionized water produced by the E-Pure deionized water system (Barnstead, USA) was used to prepare all samples. Human ceruloplasmin was purchased from R&D Systems. Anti-human ceruloplasmin antibody and biotinylated anti-nitrotyrosine antibody were produced by Abcam.

Instruments

A Safire 2 microplate reader (TECAN, Switzerland) was used to detect fluorescence, absorbance, and luminescence signals of samples. Cary 300 ultraviolet-visible (UV-vis) spectroscopy (Varian, USA) was used to detect the absorbance of samples. A Sony a-300 digital camera was used to take sample images. A Vortemp 56 thermal incubator (LabNet, USA) was used to incubate samples at a desired temperature and speed. A 5417C centrifuge (Eppendorf, Germany) was used to spin and separate samples. An orbital shaker (VWR International LLC., USA) was used to shake the plate and mix the samples during the washing step. An IQ 125 miniLab pH meter (IQ Scientific Instruments, USA) was used to determine the pH of solutions. Transmission electron microscopy (TEM) imaging and high-resolution TEM analysis were

carried out on a Jeol JEM 2010 microscope (JEOL Ltd., Japen) with a specified point-to-point resolution of 0.194 nm.

Ferritin Samples Preparation

Ferritin and apoferritin were purchased from Sigma-Aldrich. The heat-denatured ferritin sample was prepared by incubating 12.5 mg/mL ferritin at 95^{o}C in a water bath for 15 minutes. The heated denatured ferritin formed floccule after it was denatured, so it needed a supersound treatment before sampling. To obtain a ferritin core, 100 μL 25 mg/mL ferritin was mixed with 800 μL 8.0 M urea for 8 hours to completely denature the protein cage and deconstruct the nano cage to release the ferric core. The denatured sample was then centrifuged at 16,000 rpm for 45 minutes to precipitate the ferric nano-crystals, after which the supernatant was discarded. After that, 500 μL 8.0 M urea was add into the centrifuge tube followed by a vortex and supersound treatment to homogenize the sample. The sample was spun again in succession to discard the residual protein content in the supernatant. Finally, centrifuging was carried out using 500 μL phosphate buffered saline (PBS) to remove urea. The ferric core aggregates were then well dispersed in dH_2O using a supersound treatment. The ferric core was quantified by comparing its absorbance at 400 nm with the ferritin solution.

Substrates Preparation and General Assay Protocols

The tetramethylbenzidine (TMB) substrate buffer obtained from Sigma-Aldrich was used for the HRP activity assay. For the ferritin activity assay, the TMB substrate used was adapted from commercial TMB substrate (Sigma-Aldrich) by adding 0.03 mg/mL TMB and 500 mM H_2O_2. Each sample was incubated with 100 μL TMB substrate at 50^{o}C for 15 minutes before taking the image. The TMB buffer was also used in experiments testing the thermal stability and pH toleration of ferritin and HRP. The reaction was stopped by adding 20% volume of 0.5 M H_2SO_4 before measuring the absorbance at 452 nm using TECAN Safire 2.

The dihydropyrimidine dehydrogenase (DPD) substrate contains 25 mM DPD, 150 mM H_2O_2, and 50 mM NH_4OAc. The pH of the solution is adjusted to pH3.2 by adding a certain amount of HCl. Each sample was incubated with 100 μL DPD substrate at 50^{o}C for 15 minutes before taking the image or obtaining the absorbance at 610 nm using the TECAN Safire 2.

In the experiment to determine the detect limit and linear response range of ferritin using DPD, 3 mM ascorbic acid was added into the DPD substrate to decrease the background and improve the sensitivity. The final concentrations of ferritin in the samples ranged from 1.0 to 500 ng/mL. After incubating at 50^{o}C for 15 minutes, the samples were sent to get the absorbance at 610 nm using TECAN Safire 2.

The *o*-phenylenediamine dihydrochloride (OPD) substrate is composed of 30 mM OPD, 200 mM H_2O_2, 0.2 M Na_2HPO_4, and 0.1 M citric acid. Each sample was incubated with a 100 μL OPD substrate under 50°C for 15 minutes before being sent to take the image.

The *p*-hydroxyphenylpropionic acid (*p*-HPPA) catalysis substrate was prepared in 0.1 M HCl solution with 1 mM *p*-HPPA and 100 mM H_2O_2. The fluorescence assay buffer contains 0.4 M ammonia and 0.4M NH_4OAc. The ferritin samples were mixed into a 50 μL catalysis substrate and incubated at 50°C for 15 minutes to allow the catalysis reaction take place. The catalysis reactions were then stopped by adding a 10 μL fluorescence assay buffer and 50 μL dH_2O. After that, all the samples were sent to TECAN Safair 2 to obtain their fluorescence spectra with excitation at 324 nm.

Luminol was used for a chemiluminescence assay of ferritin. The luminol substrate contains 6.5 mM luminol, 400 mM H_2O_2, and 0.05 mM $NaHCO_3$-Na_2CO_3. The ferritin samples were mixed with a 100 μL luminol substrate and incubated at room temperature for 5 minutes before using the TECAN Safair 2 to count the luminescence intensity of the samples.

Negative Staining TEM and TEM

All ferritin and apoferritin samples were diluted to the final concentration of 30 μM using deionized water. Ten microliters of each sample were transferred onto the surface of the grid and placed for 2 minutes to allow the deposit of ferritin or apoferritin. The water was then removed via the absorbance of a piece of tissue to facilitate drying the sample. For a negative stain, a NanoW (Nanoprobes) solution was applied on a grid with material suspension. The operating voltage on the TEM microscope was 200 keV. All images were digitally recorded with a slow-scan charged-coupled device camera (image size 1024 × 1024 pixels), and image processing was carried out using a digital micrograph (Gatan).

Investigation of Thermal and pH Stability

To investigate the thermal stability of ferritin and HRP activities, 5.0 mg/mL ferritin and 10 μg/mL HRP samples prepared in PBS buffer and were incubated at 4°C, 20°C, 35°C, 50°C, 65°C, 80°C and 90°C using a Labnet Vortemp 56 thermal incubator for 40 minutes. Then ferritin samples were added into the TMB buffer at the concentration of 50 μg/mL and incubated at 50°C for 15 minutes. The treated HRP samples were introduced into the TMB buffer at the final concentration of 10 ng/mL and mixed well to allow the reaction for 2 minutes. All samples were added with 20 μL 0.5 M H_2SO_4 to stop catalytic reactions before obtaining their absorbance at 452 nm.

The pH tolerance of ferritin and HRP activities was explored by incubating them within a series of buffers of various pH values, including pH 2, pH 4, pH 6, pH 7.5, pH 8, pH 10, and pH 11. The buffers with a pH value lower than 7 were prepared based on a 50 mM NH_4OAc solution, while high pH buffers were prepared using 50 mM Na_2CO_3. The pH of all solutions was adjusted to desired values with 0.15 M HCl and 0.1 M NaOH. In particular, a PBS buffer was used as the pH 7.5 buffer in this experiment. Samples of 5.0 mg/mL ferritin and 10 μg/mL HRP were prepared with these pH buffers and placed at room temperature for 1 hour before testing their activity using the aforementioned protocol.

Biotinylation of Ferritin

Ferritin was biotinylated by using biotin N-hydroxysuccinimide ester (biotin-NHS, Sigma-Aldrich). Mixed 200 μL 20 mg/mL ferritin with 300 μL 1 mg/mL biotin-NHS in PBS buffer and rotated for 8 hours at room temperature. The excess biotin-NHS was removed by pass reaction solution through desalt column NAP 5 (GE Healthcare, UK) twice. The desalt column NAP 5 was thoroughly washed and saturated with PBS buffer before adding reaction sample. The collected biotinylated ferritin was then stored at 4 ^oC for future experiment.

Immunoassay of Avidin

A total of 50 μL 1.0 ppm of bovine fetal albumin (BSA), mouse IgG, human IgG, human fibrinogen, casein, and avidin were added into a 96 well plate and placed at 4^oC overnight to allow the deposit of proteins. The solutions were then discarded, and the plate was washed with PBS buffer to get rid of unbounded proteins. To completely wash the wells, 250 μL PBS buffer was introduced into each well and shaken at 250 rpm for 5 minutes before discarding the PBS buffer and tapping the plate on an absorbing paper to remove the residual solution. Subsequently, 250 μL 3% BSA solution was added into each well and incubated at 32^oC for 30 minutes to block the spare-plate surface to eliminate nonspecific adsorption. After blocking, the BSA solution was discarded, and the plate was washed with PBS buffer three times as aforementioned. Then 50 μL of 0.35 mg/mL biotinylated ferritin prepared in 0.5% BSA was added into each well and incubated at 32^oC for 15 minutes to facilitate the avdin-biotin recognition and binding. After that, the solution was removed, and the plate was washed three times. Finally, 100 μL DPD solution was added and developed at 50^oC for 15 minutes before absorbance detection. Since the avidin sample's color is too dark to detect, all samples were diluted 200 times in dH_2O immediately before reading.

Sandwich Immunoassay of nitrated human ceruloplasmin

The human ceruloplasm in phosphate buffered saline (pH 7.4) was nitrated by the addition of 1 mM authentic peroxynitrite (R&S Systems, Minneapolis, MN) according to recommendations of the manufacturers. The aliquots of nitrated human ceruloplasmin was then stored at -80 ^oC. The anti-human ceruloplasmin antibody was immobilized on 96-well microplate by incubating with 50 μL antibody 30 μg/mL solution at 4 ^oC for overnight. The nitrated human ceruloplasmin was prepared in PBS buffer with 0.5% bovine serum album (BSA) and added into antibody coated

microplate at different final concentration ranging from 33 pM to 10 nM. The solution was removed after 40 minutes incubation and washed twice with 250 µL PBS buffer. Sequentially, biotinylated anti-nitrotyrosine antibody prepared in PBS buffer with 0.5% BSA was added at the final concentration of 4.0 µg/mL. After 40 minutes incubation, the solution was removed and washed twice with 250 µL PBS. The streptavidin prepared at 1.0 µg/mL in 0.5% BSA was then introduced and incubated for 15 minutes before washing twice with 250 µL PBS. A total of 50 µL 100 µg/mL biotinylated ferritin prepared in 0.5% BSA was then added and incubated for 20 minutes to allow the ferritin to label the anti-nitrotyrosine antibody through biotin-avidin linkage. The unbound ferritin was then removed and washed three times with 250 µL PBS. The sandwich assay samples were then developed using DPD as the substrate according to the protocol aforementioned.

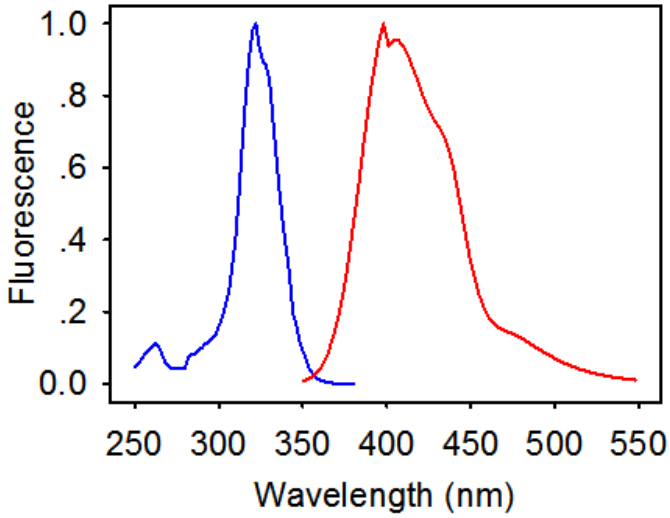

Figure S1. The fluorescence spectra of *p*-HPPA after oxidization by ferritin. Both excitation and emission spectra are normalized according to their highest value, respectively. The max excitation wavelength is 324 nm while the max emission wavelength is 398 nm.

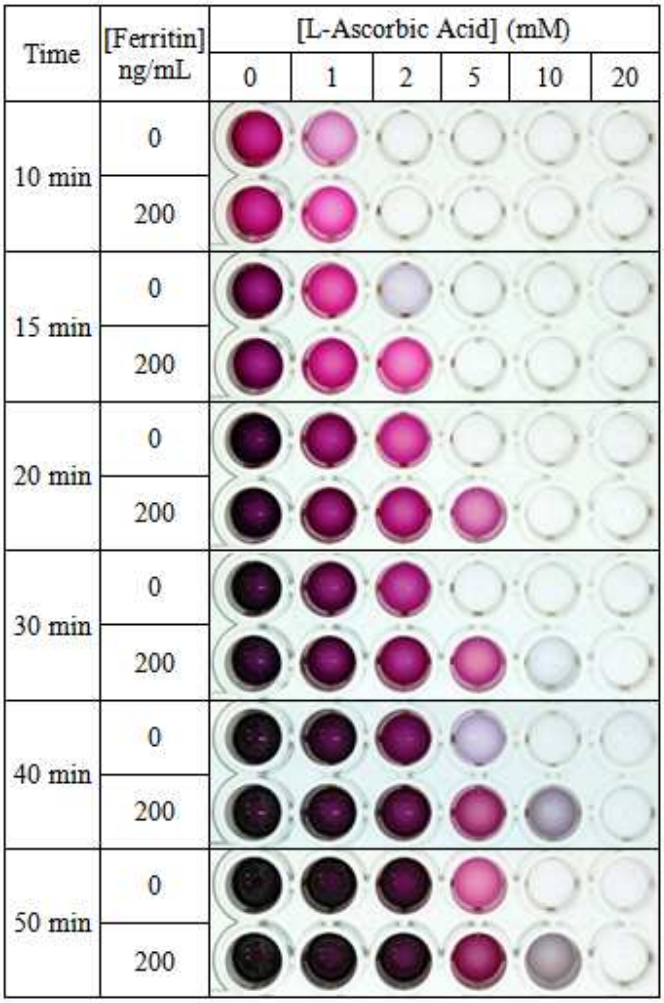

Figure S2. Optimization of L-ascorbic acid concentration in DPD substrate based ferritin assay. The addition of L-ascorbic acid into reaction buffer can decrease the background of DPD substrate and significantly improve the detect limit at the price of longer reaction time. The results suggested that the addition of 2-5 mM L-ascorbic acid can significantly improve the sensitivity of DPD substrate based ferritin assay with reasonable reaction time (15-30 minutes).