Rapid Ferritin Iron Release Using FMN Reductase

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Abstract: Iron deficiency anaemia is very common in developing countries and affects approximately two billion people worldwide. To test for iron deficiency, the common practice is to detect the body's ferritin level using an automated machine. However, ferritin level can be confounded by inflammation which does not affect the iron level that is available in the ferritin cage. Therefore, we designed a novel assay to achieve rapid ferritin iron release for the detection of iron deficiency anaemia using various biochemical compounds including reducing agents, oxygen scavenger compounds and chaotropic agents.

Key words: Iron deficiency anaemia, ferritin iron, ferritin, rapid assay.

1. Introduction

Iron deficiency anaemia is the most common cause of anaemia worldwide that affects almost two billion people in many developing countries including Malaysia [1], [2]. The clinical symptoms of iron deficiency anaemia and thalassaemia are very similar, thus misdiagnosis is easy [3]. In thalassaemia, there are either a reduced or absent production of haemoglobins but a normal or increased absorption of iron [4]. In areas without proper medical access, many anaemic individuals were misdiagnosed and treated with iron because they were thought to have iron deficiency anaemia due to low haemoglobin content, but iron in excess is toxic to the body. Excess iron is stored safely in ferritin protein and is readily available when our body needs [5].

Serum/plasma ferritin is commonly used to assess the body's iron status [6]. Serum/plasma ferritin is an acute phase protein which is elevated with any inflammatory state [7], [8]. However, studies have shown that iron available in the ferritin could clearly distinguish those with an iron overload from those with elevated ferritin due to inflammation as the iron level is not affected by inflammation [9]. Many efforts have been done to mobilize the iron from ferritin cage *in vitro*, either by direct chelation of iron(III) or by indirect reductive mobilization in the presence of iron(II) chelating agents. However, studies showed that iron mobilization by chelators such as desferrroxamine is rather slow compared to indirect mobilization using various reducing agents [10]. Some studies showed that ferritin iron could be released by manipulating the ferritin cage using chelating agents and forming coloured complex using a chromophore for spectrophotometry detection [11]. Therefore, a thorough optimisation and quantification of iron release from the ferritin cage would allow for the detection of the body's iron status without interference from inflammation.

2. Materials and Methods

Commercially available lyophilized human liver ferritin was purchased from Lee Biosolutions (Saint Louis, MO, USA). Chromophores, reducing agents and other chemicals were obtained from Sigma-Aldrich(Saint Louis, MO, USA) or Merck& Co. (Kenilworth, NJ, USA), and were of analytical reagent grade. The spectrophotometric analysis was performed in Lambda 25 UV/Vis Spectrophotometer, PerkinElmer (Waltham, MA, USA), VersaMax ELISAMicroplateReader, Molecular Devices (Sunnyvale, CA, USA) and Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc. (Waltham, MA, USA). All reactions were conducted at room temperature.

2.1. Standard Calibration Curve

Iron(II) substrate was added to the reactant (chromophore) in buffer pH 7.0. Spectrophotometric analysis between 400-700 nm was carried out immediately. A calibration curve of iron(II) constructed to optimize the optimum chromophore concentration needed to achieve spontaneous reaction. The amount of iron(II) was calculated using Beer-Lambert Law.

$$A = -\lg(I/I_0) = \varepsilon cl$$

The above equation is based on the Beer-Lambert law, which is widely used in quantitative analysis. The absorbance, A, is directly proportionate to the concentration, c, of the compound measured, the path length of the sample, l, and the molar absorption coefficient, ε, a wavelength-dependent constant characteristics of the compound. I_0, is the incident light intensity and I, is the transmitted light intensity. All compounds do absorb some of the light that falls upon them giving them the energy to excite the electrons of the compound to a higher energy level. The photometer was calibrated using a standard compound and background noise was taken into account.

2.2. Iron Release from Human Liver Ferritin

In this section, the iron content of human liver ferritin was determined by inductive-coupled plasma-mass spectrometry (ICP-MS). Iron(III) compound was used to construct the calibration curve in order to determine the optimized concentration of reducing agents. Iron release of human liver ferritin was induced and facilitated by the addition of FMN reductase at various concentrations according to previous studies and oxygen scavenger system in the presence of the chromophore for detection purposes [12]-[14]. The recovery of ferritin iron release was compared with results from elementary analysis by ICP-MS.

3. Results and Discussion

3.1. Standard Calibration Curve

By spectrophotometric analysis, the concentration of colourediron(II)-chromophore complexes in solution can be easily determined by correlating the absorbance readings with a standard curve [15]. Therefore, a standard curve was constructed as a reference to determine the iron(II) level in an unknown sample. Fig. 1 shows the absorption spectra curve of the iron(II)-chromophore system at zero time, with 10 mM chromophore. The spectra curve of iron(II)-chromophore complex showed maximum absorbance at 562 nm with concentration in the range of 5-200 μg/L iron(II). At wavelength of 562 nm, the molar absorption coefficient is 27,900 L mol^{-1} cm^{-1} according to Stookey [16]. As the concentration increased, the absorbance peak was higher, indicating the proportional relationship between iron(II) concentration and the absorbance readings. Fig. 2 shows the calibration curve of serial dilution with a range of 5-200 μg/L of iron(II). For the calibration curve, it was a straight-lined graph, obeying the Beer Lambert Law. This was

used for downstream quantification of iron release from ferritin.

3.2. Optimization of Chromophore Concentration

Concentration of chromophore was determined using iron(II) compound in order to attain the optimum concentration that is sufficient to form colourediron(II)-chromophore complexes with available iron(II) in a solution. In order to facilitate the optimization purposes, microplate well was used to perform each assay in triplicates using Multiskan GO microplate reader. Iron(II) in a range of 50-1000 µg/L was used to optimize the optimum concentration of chromophore at pH 7.4, in a range of 10-500 µM. Fig. 3 shows the stability ofiron(II)-chromophore complex within a 30 minutes time period; Fig. 3 shows the iron(II) amount ranging from 5-1000 µg/L. The recovery of each iron amount of each chromophore concentration can be seen on Fig. 4.

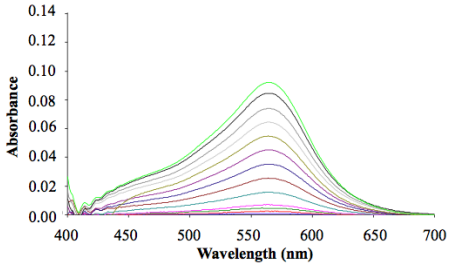

Fig. 1. Absorption spectra curve of iron(II)-chromophore complex in buffer, pH 7.0, room temperature, 10 mM chromophore, with 5-200 µg/L of iron(II), 10 mm path-light cuvette.

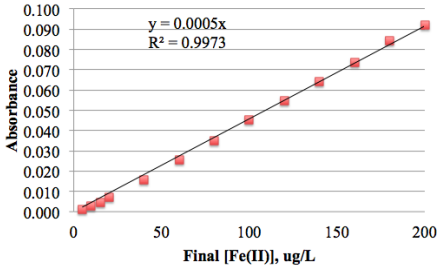

Fig. 2. Calibration curve of iron(II)-chromophore complex in buffer, pH 7.0, room temperature, 10 mM chromophore, with 5-200 µg/L of iron(II).

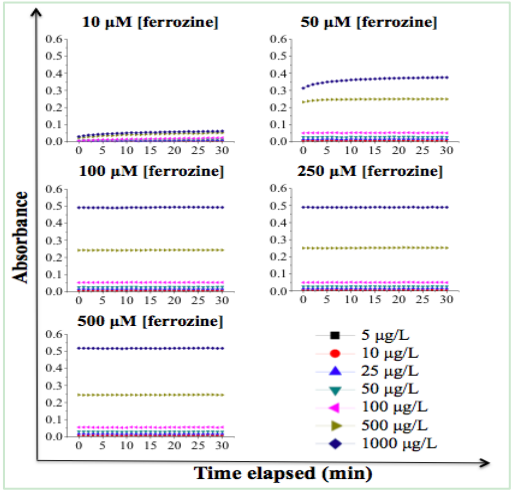

Fig. 3. The stability of iron(II)-chromophore complex in buffer, pH 7.4 room temperature at different concentration of chromophore, at 5-1000 ug/L iron(II) within 30 minutes.

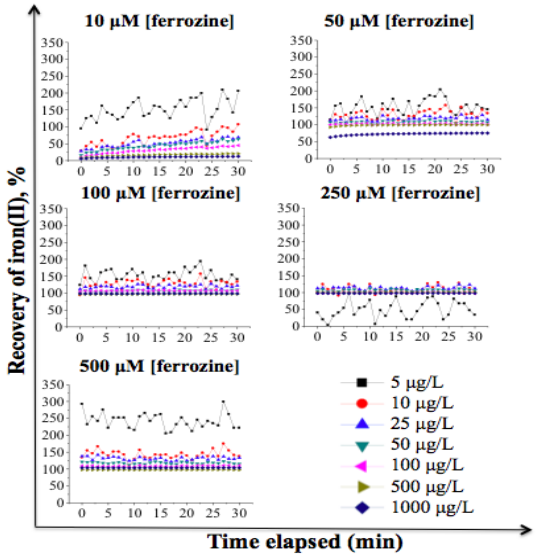

Fig. 4. Recovery of iron(II) in buffer, pH 7.4 room temperature at different concentrations of chromophore, at 5-1000 ug/L iron(II).

At 10 µM chromophore, there was significantly lowered absorbance readings as compared with others while the recovery of iron(II) was poor, suggesting insufficient chromophore concentration to complex with iron(II). At 50 µM chromophore, iron at 500 µg/L and 1000 µg/L required a few minutes to plateau and only achieved 80% Fe(II) recovery in 1000 µg/L iron(II), suggesting inadequate chromophore available for iron(II) coupling at 1000 µg/L of iron(II). On the contrary, the reaction is spontaneous at 100, 250 and 500 µM chromophore for iron(II) concentration up to 1000 µg/L of iron(II) (Fig. 3). However, for the 5 µg/L iron(II), the recovery was inconsistent within a 30 minute period, suggesting that the low iron(II) amount readings might hampered by background noise.

3.3. Reduction Effectiveness

Studies have showed that FMN reductase is effective enough to liberate iron(II) from insoluble iron(III) inside the ferritin cage, whereby FMN reductase reduces both NADH and FMN simultaneously. Therefore, the effectiveness of FMN reductase can be confirmed by examining the changes of the maximum absorption peaks, respectively [17]. Shorter path-light cuvette was used to resolve higher concentrations of the specific compound. Fig. 5 shows the changes of the absorbance readings of maximum absorption peaksin NADH and FMN, respectively. Concentrations of both NADH and FMN decreased over time indicating the production of reducing agents. Optimum concentration of both NADH and FMN was used to induce ferritin iron release.

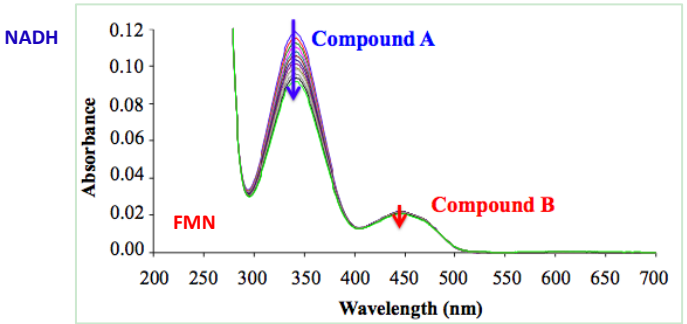

Fig. 5. Absorption spectra curve of NADH (2 mM) and FMN (0.2 mM) in buffer, pH 7.4, room temperature.

3.4. Optimization of FMN Reductase Concentration

Concentration of FMN reductase was determined using iron(III) compound and optimized chromophore concentration in order to attain the optimum concentration that is sufficient to induce the liberation of soluble iron(II) from insoluble iron(III) inside the ferritin cage. Iron(III) in a range of 50-3000 µg/L was used to optimize the optimum concentration of the reducing agents at pH 7.4. Fig. 6 shows the stability of iron(II)-chromophore complex within 30 minutes after iron(III) was converted to iron(II) by the reducing agents followed by the formation of iron(II)-chromophore complex in the presence of 1.0 mM chromophore. Lower iron(III) amount might require relatively lower concentration of reducing agents to attain the stability of the complex formed. However, in higher reducing agents concentration as in 500 µg/L of iron(III), the absorbance fluctuated, suggesting that the complex was not stable, probably due to the excessive unused reducing agents in the assay, which may be detrimental to the iron(II)-chromophore complex formed.

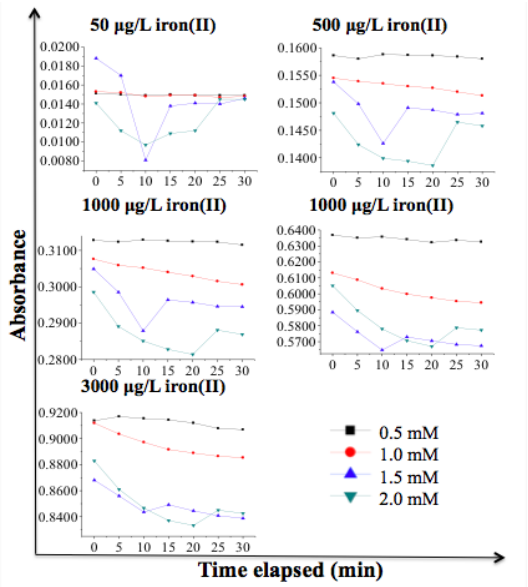

Fig. 6. The stability of iron(II)-chromophore complex in buffer, pH 7.4, room temperature at different concentration of FMN reductase over 30 minutes.

3.5. Effects of Oxygen Scavenger System

Iron(II) is readily converted back to iron(III) in the presence of oxygen [18]. To avoid this complication, oxygen scavenger was added to each assay to minimize the oxidization of iron(II) to iron(III). Additionally, the oxygen scavenger also played a crucial role to avoid FMN reductase from being oxidized in this experiment setup. Therefore, oxygen scavenger was added to remove dissolved oxygen that was introduced unintentionally during the buffer preparation and experiment setup. Iron(III) in the range of 50-3000 µg/L was used. Fig. 7 shows the stability of iron(II)-chromophore complex within 30 minutes after iron(III) was converted to iron(II) by the reducing agents followed by the formation of the iron(II)-chromophore complex in the presence of the chromophore and the oxygen scavenger. As compared to Fig. 6 (without the oxygen scavenger), Fig. 6 shows improvement in the stability of the iron(II)-chromophore complex in 50 µg/L and 500 µg/L iron(III) respectively. Also, improvement in the stability of the iron(II)-chromophore complex was found in both 2000 µg/L and 3000 µg/L iron(III), but not in 0.5 mM FMN reductase concentrations that probably overwhelmed the reaction system as time elapsed.

3.6. Recovery of Iron(II) from Ferritin

Results showed that we were able to recover significant amounts of iron(II) from using our assay within 30 minutes of testing (Fig. 8). Various amount of ferritin up to 1000 µg/L were tested.

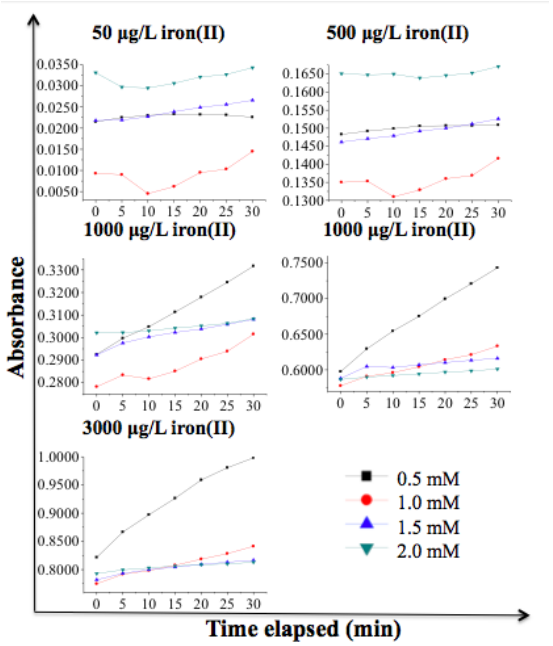

Fig. 7. The stability of iron(II)-chromophore complex in buffer, pH 7.4, room temperature at different concentration of FMN reductase (in the presence of oxygen scavenger A) over 30 minutes.

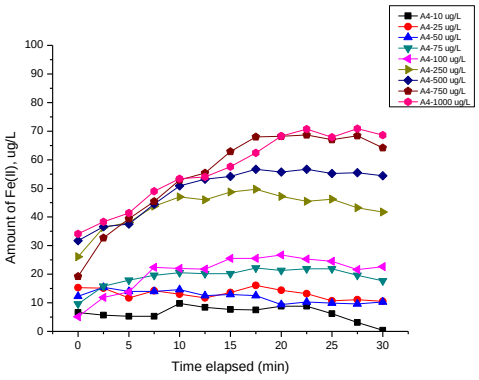

Fig. 8. The amount of iron(II) recovered from different concentrations of ferritin over 30 minutes.

4. Conclusion

Iron deficiency anaemia is the most common cause of anaemia worldwide and is curable with the supplementation of iron [1]. The symptoms of iron deficiency anaemia include tiredness, pallor, shortness of breath and palpitations which could affect ones' quality of life and work performance. Iron deficiency anaemia could be assessed by several methods and one of the common technique used is the measurement of serum/plasma ferritin level [6]. However, as ferritin level is confounded by inflammation, this method has its disadvantages in certain patients including those with thalassaemia and chronic infections [3]. A method to assess the iron status without the influence of inflammation is necessary to give a more accurate

account of the iron status level.

We have used FMN reductase to reduce the iron(III) to iron(II) in ferritin, which complexes with the chromophore to form iron(II)-chromophore complex to be detected by a spectrophotometer assisted by the oxygen scavenger system to remove any dissolved oxygen in order to avoid oxidation of both reducing agents and iron(III) in this experiment setup. We believe that this procedure would lead to a novel rapid assay that will be beneficial in quantifying the total iron levels in the ferritin without being confounded by inflammation.

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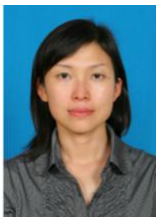

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