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Characteristics and Kinetics of Iron Release from the Ferritin under the EGCG reduction

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Abstract The mechanism of iron release from ferritin in vivo is still unclear even though it represents a key step of the metabolism of iron in vivo. Here, both interaction intensity and binding stability between epigallocatechin gallate (EGCG) from tea and liver ferritin of *Dasyatis akajei* (DALF) were investigated using UV–visible, fluorescence and circular dichroism (CD) spectrometry, respectively. The results indicated that EGCG could reduce the iron within the ferritin shell directly in the absence of chemical reducers such as $Na_2S_2O_4$, but this process was strictly pH-dependent, and the rate of iron release is faster at low pH than at high pH. The kinetic study of iron release showed that this process fitted the law of zero order reaction, which differed from that of first order reaction by various chemical reducers such as Vitamin C. In addition, Both fluorescence and CD spectrometry were further used to study the reduction mechanism of iron release in vitro, showing that there was a slight conformation change of the ferritin shell during EGCG reduction because of a complex formation of DALF–EGCG. It appears that chemical reducers with large molecular sizes reduce the iron across the protein shell by the way of an electron transfer pathway (ETP). A novel pathway for iron release from DALF with EGCG reduction is suggested to explain for a reductive route of iron metabolism by biological reducers in vivo.

Keywords Liver ferritin of *Dasyatis akajei* (DALF) · EGCG · Reduction · Fluorescence spectrometry · CD spectrometry · Kinetic of iron release

Abbreviations

DALF	Liver ferritin of *Dasyatis akajei*
EGCG	Epigallocatechin gallate
CD	Circular dichroism

Introduction

Ferritin is a ubiquitous and conserved iron storage protein which exists in animals, plants and bacteria [1–3]. The main physiological function of ferritin is considered to be sequestering free Fe^{2+} or Fe^{3+} ions in cells, thus avoiding them from catalyzing dangerous Fenton reactions, which can produce reactive oxygen species such as superoxide, peroxide and hydroxyl radicals, which are extremely powerful and cause extensive damage to cells [4]. Another important function is that it may also serve as a source of iron for in vivo biosyntheses, that is, ferritin has the dual function of storing iron in non-toxic and bioavailable forms [2].

The kinetics of iron release from ferritin in vitro has been broadly studied, and it has contributed to some knowledge of the iron release mechanism of ferritin in vivo, which, however,

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remains unclear [5]. The typical in vitro method for releasing iron from ferritin is first reducing the Fe^{3+} to Fe^{2+}, and then chelating the Fe^{2+} using chelating agents. Many kinds of reducers and chelators are reported such as $Na_2S_2O_4$, bipyridyl and ferrozine [6, 7], and this represents one model for the mechanism of iron release from ferritin as a reducer molecule small enough to pass through the ferritin channel and reduce the iron core [5, 8]. Another two major iron release models have also been proposed. One model, if the reducer is much too large to cross the channels, first involves the transfer of the electron to the ferritin shell, and then a hypothetical specific electron transfer pathway in the protein shell will transfer these electrons to the ferritin iron core [5, 9]. Another approach utilizes specific chelating agents to remove of the Fe^{3+} from ferritin [5, 10].

Epigallocatechin gallate (EGCG) is the major polyphenolic constituent found in green tea and accounts for more than 50% of the total content of polyphenolic compounds in green tea. EGCG is considered to be a type of antioxidant and is of great benefit to human health [11]. Because it has a two orthodihydroxphenyl structure, it is also considered to be a strong reducing agent, which has the ability for reducing Fe^{3+} [12].

The ferritin is purified from the liver of *Dasyatis akajei*, and it shares the common structural characteristics with most ferritins in nature, which are described by Kong et al. [6] and Huang et al. [13], respectively. The molecular size of DALF is somewhat larger than that of horse spleen ferritin (HSF), and its protein shell consists of two different type subunits, called H and L subunits [6, 13].

Since EGCG has a reducing capacity of its own, it was used in the present study to effect the release of iron from DALF in the absence of added chemical reducing agents. This choice was made in consideration of its strong intrinsic reducing property and its large molecular size, and to elucidate the mechanism of the iron release, as this might lead to a better understanding of the iron release models discussed above.

Materials and Methods

Materials

DALF was purified according to the method described by Kong et al. [6], and EGCG was purchased from the Hetian Biology Company, Hangzhou, China, the purity being 95%. Iron release from ferritin was measured using the ferrous chelator α,α′-bipyridine as a chromophore.

Iron Release of Ferritin with EGCG Reduction

In the pH-dependent iron release experiment, the reaction mixture (1.5 ml) contained 0.13 μM DALF, 5.33 μM EGCG and excess α,α′-bipyridine reagent. The reactions were carried out at 37°C; the pH ranged from 5.8 to 7.8 and 100 μl reaction solution was removed from each centrifuge tube to determine the absorbance at 520 nm after 24 and 48 h.

In the time-dependent iron release experiment, the reaction pH was fixed at pH 6.8. The reaction volume was 3.0 ml, and it contained 0.27 μM DALF, 10.67 μM EGCG and excess α,α′-bipyridine. The reaction was carried out at room temperature (25°C) against reference cuvettes containing all reactants, and was initiated by the addition of EGCG. It was scanned once every 5 h and ranged from 350 to 800 nm during the 12 times it was measured.

Ferritin (0.13 μM) and EGCG (5.33 μM) were also mixed directly and then detected using UV–visible spectrometry after 3 h.

All the above reaction mixtures were exhausted and flushed out with nitrogen.

Fluorescence Quenching of Ferritin by EGCG

DALF (0.17 μM) was dissolved in phosphate buffer at pH 6.8. The fluorescence quenching spectra of ferritin were obtained at excitation and emission wavelengths of λ_{ex}= 280 nm and λ_{em}=295–500 nm. The synchronous fluorescence spectra were recorded from 245 to 330 nm at $\Delta\lambda$= 60 nm. Fluorimetric titrations of 0.17 μM ferritin by successive additions of a solution of 0.34 μM EGCG were performed using a Varian Cary Eclipse fluorescence spectrophotometer (Varian Incorporation, America).

Circular Dichroism Measurements

Circular dichroism (CD) spectra were measured on a Jasco J-810 Spectropolarimeter (Jasco, Tokyo, Japan) using a 0.1-cm path length quartz cell at 0.1-nm data pitch intervals, with three scans averaged for each CD spectrum. The spectra were recorded in the range 190–240 nm at room temperature with a scanning rate of 50 nm/min and a response time of 4 s. The ferritin concentration was 0.1 mg/ml. The CD measurements of ferritin was made in the absence and presence of EGCG (the molar ratios of ferritin to EGCG were 1:10 and 1:20 at pH 6.0). Buffer solution (as the reference) was subtracted from the sample spectra.

Calculation of the Diameter of EGCG

The optimized geometry of the EGCG was obtained using Gaussian 09 with B3LYP/6-31 G (d), and the diameter of the EGCG was calculated from the volume using Gaussian 09 software.

Results

Release of Ferritin Iron by EGCG

Ester-type catechins such as EGCG and ECG are known to precipitate proteins from solutions, but this property depends significantly on the concentration ratio of these agents to the protein. At low ratios, the protein precipitate ion may not occur [14]. Under our experimental conditions, no precipitate ion was detectable before or after centrifugation at 12,000 rev/min for 10 min when molecular ratios of EGCG to DALF was <50.

The iron released from DALF by EGCG (at 40 times the molecular ratio of EGCG to ferritin) at different pH conditions (from pH 5.8 to pH 7.8) over 24 and 48 h is shown in Fig. 1. The concentration of DALF was constant at 0.13 μM. The figure shows that at low pH (pH 5.8) the speed of iron release was much faster than at high pH (pH 7.8). This indicated that the iron release from ferritin by EGCG was pH-dependent, at least from pH 5.8 to pH 7.8, and that it was easier to release iron at low pH than at high pH. This result is in accordance with previous studies which showed that a low pH could facilitate Fe^{3+} reduction from ferritins [15, 16]. Gálvez et al. [16] proposed that such facilitation resulted from the negative charge of the ferritin surface at high pH. It is thought that the negative charge repels the partially deprotonated fraction of the reducing agent and the amount of the iron chelator approaching the ferritin or entering the ferritin cavity therefore cuts down the effectiveness of the iron release. It could also be explained by the charge balance of the redox reactions of iron release, because about two H^+ are transferred to the core for each Fe^{3+} reduced to Fe^{2+} revealed by Watt et al. [17].

The time dependent iron release of DALF on addition of 40 times as much EGCG at pH 6.8 is shown in Fig. 2, as monitored spectrophotometrically by formation of the $Fe_{(bipy)_3}^{2+}$ complex which has a characteristic maximum absorption peak at 520 nm. It shows that compared to that of

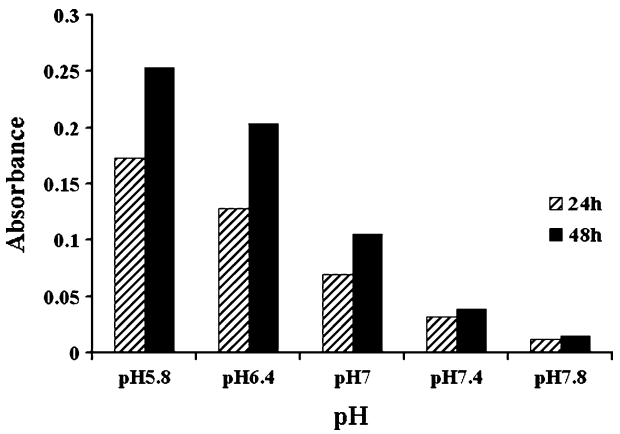

Fig. 1 pH-dependent formation of the $Fe_{(bipy)_3}^{2+}$ complex following release of Fe^{2+} from DALF (0.13 μM) using EGCG (5.33 μM)

$Na_2S_2O_4$ or vitamin C [6], the speed of iron release by EGCG was very slow. It is well known that this speed can be evidently accelerated by increasing concentration of EGCG largely in the reactive medium. In fact, the experimental results indicate that DALF brings precipitation easily while high concentration of EGCG is carried out to release the iron. It is for this reason that we have to utilize low concentration of EGCG to reduce the protein shell for iron release from the DALF.

The possible Fe^{3+}–EGCG complex which may directly have been formed by Fe^{3+} and EGCG was also investigated at pH 7.4. However no significant absorption peaks between 250 nm to 1,000 nm were detected using UV–visible spectrophotometry (see ESI 1). This result was different from the catechol interaction with ferritin in the report of Sánchez et al. [10]. They find that the release of iron from ferritin by catechol does not take place by iron (II) reduction but by direct iron (III) chelation.

In nature, most mammal ferritins show featureless absorbance peaks in visible spectral range (380–580 nm). An iron core within the ferritin shell is composed of a few thousand molecules of hydrated iron (Fe^{3+}) and hundreds of inorganic phosphate (P_i) [1]. The ferritin has no capacity to carry out iron release by adding a Fe^{2+} chelating agent such as α,α′-bipyridine in the absence of chemical reducers such as $Na_2S_2O_4$. Despite EGCG's strongly ability to reduce Fe^{3+} into Fe^{2+}, it cannot release the irons directly in ferritin in the absence of Fe^{2+} chelating agent such as α,α′-bipyridine, which means that EGCG has completely lost its capacity for iron release without the Fe^{2+} chelating agent.

Fluorescence Studies

Fluorescence Quenching of Ferritin by EGCG

The fluorescence of protein originates from its tryptophan, tyrosine, and phenylalanine residues. However, because phenylalanine has a very low fluorescence quantum yield, the intrinsic fluorescence of protein is mainly due to the tryptophan and tyrosine residues. When small molecules bind to protein, intrinsic fluorescence intensity changes of protein are induced by the micro-environmental alterations of the tryptophan and tyrosine residues [18].

The fluorescence emission spectra of DALF at various concentrations of EGCG at 298 K are shown in Fig. 3. Obviously, the ferritin had a strong fluorescence emission band at 332 nm at an excitation wavelength of 280 nm, while EGCG had no intrinsic fluorescence under our experimental conditions. The fluorescence intensity of the ferritin was decreased regularly with increasing concentration of EGCG. It is suggested that a complex was formed between ferritin and EGCG, which quenched the fluorescence of ferritin, resulting from micro-environmental changes of the tryptophan and tyrosine residues.

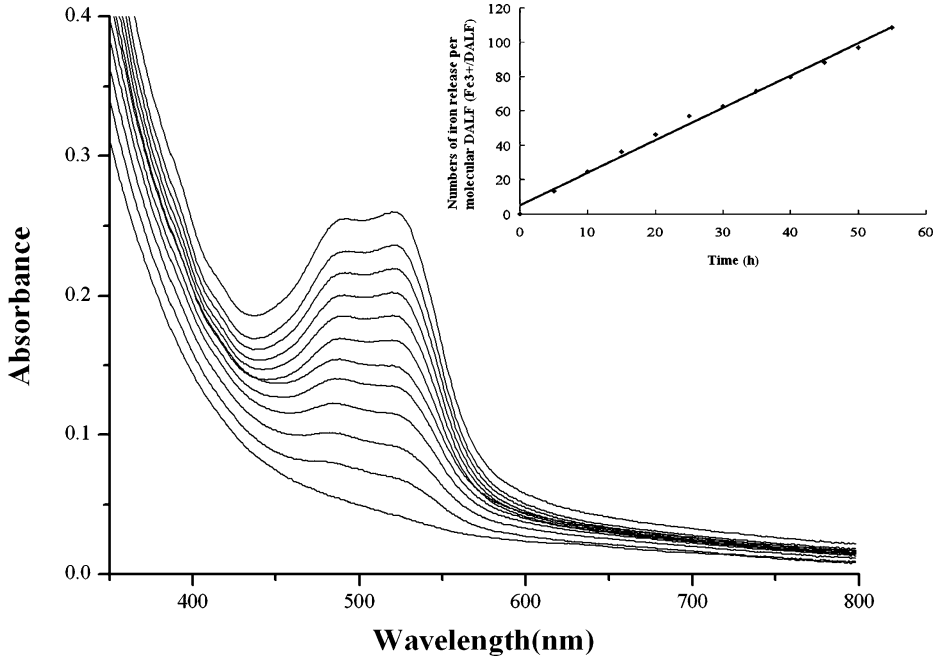

Fig. 2 Time-dependent spectra for the formation of the $Fe_{(bipy)}^{32+}$ complex following release of Fe^{2+} from DALF (0.27 μM) using EGCG (10.67 μM) (pH 6.8); $\Delta T = 5$ h. *Inset*: kinetics of iron release from DALF (0.27 μM) using EGCG (10.67 μM). The kinetics of iron release for the curve is defined as a zero-order reaction

In order to speculate on the fluorescence quenching mechanism, the fluorescence quenching data were analyzed using the classical Stern–Volmer equation:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

where F and F_0 are the fluorescence intensities in the presence and absence of quenchers, K_q is the biomolecule macromolecule quenching rate constant, τ_0 is the average lifetime of the molecule without quencher, $[Q]$ is the increasing concentration of quencher, and K_{sv} is the Stern–Volmer quenching constant. Obviously,

$$K_{sv} = K_q\tau_0 \quad (2)$$

because the fluorescence lifetime of the biopolymer τ_0 is 10^{-8} s^{-1}[19], and K_{sv} is the slope of linear regression in Fig. 3 (inset). The quenching constant K_q could be obtained from the slope in Eq. 2, and the result of linear regression was: $F_0/F = 0.9951 + 1.80245 \times 10^5$ $[Q]$ ($r^2 = 0.9986$) ($K_{sv} = 1.80245 \times$

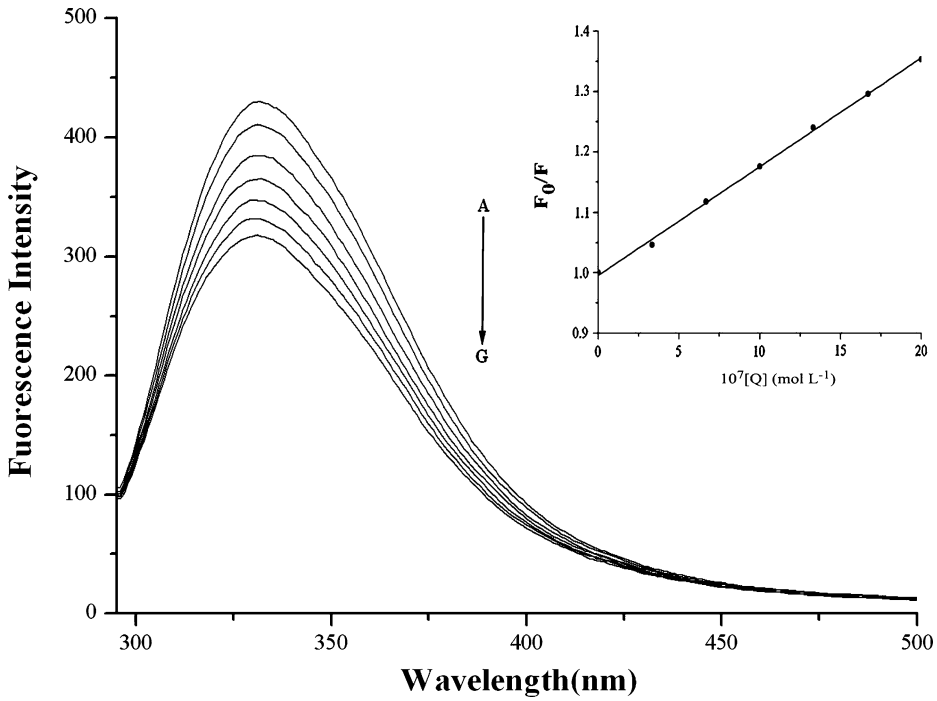

Fig. 3 Fluorescence quenching spectra of DALF in the presence of different concentrations of EGCG at 298 K with an excitation wavelength of 280 nm in phosphate buffer solution (pH 6.8). DALF = 0.17 μM, EGCG from A to G: 0, 0.34, 0.68, 1.02, 1.36, 1.70, 2.04 μM. *Inset*: Stern–Volmer plots for the quenching of DALF using EGCG

10^5 M^{-1}, k_q=1.80245×10^{13} M^{-1} S^{-1}). For dynamic quenching, the maximum scattered collision quenching constant of various quenchers to the biopolymer was 2.0×10^{10} M^{-1} S^{-1} [18]. Obviously, the rate constant of the protein quenching procedure initiated by EGCG was greater than that in the scattered procedure. This means that the above quenching was not initiated by dynamic collision but resulted from formation of a static ferritin–EGCG complex.

Characteristics of the Synchronous Fluorescence Spectra

Synchronous fluorescence spectra, which are obtained by scanning simultaneously the excitation and emission monochromators, are frequently applied to study the interaction between molecular probes and proteins, since it can provide the information about the molecular environment in the vicinity of the chromophore molecules. It is a useful method to study the environment of amino acid residues by measuring the possible shift in the wavelength emission maximum (λ_{max}), the shift in position of the emission maximum corresponding to the changes of the polarity around the chromophore molecule [20]. According to Miller [21], the distinction between excitation wavelength and emission wavelength reflects the spectra of different types of chromophores: with large $\Delta\lambda$ values such as 60 nm, the synchronous fluorescence of ferritin is characteristic of tryptophan residues and with small $\Delta\lambda$ values such as 15 nm is characteristic of tyrosine residues.

Synchronous fluorescence spectra of DALF upon addition of various concentrations of EGCG for $\Delta\lambda$=60 nm were also investigated (see ESI 2). Addition of EGCG led to a distinct decrease in the synchronous fluorescence intensity, but no apparent shift of spectral peak occurred. The interaction of EGCG with DALF did not significantly affect the environment of the tryptophan residue microregion nor the conformation of the ferritin.

CD Spectroscopic Studies

In order to investigate the possible changes in the conformation of the ferritin induced by different concentrations of EGCG, and considering in low pH the iron release is easier than in high pH, the CD spectroscopy was used at pH 6.0.

Figure 4 indicates that the CD spectra of DALF exhibited two negative bands in the far-UV region at 208 and 222 nm, consistent with ferritin's structure which is a typical four-helix bundle protein [22]. In Fig. 4, the change of CD spectra is minor. The interaction between EGCG and ferritin caused a slight decrease in band intensity at all wavelengths of the far-UV CD without any significant shift of the peaks. Calculations revealed a slight increase in the a-helix content (2.5%), clearly indicating that EGCG induced a slight increase in the a-helical structure content of ferritin. This meant that, at pH6.0, EGCG caused only minor perturbation to the overall secondary structure of ferritin.

Discussion

In the present study, EGCG was found to release Fe^{3+} from ferritin after its reduction to Fe^{2+} and subsequent captured

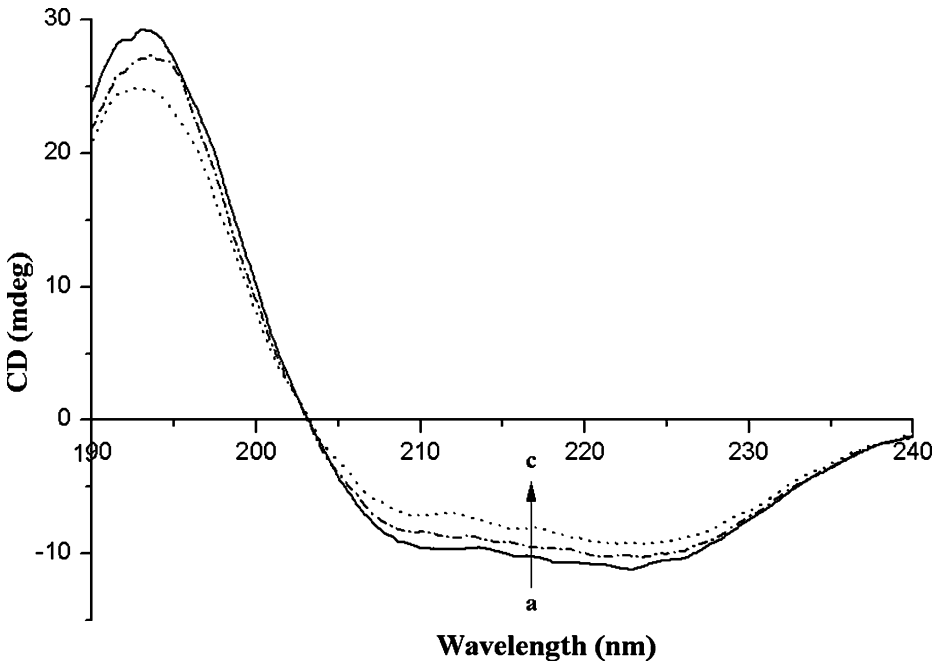

Fig. 4 CD spectra of the DALF–EGCG system (pH 6.0). c(DALF) = 0.1 mg/ml; n (EGCG)/n(DALF), (**a**–**c**) 0; 10; 20

by α,α′-bipyridine. The Fe^{3+}–EGCG complex can be hardly observed using UV–visible spectrometry ranging from 400 and 1,000 nm. Sánchez et al. [10] found that the iron released from ferritin by catechol is not a process of Fe^{2+} reduction, suggesting an explanation of it binding to Fe^{3+} chelation because of orthodihydroxphenyl structure of catechol. EGCG is one of the major components in catechin, and also has the orthodihydroxphenyl structures. However, the possible reason for zero detection of the Fe^{3+}–EGCG complex is that it is not easy to form the Fe^{3+}–EGCG complex between ferritin and EGCG because of the width restriction of the ferritin shell. Although the Fe^{3+}–EGCG complex could be directly detected using UV–visible spectrometry under the mixed condition of both EGCG and Fe^{3+} (see ESI 1), the Fe^{3+}–EGCG complex cannot be observed by UV–visible spectrometry under the mixed condition of both ferritin and EGCG, showing that EGCG has no ability to move across the shell for binding to Fe^{3+} within the ferritin shell. Here, we suggest that EGCG can utilize another novel pathway to carry out iron reduction for its release.

There are two kinds of reduction models for iron release from ferritin: one is that when the reductant molecule is small enough to pass the ferritin channels, and the reducing reaction may occur in the cavity of ferritin; and the other is that when the molecule is much too large to cross the channel, it can still transfer electrons by a specific electron transfer pathway, which exists in the protein shell, in order to reduce Fe^{3+} to Fe^{2+} such as *Azotobacter vinelandii* flavoprotein (AvFlp) and ferredoxin I (AvFdI) [5, 9].

The electron transferring model is supported by electrochemical studies, which confirm that ferritin is a kind of electron-active protein [23–25]. Huang et al. [24] found that the kinetics for complete iron release of AVBF by electrode reduction at −600 mV fits the zero-order reaction law; and the same phenomenon occurs in the kinetics of iron release from pig spleen ferritin with bare platinum electrode reduction [25]. Both of these studies show that electron transfer reduction is possible in compliance with the zero-order law. In our time-dependent iron release experiment, a good linear relationship between the released iron and time was found (r^2=0.9935). This was also in compliance with the zero order law, which was in accordance with the electrochemical studies. It indicated that the reduction of DALF with EGCG is possibly by transferring electrons by the protein shell.

The diameter of EGCG was calculated to be 12.06 Å in a water solution using Gaussian 09 software, which is much larger than the channel diameter of ferritin which is about 3–4 Å [5, 26]. However, it is reported that some molecules which are larger than ferritin channels can still permeate into the ferritin cavity [8, 16]. Gálvez et al. [16] found benzohydroxamic acid (diameter of 6 Å) and the monobenzo-hydroxamate iron(III) complex (diameter of 7 Å) were able to enter and exit the ferritin cavity. Yang and Nagayama [8] found molecules larger than the size of the ferritin channels can pass through the channels into the ferritin interior, and that the maximum size of molecules for the permeation is smaller than maltotriose (approximately 20 Å) using proton nuclear magnetic resonance relaxation. Yang and Nagayama [8] proposed that the protein shell of ferritin is sufficiently flexible. Theil et al. [27] proposed a pore gated model explaining how molecules larger than 0.4 nm can traverse the ferritin protein shell. Thus, it is somehow intricate to know the real mechanism ferritin reduction of EGCG, because the diameter of EGCG is between 4 and 20 Å. However the binding between EGCG and ferritin revealed in our fluorescence quenching study showed that a static EGCG–ferritin complex was generated. The results of the synchronous fluorescence spectral and CD spectral analyses showed that EGCG can cause a minor perturbation of the overall secondary structure of ferritin, and result a slight increase in the a-helix content (2.5%). It inspired us that the static binding of EGCG could greatly influence the flexibility or pore status of ferritin and result in preventing EGCG permeate into the protein cavity or EGCG in reality cannot enter or exit the protein cavity. This needs for further study.

A model of iron release from DALF by EGCG was proposed as follows. In step 1, EGCG binds to ferritin and induces the micro conformation change of ferritin, which gives rise to the decrease of reduction potential of the ferritin protein shell. In Step 2, EGCG transfers electrons to ferritin and electron transfer in the ferritin protein shell. In step 3, ferritin transfers electrons to the Fe^{3+} of the iron core and reduces Fe^{3+} to Fe^{2+}, and in step 4, the reduced Fe^{2+} is bound by α,α′-bipyridine and produces the $Fe_{(bipy)}3^{2+}$ complex, which can be detected by UV–visible spectrophotometry. We considered that step 3 may be the rate-limiting reaction, which is the main reason for it fitting of the zero order law.

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