Studies on the binding of fulvic acid with transferrin by spectroscopic analysis

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HIGHLIGHTS

• We studied the mechanism of interaction between fulvic acid and Tf for first time.
• The binding constant ($5.48 \times 10^7 \text{ M}^{-1}$) was nearly 1000 times than other drug molecules.
• The main interaction force of fulvic acid with Tf was hydrogen bonding.
• The fulvic acid caused conformational change of Tf with the loss of $\alpha$-helix content.

ABSTRACT

Transferrin has shown potential in the delivery of anticancer drugs into primarily proliferating cancer cells that over-express transferrin receptors. Fulvic acid has a wide range of biological and pharmacological activities which caused widespread concerns, the interaction of fulvic acid with human serum transferrin (Tf) has great significance for gaining a deeper insight about anticancer activities of fulvic acid. In this study, the mechanism of interaction between fulvic acid and Tf, has been investigated by using fluorescence quenching, thermodynamics, synchronous fluorescence and circular dichroism (CD) under physiological condition. Our results have shown that fulvic acid binds to Tf and form a new complex, and the calculated apparent association constants are $5.04 \times 10^8 \text{ M}^{-1}$, $5.48 \times 10^7 \text{ M}^{-1}$, $7.38 \times 10^6 \text{ M}^{-1}$ from the fluorescence quenching at 288 K, 298 K, and 310 K. The thermodynamic parameters indicate that hydrogen bonding and weak van der Waals are involved in the interaction between fulvic acid and Tf. The binding of fulvic acid to Tf causes the $\alpha$-helix structure content of the protein to reduce, and resulting that peptide chains of Tf become more stretched. Our results have indicated a mechanism of the interaction between fulvic acid and Tf, which may provide information for possible design of methods to deliver drug molecules via transferrin to target tissues and cells effectively.

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Introduction

Human serum transferrin (Tf) is a single-chain glycoprotein containing 679 amino acids with molecular mass of 80 kDa present in plasma at a concentration of about 35 $\mu$M [1,2]. The main function of transferrin, which has two specific Fe$^{3+}$ binding sites, is to transport iron. Transferrin in human serum is only 30% ion-saturated, and the vacant sites can bind other metal ions [3,4] and some drug molecules, such as flavanone, doxorubicin hydrochloride and vanadocene dichloride [5,6]. High levels of transferrin receptors have been found on the surface of tumor cells [7,8], possibly due to their increased requirement of iron for metabolism, growth, and development [9], which promoted
transferrin to deliver metal ions and drugs to the surface of cancer cells. Transferrin has been proposed as a potential drug carrier by three different methods, which were covalent conjugation, noncovalent binding and hydrogen bonding. Some organic molecules could bind to transferrin by hydrogen bonding [10]. Transferrin could bind with both metal compounds and organic molecules by noncovalent conjugation, which might be the case for most drug delivery in human blood. The further investigation revealed that transferrin can carry anticancer drugs into cancer cells through receptor mediated endocytosis, which make it possible to lay theoretical foundations for targeted delivery. Thus, it has great significance for understanding the mechanism of interaction between transferrin and anticancer drugs. Here we choose fulvic acid to discuss the mechanism of transferrin with the anticancer drugs.

Fulvic acid (FA, Fig. 1) is one of the most interesting natural organic matters, and it is mainly produced by the lower heat value of coal which participated in a variety of biochemical and geochemical processes in the soil [11]. Traditionally, fulvic acid is similar to humic acid, but it is worth noting that fulvic acid has some better active characteristics of smaller molecular weight, multiple active functional groups (hydroxyl, alcoholic- OH, carbonyl etc. [12]) and be absorbed easily by the human body. The pharmacological activities and biological activity of fulvic acid caused widespread concerns of the scholars [13]. Results obtained by Sherry et al. revealed that fulvic acid was shown to possess broad-spectrum and antibacterial activity via investigating the biological properties of fulvic acid [14]. Yamada et al. investigated the anti-allergic effect of fulvic acid and shown that FA may be useful for the treatment or prevention of allergic diseases [15]. The pharmacological properties of fulvic acid may make it a candidate for studies of possible anticancer agents. However, the therapeutic mechanism of fulvic acid has intensively investigated in the past, as drugs molecular, to increase the difficulty of fulvic acid in medical researches and applications, because of the complexities of the structure and composition of fulvic acid. Thus, whether fulvic acid may interact with protein and its mechanism of interaction is well worth discussing in greater depth.

Here we report a detailed investigation on the interaction of fulvic acid with Tf by using fluorescence quenching, thermodynamics, synchronous fluorescence and circular dichroism (CD) under physiological condition. The findings of this article have great significance for illustrating the therapeutic mechanism of fulvic acid in the body, and it may provide a new design of method to deliver drug molecules via transferrin to target tissues and cells effectively and make it possible to lay theoretical foundations for targeted delivery.

Materials and methods

Materials

Human serum transferrin (Tf, Catalog No. T3309, 96–99% purity) was purchased from Sigma and used without further purification. The natural fulvic acid (M, 800 g/mol, the extraction method was in accordance with the procedure proposed by IHSS) was purchased from Shanghai Tongwei Biotechnology Co., used as received. To prepare the phosphate buffered solution (PBS, pH 7.4), analytical grade NaH₂PO₄, Na₂HPO₄ and NaCl from Beijing Chemical Plant (>99%, Beijing, China) were used directly and double distilled water was used as solvents throughout the experiments. Fresh stock solutions of natural fulvic acid (200 μM) were prepared by dissolving into PBS and Tf (50 μM) was prepared by dissolving with PBS for the fluorescence and circular dichroism (CD) experiments. The measured sample was prepared by mixing a quantity of natural fulvic acid with Tf solution, and then diluted by PBS. The sample solutions were kept at 288 K, 298 K, and 310 K for more than 12-h before experiments respectively.

Apparatus

All fluorescence spectra were recorded on F-7000 spectrofluorometer (Hitachi Limited, Japan). The UV absorption spectra were performed on a TU-1901 UV–vis spectrophotometer (Beijing Puxi, China). The circular dichroism spectra were performed on a Jasco-815 automatic recording spectropolarimeter (Jasco, Japan).

Measurements of the fluorescence and circular dichroism spectrum

Fluorescence quenching spectra were obtained in a 1-cm pathlength quartz cell using an excitation wavelength of 295 nm and an emission wavelength of 305–500 nm at three temperatures (288 K, 298 K, and 310 K). Slit widths (5 nm), scan speed (12,000 nm/min) and excitation voltage (400 V) were kept constant within each data set. Excitation and emission bandwidths were 5 nm. The range of synchronous scanning was 260–340 nm and 220–340 nm, with the constant wavelength intervals (Δλ) of 15 and 60 nm, respectively. The scanning speed was kept at 1200 nm/min, and the width of the excitation and emission slits were set at 5 nm.

Circular dichroism (CD) spectra were recorded in a cell of 1-mm pathlength at 298 K. The spectra were collected with scan speed of 500 nm/min and response time of 1 s. Solution pH values were monitored with a PHS-3C pH meter.

Results and discussion

Intrinsic fluorescence quenching of Tf by fulvic acid

Fluorescence spectroscopy is an important method to study the interaction of the drugs with protein, the binding sites and binding constants of drugs and protein can be calculated by fluorescence quenching [16]. If the basic amino acid residues or surrounding environment changed, fluorescence of the protein occurred the corresponding changes. The tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) amino acid residues in Tf have intrinsic fluorescence by the excitation, when excitation at 295 nm, only tryptophan amino acid residues in Tf have intrinsic fluorescence emission [17]. The fluorescence spectra of Tf in the absence and presence of fulvic acid in phosphate buffer of pH 7.4 were measured in the range 305–500 nm upon excitation at 295 nm and showed in Fig. 2. It is evident that Tf displayed a strong fluorescence emission peaked at 345 nm on excitation at 295 nm, while the blank fulvic acid has no fluorescence emission signals at the range of 305–500 nm, thus
the influence of fulvic acid on the Tf intrinsic fluorescence intensity can be negligible. As can be seen from the Fig. 2, the fluorescence intensity was regularly reduced remarkably with the increasing concentration of fulvic acid at 345 nm, the emission peak position did not change significantly, which indicated that the binding of fulvic acid to TF changed the microenvironment around tryptophan and caused transformation of molecular conformation of the protein. We found that the most molecules which contained the reactive groups of hydroxyl group and carboxyl group can interact with the protein. Du et al. [18] explored the effects of conformational changes to transferrin induced by the binding of flavonoids with different numbers and positions of hydroxyl groups, the results showed that the binding ability increase with increasing numbers of hydroxyl groups and further conformational changes were observed. From the fulvic acid molecule (Fig. 1), we can see the aromatic groups and various hydroxyl groups, carbonyl groups and esters, in particular hydroxyl groups, the most contained hydrogen bonds. We inferred that the binding of fulvic acid to TF occurred intrinsic fluorescence quenching, may be due to a number of the reactive groups of hydroxyl groups and carboxyl groups in presence of fulvic acid molecule.

The mechanism of fluorescence quenching can be often classified as either dynamic quenching or static quenching. Dynamic quenching is a process that solution of molecular and fluorescent molecules occurs collide efficiently to produce electron transfer, whereas static quenching is a process that quencher molecule interacts with fluorescent substances produced no fluorescence properties of composites [19]. Depending on the fluorescence quenching constants can determine the type of quenching mechanism. A modified Stern–Volmer analysis was applied to study the Tf fluorescence quenching data. The Fig. 2 shows that the curves have fine linear relationships according to the quenching equation [20]:

\[
\frac{F_0}{F_0 - F} = \frac{1}{f_a K_{sv}[Q]} + \frac{1}{f_a}
\]

where \(F_0\) and \(F\) are the relative fluorescence intensities of Tf in absence and presence of the fulvic acid, respectively, \(f_a\) is the fraction of fluorophore accessible to the quencher, \([Q]\) is the concentration of quencher, and \(K_{sv}\) is the Stern–Volmer quenching constant. From the plots of \(F_0/F_0 - F\) versus \([Q]\), the values of \(f_a\) and \(K_{sv}\) were obtained from the values of the intercept and slope, respectively. The Stern–Volmer quenching constant for fulvic acid was found to be \(1.43 \times 10^{14} \text{ M}^{-1}\). The quenching rate constant of the biomolecule \(K_q\) was evaluated using the equation:

\[
K_q = K_{sv}/\tau_0
\]

where \(\tau_0\) is the lifetime of the protein without the quencher, the \(\tau_0\) value for Tf was found to be 2.5 ns. Hence the value of \(K_q\) was observed for fulvic acid to be \(5.72 \times 10^{12} \text{ M}^{-1} \text{s}^{-1}\). The maximum scatter collision quenching constant, \(K_q\) of various quenchers with the biopolymer was \(2 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}\). Thus, the rate constant of protein quenching procedure initiated by fulvic acid was greater than \(K_q\) of scatter procedure. This indicated that a static quenching mechanism was operative [21].

**Apparent binding constant and interaction force**

It was postulated that the Tf fluorescence quenching was a static quenching process. The association constants \((K_a)\) and the number of binding sites \((n)\) can be obtained from the regression curve based on the following equation [22]:

\[
\lg F_0 - \frac{F}{F_0} = \lg K_a + n \lg [Q]
\]

where \(K_a\) is the binding constant, \(n\) is the number of binding sites, and \(F_0\), \(F\) and \([Q]\) have the same meanings as in Eq. (1). The corresponding values of \(K_a\) and \(n\) are presented in Fig. 3 and Table 1. The value of \(K_a\) indicated that there was a strong interaction and formed a complex between fulvic acid and Tf. The binding constants (\(5.04 \times 10^{6} \text{ M}^{-1}\), \(5.48 \times 10^{7} \text{ M}^{-1}\), \(7.38 \times 10^{6} \text{ M}^{-1}\)) of the fulvic acid were found to be higher than the published values of drug molecules that strongly bind to same site of Tf, such as flavone (\(6.9 \times 10^{4} \text{ M}^{-1}\)) [18], doxorubicin hydrochloride (\(9.7 \times 10^{5} \text{ M}^{-1}\)), vanadocene dichloride (\(3.3 \times 10^{4} \text{ M}^{-1}\)) [6].

Considering the dependence of binding constants on the different temperatures, a thermodynamic process was considered to be responsible for the formation of the complex. Therefore, the thermodynamic parameters dependent on the temperatures were analyzed in order to characterize the acting forces between fulvic acid and Tf [23]. In general, small molecules binding to macromolecules are mainly attributed to four binding modes: hydrogen bond, Van der Waals force, electrostatic and hydrophobic interactions [24]. The thermodynamic parameters, enthalpy change (\(\Delta H\)), entropy change (\(\Delta S\)) and free energy change (\(\Delta G\)) of the interaction are important evidence to estimate the binding mode between the
Table 1
The binding constants and thermodynamic parameters for the Tf–FA system.

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$n$</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>288</td>
<td>$5.04 \times 10^8$</td>
<td>1.78</td>
<td>$-142.24$</td>
<td>$-327.94$</td>
<td>$-47.79$</td>
</tr>
<tr>
<td>298</td>
<td>$5.48 \times 10^7$</td>
<td>1.54</td>
<td></td>
<td></td>
<td>$-44.51$</td>
</tr>
<tr>
<td>310</td>
<td>$7.38 \times 10^6$</td>
<td>1.37</td>
<td></td>
<td></td>
<td>$-40.58$</td>
</tr>
</tbody>
</table>

According to the Förster’s theory [28], the efficiency of energy transfer ($E$) between the donor and the acceptor can be calculated by the following equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + R_{\text{EF}}^6}$$

(6)

where $F$ and $F_0$ are the fluorescence intensities of Tf without and with fulvic acid, $R_0$ is the binding distance between acceptor and donor, and $R_{\text{EF}}$ is the critical distance when the energy transfer efficiency equals to 50%. The value of $R_0$ could be obtained by the following equation:

$$R_0^6 = 8.8 \times 10^{-25}K^2\phi J N^{-4}$$

(7)

where $K^2$ is the orientation factor related to the geometry of the donor–acceptor of dipole, $N$ is the refractive index of medium, $\phi$ is the fluorescence quantum yield of the donor, in the present case, $K^2 = 2/3$, $N = 1.336$, $\phi = 0.118$ [29], and $J$ is the overlap integral of the fluorescence emission spectra of the donor and the absorption spectra of the acceptor and it can be evaluated by the integrating spectra in Fig. 4, $J$ is given by the following equation:

$$J = \frac{\Sigma F(\lambda)\varepsilon(\lambda)\lambda^2\Delta\lambda}{\Sigma\varepsilon(\lambda)\lambda^2\Delta\lambda}$$

(8)

As can be seen in Table 1, $\Delta G$ of the interaction was negative, indicating the spontaneous interaction between fulvic acid and Tf. The negative sign of $\Delta H$ indicated that the interaction between fulvic acid and Tf was exothermic. The values of $\Delta S$ for the binding reaction between fulvic acid and Tf was found to be negative, for Tf–FA complexes, the main source of $\Delta G$ value was derived from a large contribution of $\Delta H$ term with a little contribution from factor $\Delta S$. So, the main interaction between fulvic acid and Tf was believed to be hydrogen bonding and weak van der Waals [25]. This result was consistent with the fact that there were multiple hydroxyl groups and carboxyl groups in fulvic acid structure to form hydrogen bonds easily.

The energy transfer between fulvic acid and Tf

The distance $r_0$ between the ligand and tryptophan residue in transferrin indicated that whether energy transfer for drug molecules systems occurred in the protein [26]. Taking into account an overlap between the emission spectra of Tf and the absorption spectra of the fulvic acid (Fig. 4), an excitation energy transfer mechanism might be assumed [27]. As the fluorescence emission of protein was affected by the excitation light around 295 nm, the spectrum ranging from 305 nm to 500 nm was chosen to calculate the overlapping integral.

Changes of Tf conformation induced by interaction with fulvic acid

Because our fluorescence and fluorescence dynamics experiments indicated that the interaction between Tf and fulvic acid, it is important to examine how the structure of Tf is affected in the Tf–FA system. When drugs bind to a protein, the intramolecular forces responsible for maintaining the secondary and tertiary structures can be altered, resulting in a conformational change of the protein [32]. To verify the change of the structure of Tf binding to drugs, we can use synchronous fluorescence and circular dichroism (CD) studies in the presence of different concentrations of fulvic acid.

Synchronous fluorescence spectra give information about the molecular environment of proteins and have several advantages, such as sensitivity, spectral simplification, spectral bandwidth reduction, and the avoidance of different perturbing effects [33]. It involves simultaneously scanning of both excitation and emission monochromators of a spectrofluorometer while keeping a constant wavelength interval between them. When the wavelength interval $\Delta \lambda$ between excitation and emission wavelength is 15 nm or 60 nm, the synchronous fluorescence spectra offer the characteristics of tyrosine or the tryptophan residues of
proteins [34]. Based on the binding of Tf to fulvic acid, we employed synchronous fluorescence spectra to quantitative determine the conformational change of Tf. The Fig. 5 showed the synchronous spectra of Tf in the presence of appropriate fulvic acid.

From the Fig. 5 we can see that the emission maximum of tyrosine keeps its position, whereas there was a small red shift of tryptophan residues upon addition of the fulvic acid, indicating that the conformation of Tf had changed, the polarity around the tryptophan residues had increased and the hydrophobicity was decreased, it resulted that peptide chains of Tf became more stretched and more closed to water molecules easily.

Circular dichroism (CD) is a suitable method for monitoring the secondary and tertiary structure of proteins [35]. Far-ultraviolet CD spectroscopy is a powerful technique that is used to determine the amount of secondary structures of proteins [36]. The Fig. 6 showed the CD spectra of the Tf and Tf–FA complex obtained at pH 7.4. The blank fulvic acid has no CD signals at the range of 200–250 nm, the CD spectra of the Tf and Tf–FA complex obtained at pH 7.4. The amount of secondary structures of proteins [36]. The Fig. 6 showed the synchronous spectra of Tf in the presence of appropriate fulvic acid.

Far-ultraviolet CD spectra of the Tf–FA system (A) Δλ = 15 nm, (B) Δλ = 60 nm. Line (a–i) the ratio of CFA/Ct = 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, CFA = 10 μmol/L.

Fig. 5. Synchronous fluorescence spectra of Tf–FA system (A) Δλ = 15 nm, (B) Δλ = 60 nm. Line (a–i) the ratio of CFA/Ct = 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, CFA = 10 μmol/L.

Fig. 6. CD spectra of the Tf–FA system. Line (a) 2.5 × 10⁻⁶ mol/L Tf. Line (b) 2.5 × 10⁻⁶ mol/L Tf + 1.25 × 10⁻⁶ mol/L FA. Line (c) 1.25 × 10⁻⁶ mol/L FA.

Conclusions

In this article, the binding of a bioactive component (fulvic acid) with Tf under physiological conditions has been presented for the first time by fluorescence quenching, thermodynamics, synchronous fluorescence and CD studies. Fluorescence quenching analysis has proved the formation of the Tf–FA complex, which is further confirmed by fluorescence dynamics analysis. The results show that fulvic acid is a strong quencher of the fluorescence of Tf through a static quenching mechanism and binds to the protein with high affinity. The interaction between fulvic acid and Tf is due to hydrogen bonding and weak van der Waals forces estimated from the signs of ΔH and ΔS. The distance between fulvic acid and tryptophan residue in Tf was lower than 8 nm after interaction, indicating that the energy transfer happened. From the synchronous fluorescence and CD results, it is apparent that the interaction of fulvic acid with Tf induces a conformational change of the protein with the loss of α-helix content and thus Tf becomes more stretched association with binding to fulvic acid. We infer that the binding of fulvic acid to Tf may be due to a number of the reactive groups of carboxyl groups and hydroxyl groups which contained hydrogen bonds in presence of fulvic acid molecules. These results indicate that fulvic acid could bind to Tf and propose a new exploring for designing drugs with higher binding affinity to Tf.

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