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Research Article

Rapid Screening and Estimation of Binding Constants for Interactions of Fe^{3+} with Two Metalloproteins, Apotransferrin and Transferrin, Using Affinity Mode of Capillary Electrophoresis

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Abstract. The interaction behavior of Fe^{3+} with transferrin and apotransferrin (iron-free form) was investigated in this study using affinity capillary electrophoresis. Change in the mass and charge of protein upon binding to the metal ion in the capillary tube led to variation in its migration time and was used to measure the noncovalent binding interactions by fast screening method. Acetanilide was used as the electroosmotic flow (EOF) marker to avoid possible errors due to the change in EOF during the experiment. The binding results were calculated from the mobility ratios of protein (*Ri*) and EOF marker (Rf) using the formula $(Ri - Rf)/Rf$ or $\Delta R/Rf$. For more comprehensive understanding, the kinetics of the interaction was studied and binding constants were calculated. Results showed that the Fe^{3+} displayed insignificant interaction with both proteins at lower metal ion concentrations (5–25 μmol/mL). However, transferrin exhibited significant interactions with the metal ion at 50 and 100 μmol/mL ($\Delta R/Rf$ = 0.0114 and 0.0201, resp.) concentrations and apotransferrin showed strong binding interactions ($\Delta R/Rf$ = −0.0254 and 0.0205, resp.) at relatively higher Fe^{3+} concentrations of 100 and 250 μmol/mL. The binding constants of 18.968 $mmol^{-1}$ and −13.603 $mmol^{-1}$ were recorded for Fe^{3+} interaction with transferrin and apotransferrin, respectively, showing significant interactions. Different binding patterns of Fe^{3+} with both proteins might be attributed to the fact that the iron-binding sites in transferrin have already been occupied, which was not the case in apotransferrin. The present study may be used as a reference for the investigation of protein-metal ion, drug-protein, drug-metal ion, and enzyme-metal ion interactions and may be helpful to provide preliminary insight into the new metal-based drug development.

1. Introduction

Iron is essential for biological systems as it plays an important role as a cofactor in several important biological processes, including respiration and DNA replication. It is insoluble and toxic in the free iron (III) ion form. Transferrin is an iron-transport protein that is crucial for the delivery of iron (Fe^{3+}) to the cells. In the process of iron transportation, iron-bound transferrin (holofrom) interacts with transferrin receptors at the cell surface and gets transported to endosomes, where iron is released due to acidic pH. Iron-free transferrin (apofrom) gets back to the cell surface and dissociates from the receptor [1–3]. Iron (III) ion preferably binds to the hard functional groups, including aspartate, glutamate, tyrosine, and histidine amino acid residues [4]. Conversely, the iron ion of low oxidation state (Fe^{2+}) favorably interacts with moieties containing borderline acid groups such as porphyrin residue in hemoglobin. The hard and soft acid and base (HSAB) principle can explain the mechanism of metal ion-ligand complexation reactions in a qualitative manner and provide information regarding the stability of their complexes [5]. The classification of metal ions and other ligands into hard and soft acid-base along with their characteristics and binding preferences according to the HSAB principle is represented in Figure 1. Transferrin, an iron-transport protein, consists of iron-binding nonheme glycoproteins widely distributed in the physiological fluids and cells of organisms. The main function of transferrin is to transport Fe^{3+} through the circulatory system and deliver it to cells by endocytosis [6, 7]. It consists of two similar but nonidentical metal ion-binding locations present in N- and C-terminal domains. Generally, transferrin interacts with two Fe^{3+} ions to produce a pink-colored complex in the presence of bicarbonate (or carbonate) ions. The binding of Fe^{3+} ion to transferrin causes a significant conformational alteration in the protein structure, resulting in the conversion of the *open* (apoprotein) form to the *closed* (holoprotein) conformation. Apotransferrin is known as an iron-depleted form of transferrin. Only the holoform of transferrin can bind to the receptor, while it could not recognize the apotransferrin. Transferrin can also interact with a number of di-, tri-, and tetravalent metal ions, including those from transition, lanthanide, and actinide series, with a mechanism similar to that of Fe^{3+}-transferrin interaction. Evidence indicating the transferrin cycle as an important cellular uptake pathway for several clinically important metal ions is available. Furthermore, transferrin is often viewed as promising delivery means for cytotoxic metal ions and metal ion-based therapeutics to malignant cells [8–10].

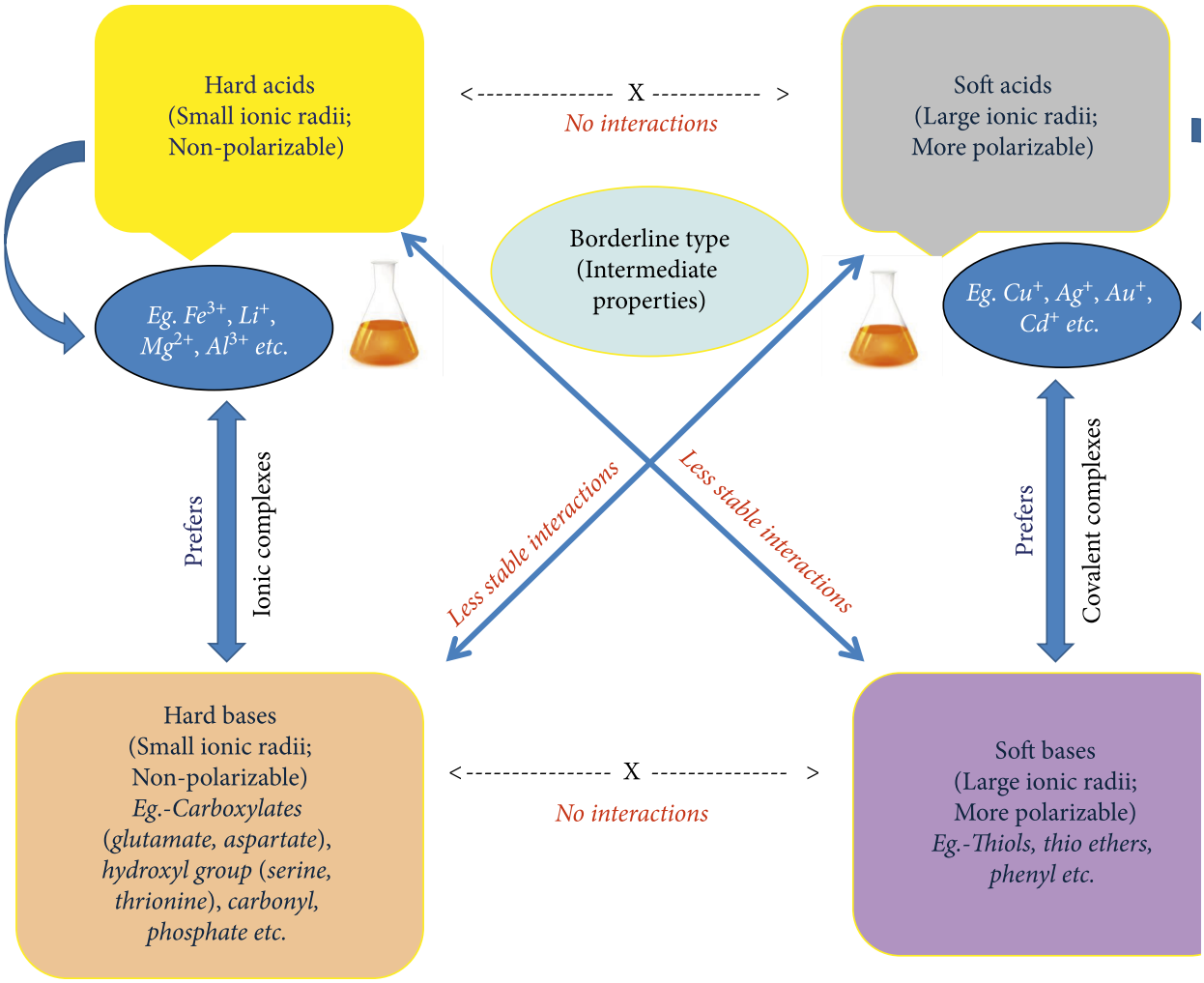


Figure 1: Hard and soft acid and base (HSAB) principle for probable complex formation between metal ions (acid) and base ligands.

1.1. Analytical Techniques for Protein-Metal Interaction Study

Proteins are generally complex in nature, rendering the investigation of protein-metal ion interaction a challenging task. Various analytical techniques, including NMR spectroscopy, FTIR spectroscopy, X-ray crystallography, atomic force microscopy (AFM), circular dichroism (CD) spectroscopy, and surface plasmon resonance (SPR), have been used for the investigation of protein-metal ion interactions [6, 7]. The interaction of proteins with molecular weights of <40 kDa can be easily analyzed with the NMR technique. However, for larger proteins (molecular weight ≥40 kDa), isotopically labeled samples are used, which make the analysis complicated [11], whereas considerably longer time (4-5 weeks) is needed in the sample preparation in X-ray crystallography; even in some cases, the crystallization is very difficult [12]. Both X-ray crystallography and NMR spectroscopy are more preferred for structure elucidation and may be used further to verify the results obtained from other techniques. Affinity capillary electrophoresis (ACE) and affinity chromatography are two important techniques used recently for studying protein-metal ion bindings. Because of their good separation capabilities, these techniques are advantageous when protein samples are impure, including a biological sample. However, with affinity chromatography, cost considerations become important owing to the use of affinity columns besides a large amount of sample for analysis [13, 14]. Recently, ACE was successfully used for studying the interaction of various proteins

with different metal ions [15–18]. Various isoforms of proteins were also investigated simultaneously using ACE, which could easily separate each isoform in relatively lesser separation times [19]. ACE-UV has been considered to be a good option for studying protein-metal ion interactions due to easy handling, cost-effectiveness, and reliable results. Additional advantages of ACE include the requirement of a very small volume of sample, high separation efficiency, and the ability to perform the experiment at pH 7.4 and 37°C, closely depicting the native environment of proteins under physiological conditions. Due to excellent separation efficiency, proteins and other biological samples can be analyzed directly in their impure form, minimizing the sample preparation time [20–24].

1.1.1. Affinity Capillary Electrophoresis (ACE)

ACE is used to study the relative affinities of two reactants using the separation power of the capillary electrophoresis. Recently, ACE gained much attention in studying protein-metal ion interactions and determining their binding constants [25–27]. It is generally utilized to study noncovalent binding interactions between an analyte and a ligand, where the alteration in electrophoretic migration (mobility shift), area, and/or height of the analyte peak before and after binding is measured. One of the three ACE modes, namely, preequilibrated, dynamic equilibrium, and kinetic modes, may be used to investigate the interaction behavior of protein and metal ions. Dynamic equilibrium mode is generally preferred when the relaxation time of metal ion binding is shorter than electrophoretic separation time. The interaction between analyte and ligand takes place in the capillary tube during the electrophoretic run; hence, the dynamic mode has the advantage of being faster than other modes (no extra time for interaction is required). Dynamic equilibrium mode using mobility shift ACE was selected for this study because it is highly sensitive and is capable of detecting even very weak interactions correctly. The precision of the method can further be increased by calculating the mobility ratio for the interaction screening [15–19]. In ACE, the mobility ratio (R), which is the ratio of the analyte mobility (μ) and can be expressed as

$$R = \frac{\mu_{\text{eof}}}{\text{pr t}}, \tag{1}$$

where R is the mobility ratio and μ_{prot} and μ_{eof} represent the mobility of protein and EOF marker, respectively. The mobility can be calculated by

$$\frac{x\ D}{Vt} \qquad 2$$

where " " represents the applied voltage and "x" and "D" are the effective and total length of the capillary tube, respectively. All these factors except migration time "t" are common for both protein and EOF marker and hence will be canceled. Thus, the mobility ratio of the interaction can be calculated using the recorded migration time by

$$\frac{\quad}{\quad} \qquad 3$$

where t_{eof} and t_{prot} represent the migration time for EOF marker and protein, respectively [19, 22].

Although the interaction of Fe^{3+} ion with transferrin has been investigated using various analytical techniques [28–30], no study based on ACE has been conducted so far. Therefore, the present work was commenced to study the interaction behavior of Fe^{3+} with transferrin and apotransferrin using fast screening method by mobility shift affinity capillary electrophoresis and determine the binding constants for better understanding of interaction behavior. The current study will provide a reference for the application of ACE in the investigation of different types of interactions such as enzyme-metal ion, drug-protein, drug-enzyme, and protein-protein interactions, in addition to determining the binding behaviors of biologically significant metal ions to various metalloproteins. Consequently, this study may prove to be useful to afford preliminary information in the development of new metal-based therapeutic agents.

2. Materials and Methods

2.1. Chemicals and Reagents

Human transferrin (98%), apotransferrin (97%), tris powder, ferric chloride, and ferrous chloride were purchased from Sigma-Aldrich, Germany. Malonic acid and HPLC grade acetonitrile were procured from Riedel deHäen (Hannover, Germany) and Fluka (Steinheim, Germany), respectively. Double distilled water was produced in-house using the Millipore Milli-Q water purifier system (Molsheim. France).

2.2. Instrumentation

Agilent Capillary Electrophoresis (CE) system, model no. G1600A (Agilent Technologies, Germany), was used to screen protein-metal ion interactions by recording electrophoretic mobility of the analyte protein before and after complexation. The CE instrument was equipped with a capillary cooling system, an autosampler, and a diode-array detector which was operated at 214 nm wavelength. Electrophoretic migration was performed on a bare-fused silica capillary tube with an effective and total length of 22 cm and 31 cm, respectively (short capillary), and an internal diameter of 50 μm. The capillary was procured from Polymicro Technologies (Phoenix, AZ, USA). A normal air plug was utilized to apply high pressure (2.5 bars) at the laboratory. The pH of the solutions was adjusted using the Jenway pH meter (Cole-Parmer, Staffordshire, United Kingdom). ROTILABO CME syringe filters (25 mm dimension and 0.22 μm pore size) were procured from Carl Roth, Germany. The instrument was monitored, screening data was processed by ChemStation software (Agilent Technologies, Germany), and Microsoft Excel version 2010 (Microsoft Corporation, USA) was used for simulation of different interaction parameters, including mobility ratios (R), $\Delta R/Rf$, and confidence interval.

2.3. Rinsing Procedure for Regeneration of Capillary

Initially, the new capillary tube was conditioned for 20 min at 1 bar with 1 N sodium hydroxide solution followed by ultrapure water. At the beginning and end of each working day, extra flushing with 0.1 N NaOH solution for 10 min followed by water for 5 min at 2.5 bar was performed. Furthermore, at the beginning of each analysis, capillaries were conditioned with a mixture of 0.1 M EDTA and 0.1 N NaOH solutions at 2.5 bar for 2.5 min, ultrapure water for 1.0 min, and running buffer for 1.5 min.

2.4. Electrophoretic Conditions

Electrophoretic migration of analyte was achieved by applying a voltage of 10 kV at 23°C capillary temperature. The separation was performed at electrophoretic mode, where anode was at the inlet and cathode at the outlet of the capillary (normal mode). Sample solutions were injected into the capillary at 50 mbar using the hydrodynamic mode, and after sample injection, the running buffer was injected at 50 mbar for 2.5 s. Overall, twelve runs were completed for each sample, including six runs for analyte protein with Fe^{3+} and six runs without metal ion.

2.5. Preparation of Analytical Solution

2.5.1. Tris Buffer (pH 7.4, 20 mmol/L)

Accurately weighed 2.42 g of tris powder was dissolved in approximately 200 mL double distilled water. The pH of the solution was adjusted with dilute HCl to 7.4, and the volume was adjusted to 1000 mL using water.

2.5.2. Acetanilide Stock Solution

Accurately weighed 37.5 mg of acetanilide was transferred in 50 mL tris buffer (pH 7.4) and dissolved completely by sonication to obtain a concentration of 750 μg/mL.

2.5.3. Protein Working Solutions

Appropriate quantities of transferrin and apotransferrin were taken separately into 25 mL volumetric flasks and dissolved in small amounts of tris buffer. Aliquots of 5 mL of acetanilide stock solution were added to each flask, and the volumes were completed with tris buffer to obtain solutions having 15 μmol/L concentrations for both proteins. To minimize protein adsorption on the wall of the capillary and band broadening, the above protein solutions were diluted threefolds in tris buffer.

2.5.4. Iron (III) Ion Solution

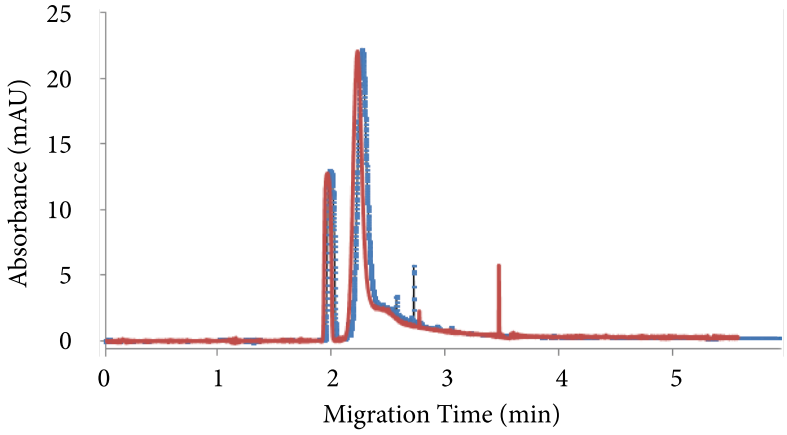
An appropriate amount of ferric chloride was dissolved in a solution containing 20 mmol/L tris buffer (unadjusted pH) and 15 mmol/L malonic acids to achieve a stock solution of 5 mmol/L concentration of metal ion (Fe^{3+}). As iron (III) is not properly soluble in tris buffer and tends to precipitate; hence, malonic acid was added to assist the dissolution of ferric chloride. Malonic acid weakly chelates the iron (III) ions leading to the formation of a complex which could exchange the ligand metal ions with protein residues [19]. The metal ion solution was then diluted in tris buffer (pH 7.4, 20 mmol/L) to obtain working solutions of 5, 10, 25, 50, 100, and 250 μmol/L concentrations. The protein and metal ion solutions could be used for only one day; consequently, all the solutions were freshly prepared at the beginning of each working day. All the prepared solutions were filtered using 0.22 μm syringe filter before analysis.

3. Results and Discussion

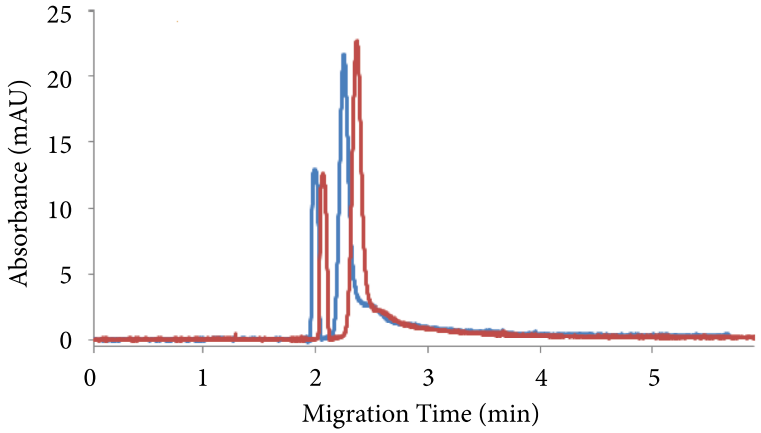
3.1. Fast Screening by Normalized Difference of Mobility Ratios

In ACE, migration time (mobility) of analyte protein is changed because of the variation in overall mass and charge following complexation with metal ions. This has been used as a measure of the extent of interaction between the metal ions and test proteins. Protein adsorption on the inner surface of the capillary tube often reduces the precision of binding results. Furthermore, binding of protein analyte with the inner capillary surface may lead to alteration in the electrophoretic mobility during electrophoretic run [31, 32]. Therefore, to minimize possible errors in the results caused by variation in the electrophoretic mobility of the protein, an EOF marker is generally used. In this work, acetanilide, which is neutral at physiological pH (7.4), was used as an EOF marker, and mobility ratios of proteins and EOF marker (μ_{prot}/μ_{eof}) were used to measure the interaction. In order to reduce protein adsorption and to obtain good interaction results, an appropriate rinsing procedure as mentioned above in the rinsing protocol section was implemented.

In the current study, the interaction between Fe^{3+} ion and two transport proteins, transferrin and apotransferrin, was screened, and the interaction results were then evaluated by their mobility ratios with precise results. The presence of consistent noncovalent interactions was confirmed by a change in the electrophoretic migration of proteins after interaction with Fe^{3+} ion (Figure 2). The strength of interactions was calculated as the difference of mobility ratios of protein before and after interaction with a metal ion (*Rf* and *Ri*, resp.) normalized by *Rf* ($\Delta R/Rf$; $\Delta R = Rf - Ri$) and confidence intervals (*cnf*), by the calculation method described by Alhazmi et al. [19]. Even a small change in protein mobility is enough to detect an interaction with a ligand metal ion, and a calculated value of $\Delta R/Rf \geq 0.01$ either in positive or negative direction proves a significant interaction [19].


Absorbance (mAU)
25
20
15
10
5
0
0
1
2
3
4
5
Migration Time (min)

(a)


Absorbance (mAU)
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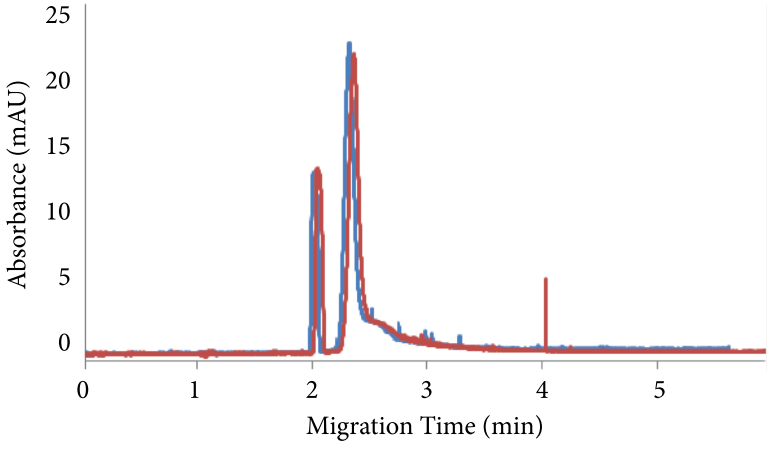
Migration Time (min)

(b)


Absorbance (mAU)
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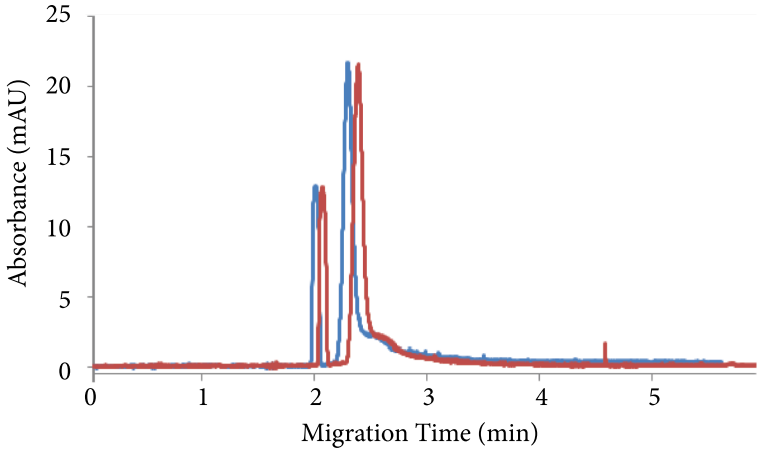
5
Migration Time (min)

(c)



(d)

Figure 2: Representative electropherograms showing the change in the electrophoretic mobility (migration time) of transferrin and apotransferrin after iron (III) ion interaction. The first peak is due to acetanilide (EOF marker) and the second peak belongs to the protein analyte. (a) *Blue line:* transferrin without Fe^{3+}; *red line*: transferrin with Fe^{3+} at 50 μmol/L iron (III) ion concentration. (b) *Blue line*: transferrin without Fe^{3+}; *red line:* transferrin with Fe^{3+} at 100 μmol/L concentration. (c) *Blue line:* apotransferrin without Fe^{3+}; *red line*: apotransferrin with Fe^{3+} at 50 μmol/L iron (III) ion concentration. (d) *Blue line*: apotransferrin without Fe^{3+}; *red line:* apotransferrin with Fe^{3+} at 100 μmol/L concentration.

Transferrin and apotransferrin interacted with iron (III) ion and electropherograms thus obtained were recorded. Metal ion concentrations of 5, 10, 25, 50, 100, 150, and 250 μmol/L were used for the study, and the results are summarized in Tables 1 and 2. At each metal ion concentration, 12 runs were performed for both protein samples (six without metal ion and six with metal ion), and corresponding mobility ratios (*Rf* and *Ri*) were calculated. It is evident from Table 1 that the % relative standard deviations (%RSDs) of the interaction results were less than 2% for both test proteins at most of the metal ion concentrations, demonstrating high precision results. Among the investigated metal ion concentrations, significant binding interactions ($\Delta R/Rf \geq 0.01$) were observed at 50 and 100 μmol/L with transferrin, while at concentrations of 100 and 250 mmol/L, apotransferrin showed the highest interactions. At other concentrations, the metal ion showed weaker affinities ($\Delta R/Rf < 0.01$) towards both test proteins; however, significantly smaller confidence intervals were observed at all the tested concentrations of the metal ion. The negative and positive signs of $\Delta R/Rf$ values as recorded in the screening results are also important as they could give valuable information about the coordination of bound metal ions with other protein residues and/or surrounding anions in the capillary tube. In general, an absolute value of $\Delta R/Rf \geq 0.01$ is enough to detect remarkable interactions [19, 33]. More importantly, for a significant protein-metal ion interaction, the value of *cnf* of $\Delta R/Rf$ should not overpass the zero values in $\Delta R/Rf \pm cnf$. In normal circumstances, a positive value of $\Delta R/Rf$ is achieved because the overall charge on the protein will get more positive (less negative) after binding to the metal ion. However, a negative value of $\Delta R/Rf$ could alternatively be obtained when the bound metal ions on the protein further interact coordinatively with the surrounding anions present in the medium, which may result in a less positive (more negative) overall charge on the protein molecule.

Table 1: Percent relative standard deviation (% RSD) of mobility ratios recorded from transferrin- and apotransferrin-iron (III) interactions at different iron (III) ion concentrations.

Iron (III)	Percent relative standard deviation*

concentrations (μmol/L)	Transferrin		Apotransferrin	
	Rf	*Ri*	*Rf*	*Ri*
5	1.28	0.60	0.19	0.11
10	0.61	0.70	0.18	0.66
25	0.37	0.16	0.13	0.37
50	1.22	0.94	0.91	0.54
100	0.84	0.54	0.03	0.53
150	1.08	0.28	0.88	0.10
250	1.23	1.41	0.14	0.18

$n = 6$.

Table 2: Average $\Delta R/Rf$ values and their confidence intervals (*cnf*) for transferrin- and apotransferrin-iron (III) interactions at different iron (III) ion concentrations.

Iron (III) ion concentrations (μmol/L)	Transferrin-iron (III) ion interaction $\Delta R/Rf \pm cnf$	Apotransferrin-iron (III) ion interaction $\Delta R/Rf \pm cnf$
5	−0.0007 ± 0.0128	−0.0042 ± 0.0019
10	0.0012 ± 0.0085	0.0021 ± 0.0062
25	−0.0007 ± 0.0037	−0.0008 ± 0.0035
50	0.0114 ± 0.0141	0.0035 ± 0.0096
100	−0.0201 ± 0.0090	−0.0254 ± 0.0047
150	−0.0075 ± 0.0101	0.0070 ± 0.0080
250	−0.0012 ± 0.0169	−0.0205 ± 0.0020

Average values; $n = 6$.

As shown in Table 2, some interesting results were obtained for the interaction of test proteins with iron (III) ions. At lower concentrations (5, 10, and 25 μmol/L) of metal ion, insignificant interaction with transferrin ($\Delta R/Rf = -0.007$, 0.0012, and −0.007, resp.) and apotransferrin ($\Delta R/Rf = -0.0024$, 0.0021, and −0.0008, resp.) was observed. A significant interaction between iron (III) ion and transferrin was recorded at 50 and 100 μmol/L of metal ion concentrations ($\Delta R/Rf = 0.0114$ and 0.0201, resp.), whereas the test metal ion exhibited significant binding affinities with apotransferrin at 100 and 250 μmol/L concentrations ($\Delta R/Rf = -0.0254$ and 0.0205, resp.). Interestingly, both proteins showed the strongest binding interactions at 100 μmol/L iron (III) ion concentration. For most of the interactions, negative $\Delta R/Rf$ values were observed, indicating that the protein-bound metal ions further coordinate with the adjacent anions present in the medium rather than other residues at the binding site of the protein and therefore the overall charge on the proteins get more negative. Furthermore, metal ion interaction with other high-affinity amino acid residues at the binding site is also possible. Protein-metal ion complexes are formed by interaction at more than one amino acid residue, and the binding sites for metal ions can be confirmed using NMR spectroscopy and X-ray crystallographic techniques [34]. At 50 μmol/L metal ion concentration, the overall charge on both proteins becomes more positive after interacting with the Fe^{3+} ion, and therefore, positive values of $\Delta R/Rf$ were obtained. This can be explained by the fact that the number of bound metal ions coordinating with binding site residues is higher than those with the surrounding anions. The variation of results from negative to positive $\Delta R/Rf$ values and vice versa is might be due to the availability of multiple metal ion-binding sites on

proteins with different binding properties. In addition to alteration in the migration time, slight variation in size and shape of the peaks due to protein-metal ion complex was also recorded at certain metal ion concentrations. This may be due to the conformational changes in protein structure as a result of interaction with metal ion (Fe^{3+}). Change in peak shape may also be attributed to variation in pH or ionic strength of the medium [31, 32].

3.2. Kinetics Binding Constants

Upon confirmation of significant noncovalent interactions between Fe^{3+} and test proteins, binding constants of the interactions were estimated. Four mathematically plotted models are used to estimate the binding constants of ligand-biomolecule interaction, which are nonlinear regression, X-reciprocal, Y-reciprocal, and double-reciprocal methods as shown in equations (4)–(7) [22, 35]:

(a) Nonlinear regression:

$$Kc\,(L) \quad \frac{f - i}{c}. \tag{4}$$

(b) X-reciprocal:

$$\frac{1}{\quad} \quad \frac{\quad}{(\quad)} \times \frac{\quad}{\quad} \quad + \frac{\quad}{\quad} \tag{5}$$

(c) Y-reciprocal:

$$\frac{\quad}{\quad} \quad \frac{\quad}{\quad} \quad \frac{\quad}{\quad} \tag{6}$$

(d) Double-reciprocal:

$$\frac{\quad}{\quad} \tag{7}$$

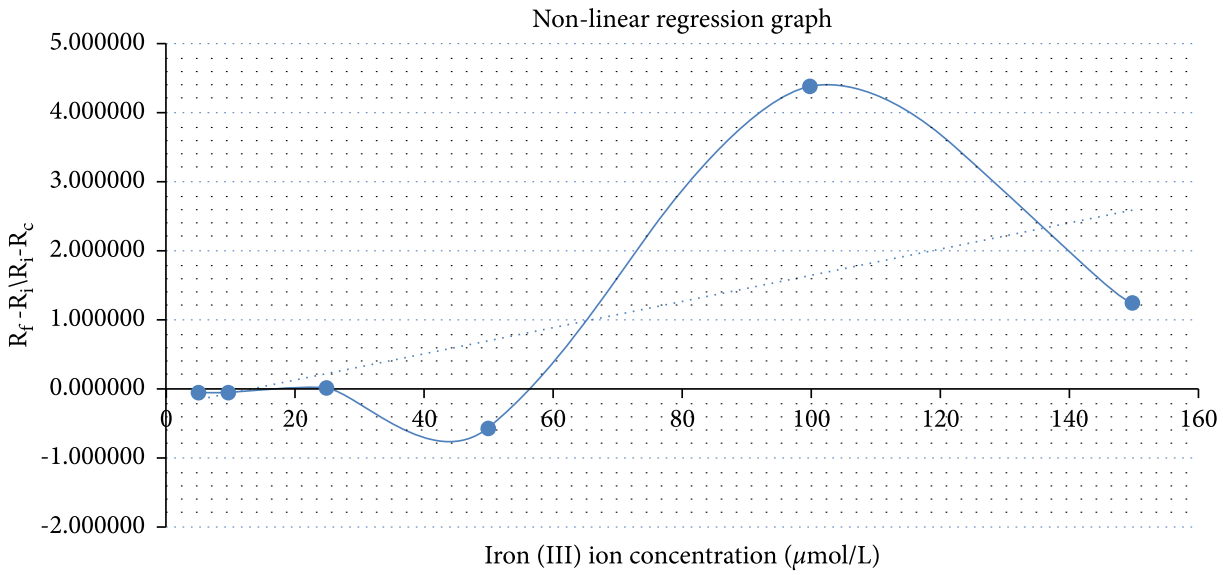
where K is the binding constant; c (L) is the micromolar concentration of ligand (metal ion); Rf and Ri are mobility ratios of the protein before and after interacting with the ligand (metal ion) in the capillary tube respectively; Rc represents the mobility ratio of analyte protein at saturated ligand concentration. All these plotting methods have different statistical treatment of data points as depicted in Table 3. Since a ligand especially metal ion can bind to multiple target sites on the proteins, 1 : 1 binding stoichiometry is uncommon. Therefore, in the screening of binding interaction between protein and metal ions using the ACE technique, nonlinear regression analysis was found to be the most appropriate method. Moreover, in ACE analysis, estimation of association and dissociation constants is less sensitive to random error while using the nonlinear regression method. The literature also revealed that the cumbersome weighted regression procedure is avoided in the nonlinear regression method, which is necessary for the statistical analysis of the other three methods (linearized plots) [36]. Consequently, in the present investigation, the nonlinear regression method was used for the estimation of binding constants.

Table 3: Statistical components of four plotting methods used for the calculation of binding constant (K).

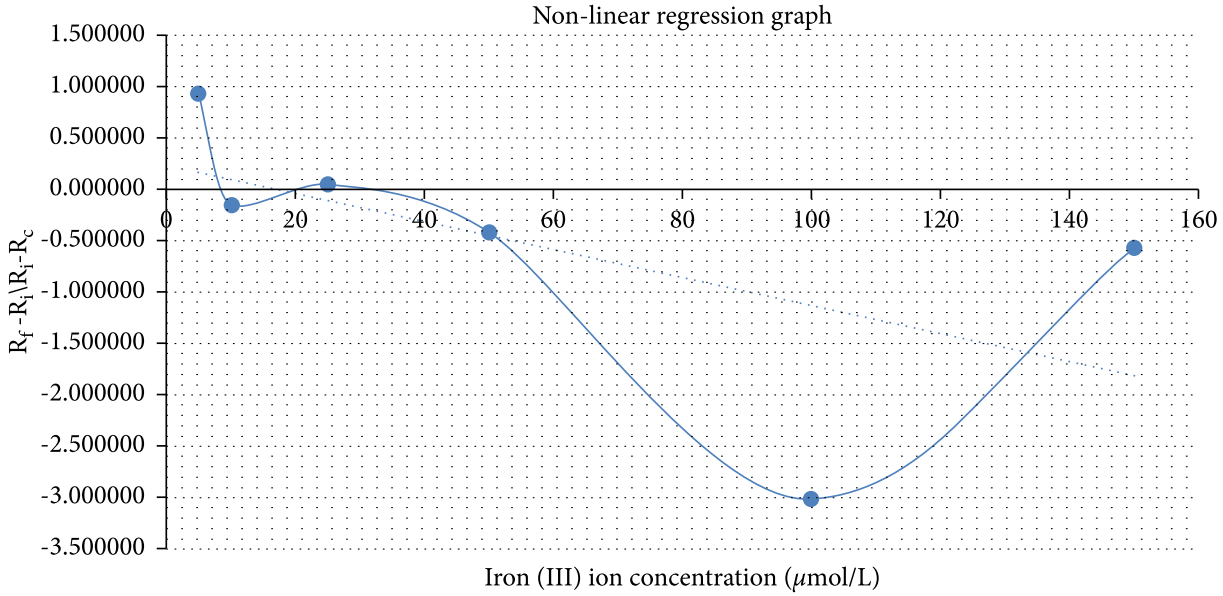
Plotting methods	Fitting method	K	Reference
Nonlinear regression	/ v su	Slope	[22, 35]
X-reciprocal	versus	Slope	[22, 35]
Y-reciprocal	versus c (L)	In c Sl	[22, 35]
Double-reciprocal	versus		[22, 35]

K: binding constant; c (L): micromolar concentration of ligand (metal ion); Rf and Ri: mobility ratios of the protein before and after interaction with the ligand in the capillary tube, respectively; Rc: mobility ratio of analyte protein at saturated ligand concentration.

Metal ion concentrations in the range of 5–250 μmol/L were used to calculate the binding constants. As shown in Table 3, the binding constant can be calculated using Rc, which is the mobility ratio of saturated protein measured at the highest metal ion concentration. A Fe^{3+} ion concentration of 250 μmol/L was used for both transferrin and apotransferrin. The mobility ratios obtained at 250 μmol/L Fe^{3+} concentration were used in the nonlinear regression analysis, and the binding constants were calculated to be 18.968 $mmol^{-1}$ and −13.6031 $mmol^{-1}$ for transferrin and apotransferrin, respectively. The binding plots were drawn between $(Rf - Ri)/(Ri - Rc)$ and ligand (metal ion) concentration [22, 35], and the progress of interaction was then analyzed using respective binding curves. Interestingly, the Fe^{3+} ion was observed to behave differently with the two test proteins (Figure 3). As discussed in the case of $\Delta R/Rf$, values, the positive and negative signs of the binding constant are attributed to the behavior of metal ions at different binding sites in the tested proteins and the additional coordination with surrounding background ions. The negative and positive values of binding constants indicate the direction of the binding interaction between the metal ion and protein. The negative sign does not mean that the value is less than zero and be considered as noninteraction. Hence, the binding constant value can be used for the characterization of protein-metal ion interaction [22]. The nonlinear regression curve revealed that the transferrin exhibited insignificant binding interaction with Fe^{3+} at initial concentrations (5, 10, and 25 μmol/L), whereas linearly increasing affinity was observed at metal ion concentrations of 50 and 100 μmol/L and a positive value of binding constant (18.968 $mmol^{-1}$) was obtained. On the other hand, a negative value of the binding constant (−13.603 $mmol^{-1}$) was obtained for the interaction between Fe^{3+} and apotransferrin. At the initial metal ion concentration (5 μmol/L), a positive and slightly stronger binding than transferrin was observed in case of apotransferrin, while it changed to be negative at 10 μmol/L and again positive at 25 μmol/L. Finally, the binding affinity between the metal ion and apotransferrin sharply increased at 100 and 250 μmol/L metal ion concentrations, and the binding pattern was shifted to a less negative direction. Different signs of the binding constants, positive for transferrin and negative for apotransferrin, and nonidentical patterns of binding curves could be attributed to different target binding sites on the tested proteins. The tested metal ion binds to nonspecific binding sites on transferrin (specific binding sites already occupied by Fe^{3+}), whereas both specific and nonspecific binding sites in case of apotransferrin lead to different interaction behaviors of Fe^{3+} on both tested proteins.



(a)



(b)

Figure 3: Binding curve showing (a) transferrin-Fe^{3+} and (b) apotransferrin-Fe^{3+} interactions using nonlinear regression analysis. *Ri* and *Rf* represent the mobility ratios of proteins (t_{eof}/t_{prot}) recorded in the presence and absence of metal ions at different concentrations, respectively. *Rc* represents the mobility ratio of the protein (t_{eof}/t_{prot}) measured at a saturated concentration of metal ion (250 μmol/L).

Overall, the use of fast screening by the normalized difference of mobility ratios is inadequate for studying and comparing the binding of metal ions to proteins. Consequently, in the present study, the fast screening method showed unclear binding results between the tested proteins and Fe^{3+}. An insignificant binding interaction was observed at lower iron (III) ion concentration with both tested proteins. Transferrin exhibited greater affinities at

medium concentrations (50 and 100 μmol/L), whereas apotransferrin was found to show stronger interactions at higher metal ion concentrations (100 and 250 μmol/L). The interaction behavior became more evident from the kinetic study of the interaction. The calculation of binding constants and curve patterns for the interaction between proteins and iron (III) ion revealed that the metal ion exhibited significant binding affinities with both proteins; however, it behaved differently due to differences in the availability of the specific binding sites on both proteins.

4. Conclusion

In the present study, noncovalent interactions between two important metal transport proteins and iron (III) ions were investigated using the ACE technique. During the analysis, inner capillary wall was intermittently regenerated by applying proper rinsing protocol, the low voltage was applied to decrease joule heat, and use of a short capillary tube has offered a fast and precise analysis. The fast screening method was based on the calculation of the normalized difference of mobility ratios ($\Delta R/Rf$) of protein and electroosmotic flow marker, representing the binding results (a value of $\Delta R/Rf \geq 0.01$ indicates significant binding). Transferrin showed significant binding interaction at 50 and 100 μmol/L concentrations of iron (III) ion, whereas apotransferrin exhibited remarkable binding affinity to metal ion at 100 and 250 μmol/L concentrations. Both proteins showed insignificant affinities at lower metal ion concentrations (5–25 μmol/L). In addition to that, binding constants of the interaction of iron (III) ion and test protein were estimated to obtain a more comprehensive understanding of the affinity of metal ions to the proteins. The binding constants of 18.968 $mmol^{-1}$ and −13.603 $mmol^{-1}$/L for iron (III) ion interaction with transferrin and apotransferrin, respectively, were obtained. The positive and negative values of binding constants indicated different behaviors of Fe^{3+} ion with transferrin and apotransferrin, which may be attributed to the difference in the availability of specific and nonspecific binding sites on both proteins. The outcomes of the present investigation recommended the use of kinetic study in combination with the fast screening method in the ACE technique to understand the interactions between proteins and metal ions.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no potential conflicts of interest related to this manuscript.

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