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Measurement of interaction behavior of six biologically important noble metal ions with the iron(III) binding protein, apo-transferrin, using mobility-shift affinity electrophoresis

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Transferrin is iron (III)-binding protein that transports Fe (III) ion to cells in different parts of the body. At saturation level 70% of transferrin remains free from iron (apo-transferrin), suggesting a broader scope of binding capabilities with non-iron (III) metal ions. Our previous work demonstrated that transferrin can effectively coordinate with noble metal ions. Hence to improve our understanding of the *in-vitro* metal-interaction behavior of the protein and how it correlates with its functions *invivo*, the interactions between apo-transferrin and medicinally important noble metals, Au (I), Au (III), Os (III), Pt (IV), Ir (III) and Ru (III) were investigated in the mobility shift mode of affinity capillary electrophoresis. The binding interactions were estimated according to the variation in electrophoretic mobility of the protein after coordination with the metal ions at physiological pH 7.4. $\Delta R/Rf$ values and confidence intervals were used to express the interaction results, which were calculated by using mobility ratios of protein with and without metal ions (R_f and R_i respectively) with respect to an electrophoretic marker (acetanilide). All the tested metal ions were found to be coordinated well with apo-transferrin, out of those Ir (III) exhibited the strongest interaction followed by the Au (I) ion. Noticeable variations in the shape and intensity of the protein peaks have been observed after interaction with some of the metal ions, especially those showing high binding affinities, which is probably due to conformational changes in the protein structure. The screening results suggest that apo-transferrin can interact with a variety of non-iron (III) metal ions and be the medium of transport for those metal ions into the biological system. This extended binding capability of apo-transferrin can be utilized for the targeted delivery of therapeutically significant metal ions. It can also be helpful in the development of new metal-based drugs for the treatment of a variety of ailments.

1. Introduction

The human serum transferrin (TF) is a single chain glycoprotein comprising 680-690 amino acid residues together with two glycan moieties and has a molecular weight of approximately 80 kDa. In healthy human serum, TF is present at a concentration of 25-50 μM and its main function is to transport Fe^{3+} to the cells throughout the body from the sites of intake in the systemic circulation (MacGillivray et al. 1983; Sun et al. 1999; Williams and Moreton 1980). It contains two structurally similar lobes known as N-lobe and C-lobe located in the N-terminal and C-terminal domains of the protein, respectively. The N- and C-lobes contain similar amino acid residues, though they are different in kinetic and thermodynamic behaviors towards iron uptake and release. The C-terminal binding site shows greater binding affinity for iron than the N-terminal, furthermore, at the C-terminal site of TF, iron remains attached at pH values down to 4.8, whereas the N-site cannot bind below pH 5.7. In addition, at physiological pH (7.4) the stoichiometric binding constant of N-terminal site is 5 times lower than the C-terminal site. From the results of various physicochemical experiments, it has been accepted that the iron binding sites in transferrin consists of two tyrosine and two histidine residues, a carbonate/bicarbonate ion and a hydroxide ion (from water) forming six coordinate complex altogether with iron (Bellounis 1996; Chung 1984).

Each lobe makes a cleft providing a binding site for one Fe^{3+} ion, forming two binding sites for Fe^{3+} in TF molecule. Iron is positioned in these cleft of N- and C-lobes which get closed around the metal ion and a synergistic anion (a bidentate carbonate ion). As a result transferrin adopts two conformational forms, a closed or 'holo' (diferric) form and an open or 'apo' (iron-free) form. It has been reported that the diferric transferrin (holo form, TF-2Fe) is taken up into cells through receptor-mediated endocytosis, in which the TF-metal complex interacts with the transferrin receptor present on the cell surface forming a TF-TF receptor complex at pH 7.4. Afterwards, the TF-TF receptor complex is internalized into the cell, where the pH is 5.5. As a result of reduction in the pH value, iron is released from the complex and transferrin changes to open (apo-transferrin) from closed conformation. The apo-transferrin together with its receptor returns to the plasma membrane to eventually be released into plasma and reutilized (Keshavarz et al. 2015; Harding et al. 1983).

In normal human serum, TF is only 30% saturated with iron and the remaining 70% exists as apo-transferrin, indicating that there is a considerable binding capacity for metal ions that enter the systemic circulation (Kovac et al. 2009; Xiu-Lian et al. 2004). Transferrin can also bind a number of di-, tri- and tetravalent metal ions from lanthanide, actinide and transition groups with mechanism of reaction which appear to be similar to that for Fe^{3+} binding (Chung 1984). For example, transferrin interacts effectively with aluminium(III), indium(III), ruthenium(III), gallium(III) and Zn(II) and may play a role in their transport to different compartments of the body (Harris and Messori 2002; Vincent and Love 2012; Harris and Pecoraro 1983). Hence, apo-transferrin may be one of the most important serum transport agents for therapeutically, diagnostically and toxicologically important metal ions.

During the last few decades, noble metals based drugs have been studied extensively. The attention of researchers have been shifted to these metals following the discovery of cisplatin, a platinum-based complex, as strong chemotherapeutic agent for variety of cancers, mainly testicular and ovarian cancers (Rosenberg et al. 1965; Medici et al. 2015). Gold based drugs have been used for the treatment of rheumatoid arthritis since long and also being tested for anticancer activity (Healy et al. 2009). Hence, in recent years, a significant amount of research has been done on the investigation of these metals resulting in the development of a vast library of noble metal-complexes with remarkable biological activities. Although these metal complexes were mainly studied for anticancer activities, they also showed promising antimicrobial, anti-rheumatic, anti-inflammatory and anti-malarial activities. The antitumor action of noble metal complexes is primarily based on their strong affinity to DNA; however, interaction with other molecular sites such as thiol groups in certain proteins and redox processes have also been proposed (Medici et al. 2015).

In spite of cisplatin, other platinum complexes based on camptothecin (Cincinelli et al. 2013), podophyllotoxin (Liu et al. 2013) and andendoxifen (Ding et al. 2013) have been developed as strong cytotoxic agents that act through DNA cleavage. Ruthenium complexes are studied as potential anticancer (Suss-Fink 2010; Ganeshpandian et al. 2014), antibacterial (Lam et al. 2014, Li et al. 2012) and anti-parasite (Iniguez et al. 2013) agents. Ru-based drugs are less effective than Pt-based complexes but have advantage of fewer side effects. Ruthenium is selectively transported to tumor cells by transferrin which is often over-expressed in these cells. In the process of interaction with transferrin, Ru is able to mimic iron. The mechanism of anticancer activity of Ru-compounds is not fully understood and it is thought to be more complex than that of platinum drugs, however, binding with DNA is thought to be the main action (Bergamo et al. 2012).

Gold is among the first metals used in medicine, most popularly in the treatment of rheumatoid arthritis (eg., auranofin, an Au(I)-thiolate-triethylphosphine complex). Since, Au(III) has the same electronic configuration and forms structurally similar complexes (square-planar arrangement) as Pt(II), its complexes may be good alternatives for platinum compounds for the treatment of cancer and are expected to interact with DNA in the same way. Consequently, a number of gold complexes have been prepared and reported to possess remarkable cytotoxicity against a variety of tumor cells (Medici et al. 2015; Nardon et al. 2014). Iridium-based drugs have been intensively investigated for a wide range of biological applications such as anticancer, cellular imaging and protein staining (Liu and Sadler 2014; Li et al. 2011). Recent studies on osmium complexes have suggested them as potential chemotherapeutic agents. Significant research on osmium-based drugs have been started from the last ten years resulting in the development of various osmium-based organometallic compounds with promising anticancer activity (Peacock and Sadler 2008; Van Rijt 2010) .

The most striking feature of metals is their ability to form complexes with a variety of ligands. It is also noteworthy to mention that noble metals display greater biological activities in the form of complexes than the free metal ions. Indeed, it is the complex form which governs their distribution in the biosphere as well as their use as medicinal agents (Medici et al. 2015; Brooks 2000). Furthermore, almost all proteins can interact with metal ions and some of these metals govern their biological roles in the living system, also the medicinally important metals are selectively transported to their target sites using these proteins as transport medium and released through redox or ligand exchange processes (Swart 2013; Mounicou et al. 2009; Romero-Canelon and Sadler 2013). Hence, the study of metal ion interactions with proteins as well as other ligands is not only important to develop new metal-based therapeutic agents but also to understand the mechanism of their action within a biological system.

Various modern analytical techniques have been used to investigate protein-metal ion interactions. Few of the most commonly used techniques are UV-Visible absorption spectroscopy (Hegde et al. 2011), FTIR (Gerbino 2011; Nara et al. 2013), ESI-mass spectrometry (Kaluarachchi et al. 2011; Delobel et al. 2005), NMR spectroscopy (Binolfi et al 2006), circular dichroism spectroscopy (Hegde et al. 2011), fluorescence spectroscopy (Sacco et al. 2012; Hegde et al. 2011) and capillary electrophoresis (Redweik et al. 2012; 2013; Alhazmi et al. 2014). Binding investigations of TF and apo-TF with metal ions have been carried out with various analytical techniques including spectroscopy (Elham Keshavarz 2015; Moshtaghie and Gaffari 2003), calorimetry (Lin et al. 1993), ESI-mass spectrometry (Zhang et al. 2004) and capillary electrophoresis-MS (Groessl et al. 2009). The binding of apo-TF with metal ions such as Bi^{3+} (Li et al. 1996), Al^{3+} (Fatemi et al. 1991), Tb^{3+} (Abdollahi and Harris 2006), Co^{2+} (Smith 2005) and Ga^{3+} (Elham Keshavarz 2015) were studied using UV-Vis spectrophotometry and the complexation behavior of Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} was investigated by isothermal titration calorimetry (Wyrzykowski and Chmurzyn´ski 2010).

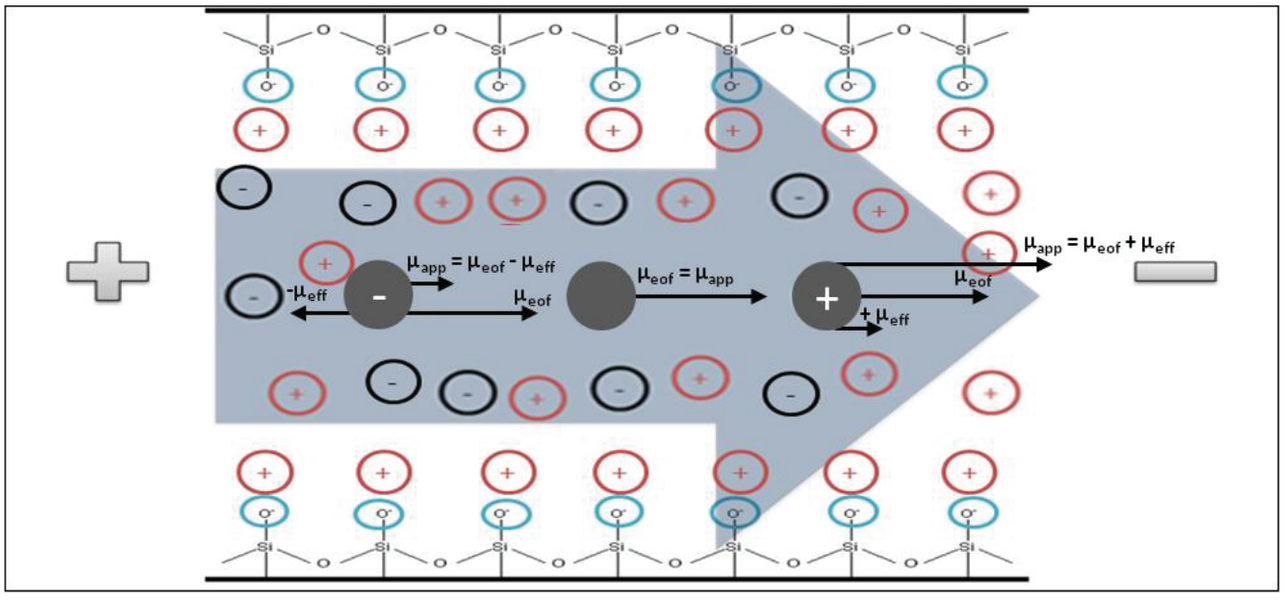

Fig. 1: Apparent mobilities of positively, negatively charged and uncharged analytes under the influence of eletroosmotic flow inside the capillary of an electrophoresis. M_{app} = apparent mobility; μ_{eof} = mobility due to electroosmotic flow; μ_{eff}: mobility due to ionic effect.

Capillary electrophoresis is a powerful technique used in biological and pharmaceutical research. It is gaining more popularity from the last few years due to its wide application in measuring the interactions between proteins and other ligands such as metal ions (Redweik et al. 2012; 2013, Alhazmi et al. 2014, 2015a, b; El-Hady et al. 2010). In comparison to many other techniques used to study protein-metal ion binding, capillary electrophoresis is advantageous due to its simple handling, easy sample preparation, fast analysis, small sample and reagent consumption (from picolitre to nanolitre), excellent separation efficiency that allows analysis of impure samples, even biological samples (El-Hady et al. 2016). In addition to that, the interaction results obtained from capillary electrophoresis are found to be accurate and in agreement with those from other techniques (Alhazmi et al. 2015b).

Due to important roles of TF and apo-TF in the metal ion transport in the biological system, in the present work we have investigated the interaction behavior of some medicinally important noble metal ions: Au(I), Au(III), Os(III), Pt(IV), Pt(III) and Ru(III) with human apo-transferrin (iron-free transferrin) using affinity capillary electrophoresis (ACE) and compared the binding results with our previous results of interaction study between the same metal ions and human transferrin, using ACE (Alhazmi et al. 2017). This study will provide an approach to understand the behavior of protein-metal ion interactions better and will also improve insight in the development of target oriented metal-based therapeutic agents and help in understanding the mechanism of therapeutic actions and toxicities of metal ions.

2. Investigations, results and discussion

2.1. Electrophoretic mobility shift and mobility ratio (R)

The interaction between a protein and metal ions results in the alteration of certain properties of the test protein such as size, charge and mass, leading to a shift in electrophoretic mobility in the running buffer within the capillary tube (Fig. 1). This small change in the electrophoretic mobility of the test protein under the influence of a metal ion is being exploited to measure the protein-metal ion interaction by affinity capillary electrophoresis (ACE). However, the screening of interaction is done by using the mobility ratio (R), which is the ratio of mobilities of the test protein ($\mu prot$) and an electroosmotic flow (EOF) marker (μeof) at the same time ($R = \mu prot/\mu eof$). The apparent mobility is represented as: $\mu = lL/Ut$, where l is the effective and L is the total lengths of the capillary, U is the applied separation voltage and t is the migration time of analyte. Except migration time (t), all the parameters are same for the test protein and EOF marker screened during the same run and hence will be cancelled. Thus, the mobility ratio (R) can be calculated using the following equation:

$$R = \frac{t_{eof}}{t_{prot}} \quad (1)$$

Where, *teof* and *tprot* are the migration time for EOF marker and protein, respectively. The change in migration time of test protein after metal ion binding can be seen in the electropherograms (Fig. 2).

The electrophoretic mobility ratios *Rf* and *Ri* were calculated according to the above relationship. The *Rf* is the mobility ratio of test protein without metal ion interaction and *Ri* represents the mobility ratio of the protein after interaction with a metal ion (protein-metal ion complex). Finally the results for every metal ion-protein interactions were expressed in terms of the difference of the mobility ratios as, $\Delta R\ (Ri\text{-}Rf)$ normalized to *Rf,* i.e., $\Delta R/Rf$, which reflects the change in the charge/size ratio of the protein after binding to a metal ion. After that, the confidence interval *{cnf(ΔR/Rf)}* for every metal ion-protein interaction was calculated using the formula as per the reported method (Alhazmi et al. 2015) to evaluate if the variation in the mobility ratios are due to the interaction between protein and metal ions under investigation. It is important to note that, for a significant interaction,

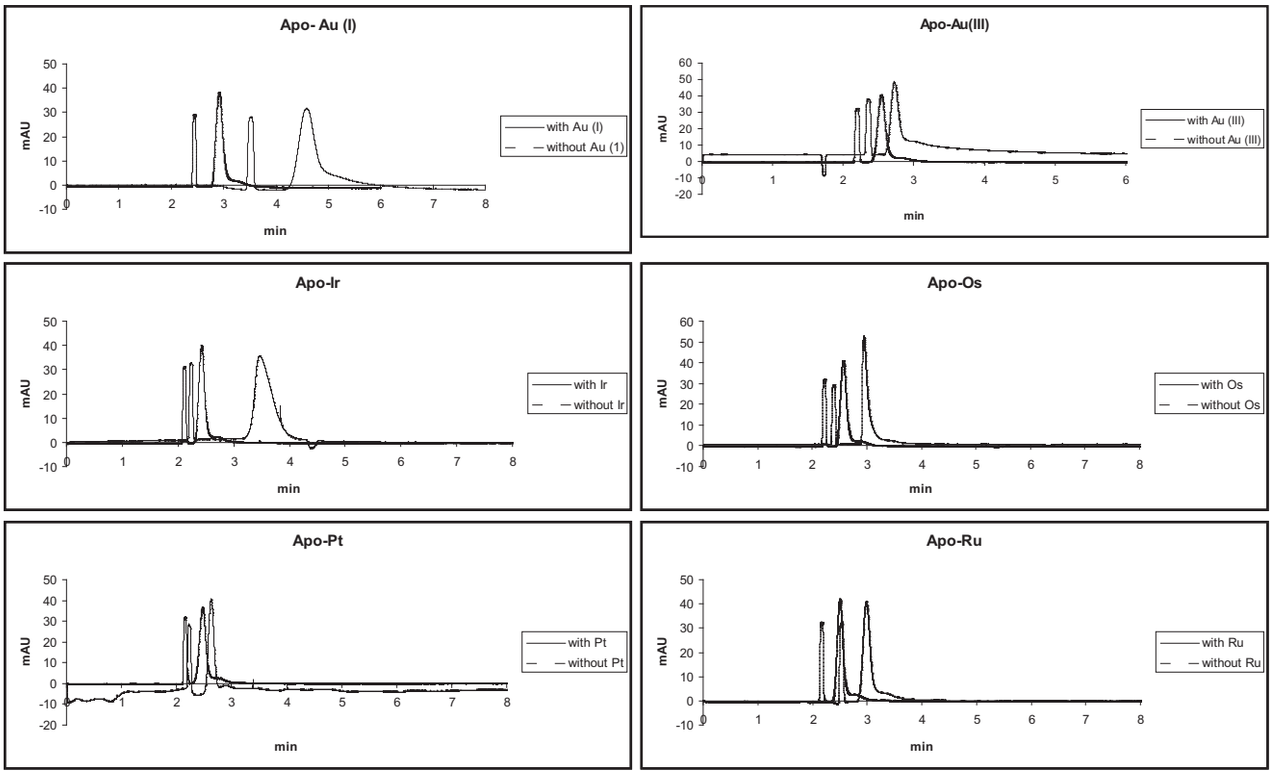

Fig. 2: Electropherograms of apo-transferrin-noble metal ion interaction showing variation the electrophoretic mobility of protein after metal ion binding. *Dotted line:* apo-transferrin without metal ion; *solid line:* apo-transferrin with metal ion. *Electrophoretic conditions:* tris-buffer (20 mM, pH 7.4), 214 nm wavelength, effective capillary length 22 cm with total length 31 cm, internal diameter 50µm, capillary cartridge temperature 23°C, applied voltage 10 kV and samples injected for 4.5s at 0.5 bar followed by buffer for 2.5s.

the confidence interval values should not intersect the zero line in the Δ*R/Rf* chart (Fig. 3). Furthermore, the value of Δ*R/Rf* may be positive or negative depending on the direction of the influence of particular metal ion on the protein. The positive value of Δ*R/Rf* suggests that after metal ion binding, the overall charge on the protein metal ion complex becomes more positive (less negative), as a consequence, the electrophoretic mobility of the protein after interaction increases (anode at the inlet and cathode at the outlet of the capillary). On the other hand a negative Δ*R/Rf* value indicates that the overall charge on the metal-protein complex becomes more negative, leading to reduced electrophoretic mobility of the protein after interaction. The positive and negative signs of Δ*R/Rf* are considered to be highly significant for the preliminary investigation of protein-metal ions interactions because it may provide an initial understanding of the coordination between metal ions and the protein residues.

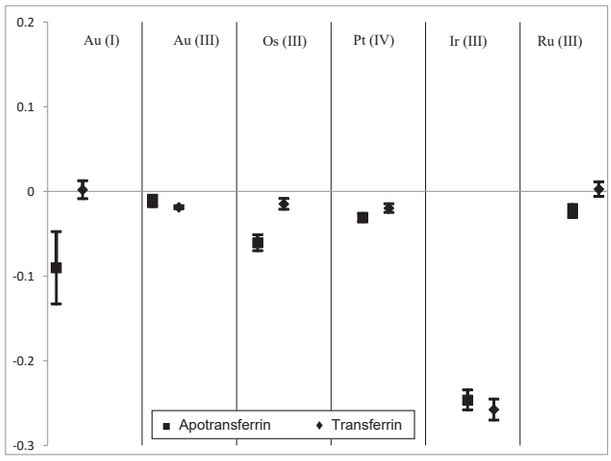

Fig. 3: Comprehensive Δ*R/Rf* chart displaying binding affinities of two proteins; apo-tranferrin and transferrin with Au(I), Au (III), Os (III), Pt (IV), Ir (III) and Ru (III) ions. Metal ion concentration: 250μM. *Electrophoretic conditions:* tris-buffer (20 mM, pH 7.4), 214 nm wavelength, effective capillary length 22 cm with total length 31 cm, internal diameter 50μm, capillary cartridge temperature 23°C, applied voltage 10 kV and samples injected for 4.5s at 0.5 bar followed by buffer for 2.5s. The data for transferrin inteaction was taken from our previous study (Alhazmi et al 2017)

A value of Δ*R/Rf* ≥ 0.01 is often adequate to consider it as a significant interaction, even with an unusually broad confidence interval. The mobility ratio was used to calculate the binding results because generally it provides very high precision. In this study, the % RSD of mobility ratios (*Rf* and *Ri*) for six runs were achieved to be less than 1% for all the tested metal ions interaction with apo-transferrin, except with Au^{+} (3.6%). It can be stated that the interactions for which highly precise mobility ratios were recorded for repeated runs, indicate significant, if not necessarily strong interactions. In exceptional cases, the electrophoretic mobility might not be affected and the recorded ΔR value may become close to zero (ΔR ≈ 0), even there is an interaction between protein and ligand metal ion. This can give a false interpretation of no interaction; however, it is definitely a rare exception.

It has been observed from previous studies based on ACE that proteins and positively charged metal ions are adsorbed on the surface of the stationary phase (Towns and Regnier 1991, 1992) in the capillary. This affects the electroosmotic flow (EOF) and results in the change in electrophoretic mobility of the analyte protein, which actually is not due to interaction between the protein and metal ions under investigation. This problem in ACE is being overcome by two ways: first using a rinsing protocol to remove the adsorbed substances from the capillary wall and secondly by using an EOF marker to avoid errors due to change in the mobility (Alhazmi et al 2015; El-Hady et al. 2010; Gomez et al. 1994). The use of ethylenediamine tetraacetic acid (EDTA) in the rinsing protocol in this analysis was proved to be very helpful in quick regeneration of the capillary for further screenings. Acetanilide (neutral at physiological pH 7.4) was used as EOF marker throughout the course of present screening.

2.2. Protein-metal ions interactions

Capillary electrophoresis has been proved to be one of the most efficient tools for the fast screening of binding interactions between a protein and other ligands such as metal ions. The current work is the investigation of interaction between apo-transferrin and medicinally significant noble metal ions, Au(I), Au(III), Os(III), Pt (IV), Ir(III) and Ru(III). Apo-transferrin is the iron free form of human transferrin, a protein that transfers iron from its sites of entry in the blood circulation to the cells at different locations of the human body. The interaction screening was performed by using an affinity mode of capillary electrophoresis known as mobility shift-affinity capillary electrophoresis, in which the change in electrophoretic mobility of a protein after binding with a ligand metal ion has been measured.

Because most of the proteins possess multiple binding sites for ligand metal ions, a high concentration of metal ions was applied to attain saturation for fast screening. For the interaction screening with apo-tranferrin, all the metal ions were used at a concentration of 250 μmol/L. Furthermore, two running solutions were used; one without metal ion and the other with metal ion at physiological pH 7.4. Twelve runs were performed for each metal ions (six without and six with metal ion) under the same electrophoretic condition and the recorded electropherograms were used to calculate Δ*R/Rf* values and their confidence intervals {*cnf(ΔR/Rf)*} for each tested metal ion using the mathematical equations as discussed above.

The migration times of apo-transferrin after interaction with all the metal ions in the present screening were increased (Fig. 2) as compared to acetalinide (EOF marker). This indicates that the electrophoretic mobility of apoTF-metal ion complexes were less than the apo-transferrin without metal ion interaction. The reduced electrophoretic mobility suggests that the overall charge on the test protein becomes negative as a result of interactions with metal ions, as a consequence, negative Δ*R/Rf* values were obtained. In the binding process, a metal ion usually interacts with more than one amino acid residue of the protein (Bal et al. 1998). Since metal ions bind to the deprotonated form of amino acids, the net charge on the protein remains unchanged at the first instance, because the loss of protons are compensated by the new positive charges of the metal ions. However, due to the multifaceted behavior of metal ions, after initial interaction with protein, it probably coordinates with the surrounding anions present in the solution within the capillary, as a result, the net charge on the protein becomes more negative.

The observed behavior of apo-TF in this experiment was in agreement with that reported in the literature, which has shown that when a Fe^{+3} ion interacts with transferrin, three protons ($3H^{+}$) are released from deprotonation of tyrosine residues and a bicarbonate ion (HCO_3^{-}) is concomitantly get coordinated, producing a negatively charged iron-transferrin complex $[Fe\text{-}TF\text{-}HCO3]^{-}$. Transferrin binds with two Fe^{3+} ions releasing a total of six protons and the diferric complex of transferrin gains two negative charges $[2Fe\text{-}TF\text{-}HCO3]^{--}$. The mechanism of interaction of transferrin with other metal ions appears to be similar as Fe^{3+} ion binding. However, the number of protons released and metal ions involved in the overall binding process may be different and depends on the ionic radii of the metal ions. For example, six protons are released when two Al^{3+} ions interact with transferrin, whereas, interaction with Cu^{2+} by a similar reaction releases only four protons (Chung 1984).

The results of interaction between apo-TF and the tested noble metal ions are expressed as Δ*R/Rf* values and their confidence intervals and are summarized in Table 1. However, easier and more precise comparison of interaction data was achieved by making a Δ*R/Rf* chart (Fig. 3), which is considered to be a comprehensive platform to interpret the interaction between a protein and ligand metal ions. From the interaction data and the Δ*R/Rf* chart, it can be deduced that, in the present study, highest affinity towards

Table 1: Δ*R/Rf* values and their confidence intervals (*cnfs*) for Apo-transferrin-noble metal ions interactions

Injection No.	Δ*R/Rf* values*					
	Au (I)	Au(III)	Os (III)	Pt (IV)	Ir (III)	Ru (III)
1	-0.0820	-0.0099	-0.0655	-0.0274	-0.2621	-0.0140
2	-0.1051	-0.0092	-0.0637	-0.0311	-0.2374	-0.0229
3	-0.1013	-0.0106	-0.0560	-0.0311	-0.2502	-0.0220
4	-0.0908	-0.0117	-0.0566	-0.0347	-0.2604	-0.0266
5	-0.0803	-0.0128	-0.0736	-0.0327	-0.2345	-0.0251
6	-0.0823	-0.0140	-0.0489	-0.0290	-0.2333	-0.0291
Mean ± confidence interval	-0.0903 ± 0.0427	-0.0114 ± 0.0068	-0.0607 ± 0.0094	-0.0310 ± 0.0050	-0.2463 ± 0.0119	-0.0233 ± 0.0077
SD	0.0375	0.0059	0.0090	0.0048	0.0113	0.0073

*$\Delta R = (R_i - R_f)$, where R_i and R_f are the mobility ratios with and without metal ion respectively.

apo-transferrin was shown by the Ir(III) ion followed by the Au(I) ion. The Ir(III) ion has exhibited extremely strong interaction with test protein because a high Δ*R/Rf* value with a remarkably narrow confidence interval (-0.2463±0.0119) was obtained in the interaction screening. Au(I) has exhibited strong interaction with the test protein (ΔR/*Rf* = -0.0903±0.0427), which is stronger than that displayed by Au(III) (Δ*R/Rf* = -0.0114±0.0068). Although, Au(III) displayed the poorest binding interaction among the analyzed metal ions with apo-TF, the observed Δ*R/Rf* value is still greater than 0.01 and its confidence interval is not intersecting the zero line of the Δ*R/Rf* chart, suggesting it to be a significant interaction. The remaining metal ions, Os(III), Pt(IV) and Ru(III) were found to interact significantly with apo-transferrin (Δ*R/Rf* = -0.0607±0.0094; -0.0310±0.0050 and -0.0233±0.0077 respectively) under the experimental conditions. Overall, the interaction between apo-transferrin and all the analyzed noble metal ions are considered to be significant and found to be in the following order of affinities: Ir(III) > Au(I) > Os(III) > Pt(IV) > Ru(III) > Au(III) (Fig. 3).

The interaction behavior between apo-TF and tested noble metal ions were compared with the binding screening results of the same metal ions with transferrin, studied under identical electrophoretic conditions in our previous study (Alhazmi et al. 2017). It was observed that most of the metal ions showed similar binding behavior with both the proteins, except Au(I) and Ru(III), which have demonstrated insignificant interaction with transferrin (Δ*R/Rf* = 0.0019±0.0106 and 0.0026±0.0086), in addition to positive Δ*R/Rf* values in contrast to negative and significant Δ*R/Rf* values due to interaction of these metal ions with apo-TF. The metal ions Au(III), Os(III), Pt(IV) and Ir(III) displayed similar binding behavior with both the proteins, showing overall stronger affinities with apo-TF. The interaction with both proteins resulted in the decreased mobility of the proteins leading to negative Δ*R/Rf* values. Furthermore, like apo-TF, transferrin showed strongest binding affinity with Ir(III) (Δ*R/Rf* = -0.2577±0.0124). The comparison of interaction results of transferrin and apo-transferrin is made in Table 2 and in Fig. 3.

Apart from variation in the electrophoretic mobilities, which has been exploited to investigate the interaction behavior of the test protein with metal ions, other changes such as shape and intensities of the peaks due to protein-metal ion complexation were also observed. Remarkable changes in the peak shape and intensity were observed after the interaction of apo-TF with Au(I) and Ir(III) ions, in that significantly broader and larger peaks were obtained due to apo-TF-Au^{+} and apo-TF-Ir^{3+} complexes. Peaks with greater height were observed due to apo-TF-Au^{3+} and apo-TF-Os^{3+} complexes, though the sharpness of the peaks remains almost intact. On the other hand, no marked change in the peak shape and intensity was seen in the electropherograms obtained from apo-TF after interaction with Pt (IV) and Ru (III). In general, the metal ions which showed stronger interaction brought major changes, whereas no marked variation in the peak shape and intensities were noticed with the metal ions showing relatively weaker binding affinities. These changes are probably due to conformational alteration in the protein molecule after interaction with metal ions. However, the degree of alteration depends on the strength of the binding reaction between protein and metal ions, which can be seen from the variation in the peak shape and intensity due to analyte protein in the respective electropherograms (Fig. 2).

From the screening results, one can state that apo-transferrin has the capability to bind a range of metal ions other than iron (III) and can be utilized as a medium for transport of these metal ions into the biological system. Hence, apo-transferrin can serve as a ligand for the delivery of biologically important metal ions selectively to their sites of action. An investigation like this can be valuable in providing preliminary insight in the screening of different types of interactions such as drug-metal ions, enzyme-metal ions and protein-metal ions interactions and can provide the starting points to development of new metal-based drugs to fight a variety of ailments such as cancer and serious infections.

In conclusion, the interaction of apo-transferrin with medicinally significant noble metal ions was screened using mobility-shift mode of ACE technique, in which the interactions were carried out by mixing the metal ions directly in the running buffer. The interaction study was performed using a small injection volume of relatively low sample concentrations and a short capillary. A proper rinsing protocol was applied to regenerate the capillary which helped to give very precise interaction results. The precision

Table 2: Comparison of results of tested noble metal ion interactions with transferrin and apo-transferrin analyzed under identical electrophoretic conditions

Protein	*ΔR/Rf* values and their confidence intervals						
	Au+	Au3+	Os3+	Pt4+	Ir3+	Ru3+	
Apo-TF	-0.0903± 0.0427	-0.0114 ± 0.0068	-0.0607 ± 0.0094	-0.0310 ± 0.0050	-0.2463 ± 0.0119	-0.0233 ± 0.0077	This study
TF	0.0019 ± 0.0106	-0.0188± 0.0017	-0.0148 ± 0.0064	-0.0198 ± 0.0051	-0.2577 ± 0.0124	0.0026 ± 0.0086	Our Previous study*

* Alhazmi et al 2017. Apo-TF: Apo-Transferrin; TF: Transferrin.

was further enhanced significantly by the use of 0.1M EDTA in the rinsing protocol. The binding interactions were assessed by exploiting the shift in electrophoretic mobility of the analyte after interaction with metal ions. The variation in the electrophoretic mobility is proportional to the intensity of interaction between the test protein and metal ions. Apart from mobility shift, marked changes in the shape and intensity of the peaks due to the protein were noticed, which is probably due to conformational changes in the protein molecule after metal binding. The mobility ratios of apo-tranferrin with and without metal ion interactions were calculated with respect to acetanilide, an EOF marker using mathematical equations. Finally the interaction results were expressed in normalized difference in the mobility ratios (Δ*R/Rf)* of test protein and its confidence interval (*cnf* of Δ*R/Rf).* The tested metal ions exhibited significant binding with apo-transferrin, the interaction with Ir(III) being the strongest one. In general, for all the interactions, negative Δ*R/Rf* values were achieved, which could be due to further coordination of a bond metal ion with the surrounding anions inside the capillary tube. This study would be useful for other future protein-metal ion interaction screenings using the mobility-shift ACE method. In addition to protein-metal ion interactions, it can offer preliminary insights into other related studies such as protein-protein, drug-protein, drug-metal ions and enzyme-metal ions interactions. Moreover, this study may be helpful in understanding the complexing behavior of medicinally valuable metal ions and hence, in the development of novel metal based compounds as potential therapeutic agents.

3. Experimental

3.1. Chemicals and reagents

Apo-Transferrin Human (≥ 95%, 76-81 kDa), tris powder, gold (I) chloride (AuCl), gold (III) chloride ($AuCl_3$), osmium (III) chloride hydrate ($OsCl_3$), platinum (IV) chloride ($PtCl_4$), iridium (III) chloride ($IrCl_3$) and ruthenium (III) chloride ($RuCl_3$) were purchased from Sigma-Aldrich (Steinheim, Germany). Silver (I) nitrate ($AgNO_3$) was obtained from Grüssing (Filsum, Germany). Disodium salt of ethylenediamine tetraacetic acid dihydrate (EDTA-Na_2.$2H_2O$) and sodium chloride (NaCl) were procured from Riedel de Häen (Hannover, Germany). Acetanilide was purchased from Fluka Chemicals (Steinheim, Germany) and concentrated hydrochloric acid (Conc. HCl) was purchased from Merck (Darmstadt, Germany). Double distilled water (ddH_2O) was produced in our laboratory.

3.2. Apparatus and instruments

Agilent CE system model G1600A (Agilent Technologies, Germany) was utilized for the investigations of all apo-transferrin-metal ion interactions that was equipped with an autosampler, a capillary cooling system, and a diode array detector (λ = 214 nm). An external pressure of 2.5 bar was applied through a normal air plug of the laboratory. Bare fused silica capillaries of internal diameter 50 µm and total length 31 cm (effective length to the detector window = 22 cm) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The electropherograms obtained were analyzed by Agilent ChemStation software. To measure the pH of the buffer solutions, Mettler Toledo FE20/EL20 pH-meter (Carl Roth, Karlsruhe, Germany) was used throughout the study. For the filtration of sample solutions, Rotilabos-syringe filters (CME 022 mm) were utilized that were purchased from Carl Roth (Karlsruhe, Germany). All the statistical analyses including the calculation of mobility ratios (Δ*R/Rf*) and their confidence intervals were performed by Microsoft EXCEL™ software (Microsoft Corporation, Version 2007).

3.3. Rinsing protocol and separation modes

At the start of study, conditioning of new capillaries was performed by flushing with 1M NaOH solution for 20 min followed by ddH_2O for 10 min at a pressure of 1 bar. Capillaries were again flushed at 2.5 bar with 0.1M NaOH solution for 10 min followed by water for 5 min at the start and was repeated at the end of each day of analysis. Finally, before the start of each run, the capillary was flushed with a mixture of 0.1 M NaOH and 0.1M EDTA (1:1) solutions for 2.5 min, ddH_2O for 1 min and running buffer for 1.5 min at 2.5 bar (Alhazmi et al. 2014). At the end of each screening, capillary was again rinsed at 2.5 bar with 0.1M NaOH solution for 10 min and then by water for 5 min to get the precision in next analysis. It also helped to minimize the background noise.

All the samples were injected into the capillaries hydrodynamically at a pressure of 0.5 bar for a period of 4.5 s. These were further pushed by injecting running buffer at the same pressure for 2.5 s. All the electrophoretic separations were achieved at temperature 23 °C, voltage 10 kV, and a maximum current at 20 µA. If the maximum current is reached; the instrument shuts down automatically. Since, a stable current of ≤ 13 µA was observed throughout the study, the joule heating effect was insignificant (joule heating is directly proportional to produced current). Normal mode was selected for all the separations where capillary inlet was at anode and the outlet was at cathode. Each sample with and without metal ions was repeated six times and their mean was calculated.

3.4. Preparation of solutions

3.4.1. Tris buffer (20 mM, pH 7.4)

Tris buffer was prepared by weighing 2.42 g of tris and dissolving into 200 mL of ddH_2O in a volumetric flask. pH was adjusted to 7.4 with the help of dilute hydrochloric acid solution and the final volume was made up to 1000 mL with ddH_2O.

3.4.2. Acetanilide stock solution (750 µg/mL)

An amount of 37.5 g of acetanilide was weighed accurately and dissolved in 50 mL of tris buffer followed by sonication to get the clear solution.

3.4.3. Protein solutions (20 µM and 6.66 µM)

Protein solutions were prepared by dissolving 39.25 mg of apo-transferrin in 5 mL of tris buffer into a 25 mL volumetric flask followed by the addition of 5 mL of acetanilide stock solution. The final volume was made up to 25 mL with tris buffer. It was diluted three times to get the concentration of 6.66 *µM* that was used before the injection in order to minimize the band-broadening and protein adsorption.

3.4.4. Metal ion solutions

Metal ions stock solutions were prepared in tris buffer of pH 7.4 at a concentration of 250 µmol/L. The protein and metal ions solutions were prepared fresh in the same tris buffer at the start of each day of analysis. These solutions were previously filtered through 0.22 µm membrane filter and were degassed by sonication for 5 minutes before the injection.

Conflicts of interest: none declared.

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