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Investigation of transferrin interaction with medicinally important noble metal ions using affinity capillary electrophoresis

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Transferrins (TFs) consist of a large group of glycoproteins, whose function is to transport iron across the cell membrane. Apart from iron, serum transferrin can also bind several other metal ions and hence can offer a potential route for the delivery of these metal ions into the cellular fluids. In the present study the interaction behavior of nine noble metal ions, Ag^{+}, Au^{+}, Au^{3+}, Os^{3+}, Pd^{2+}, Pt^{4+}, Rh^{3+}, Ru^{3+} and Ir^{3+} with transferrin was investigated by affinity capillary electrophoresis (ACE) using the dynamic mobility shift mode. A proper rinsing procedure was applied to regenerate the capillary tube. The influence of these metal ions on transferrin was studied through comparison of the mobility ratios of free protein and protein-metal ion complex. The interaction results were expressed by the normalized difference of the mobility ratios ($\Delta R/Rf$) and its confidence intervals. Most of the tested metal ions showed significant interaction with transferrin with small confidence intervals, except Ag^{+}, Au^{+} and Rh^{3+} that exhibited very weak interactions. Maximum interaction was observed between transferrin and Ir^{3+}, followed by Pd^{2+} that also showed strong affinity towards the test protein. The screening results were compared with Bovine Serum Albumin (BSA)- and Human Serum Albumin (HSA)-noble metal ions interactions. An excellent precision (% RSD of mobility ratios were less than 1%, except for transferrin-Pd^{2+} interaction ≈ 4%) was recorded for repeated runs of transferrin-metal ions interactions. This study contributes to the understanding of the affinity of transferrin to the tested metal ions and will provide preliminary information for the investigation of other protein-ligands interactions.

1. Introduction

1.1. Protein-metal interaction

The roles of metalloproteins in the biological systems have been studied extensively; they constitute approximately one-third of all known proteins (Tainer et al. 1991). Each metalloprotein contains a specific metal ion as cofactor which plays an important role to control their biological functions in the body. Basically all proteins can interact with metal ions, so their selectivity for specific metal ions is of particular concern because some of these interactions are proved to be important (Swart 2013; Mounicou et al. 2009). Several organometallic complexes have been developed as medicinal agents to treat a variety of serious disorders. Generally these complexes are pro-drugs selectively transported to the target area where metal ions are released by ligand exchange or redox process (Romero-Canelon and Sadler 2013; Frezza et al. 2010; Chen et al. 2009; Hannon 2007; Desoize 2004).

In the development of new metal ion-based prodrugs, it is important to investigate the effects of the metal ion partner which makes the characterization of protein-metal ion interaction vital. However, in biological systems this remains challenging task. Among the most important factors affecting the protein-metal ion interactions are the properties of proteins such as dipole moment, net charge, acceptance and donation of charges and number of potential ligands for metal ion binding inside a binding cleft and metal ions properties such as valency, charge accepting capacity and atomic radius. An understanding of the basic mechanisms of complex formation will give a preliminary approach for the study of protein-metal ion interactions (Swart 2013; Dudev and Lim 2014). In general, the ion-ion and ion-dipole interactions increase with the valency of the metal ions. Furthermore, a metal ion can accept more charges from biological ligands if it is a good electron acceptor (soft) like Zn^{2+} and forms more stable complexes than a hard metal ion (such as Mg^{2+}), regardless of both the metal ions are having the same charge and ionic radius. The soft metal ions are more selective towards nitrogen and sulfur containing groups, while hard metal ions show more affinity to oxygen containing groups (Glusker et al. 1999; Gamer and Gresh 1994; Bock et al. 1995). Overall, the metal ions interact with proteins of similar hardness or softness. Hence, the Hard and Soft Acid and Base Theory (HSAB) can be used to estimate the interaction selectivity (Pearson 1963; Lemire et al. 2013; LoPachin et al. 2012; Hoeschele et al. 1991). The binding sites in calmodulin, carboxypeptidase and multicopper oxidase are good examples where different lewis acids such as Ca^{2+} (hard), Zn^{2+} (borderline) and Ag^{+} (soft) respectively can bind (Babu et al. 1988; Greenblatt et al. 1998; Singh et al. 2011). In Fig. 1, it can be seen that soft metal ions Ag^{+} and Zn^{2+} (borderline) bind selectively to thioether and imidazole groups respectively, while the binding site for hard metal ion Ca^{2+} consists of carboxylate groups only. Moreover, the distance between the metal ion in the centre and the functional groups in the binding site is also an important factor in the structural characterization of the metal ion-protein interactions. The coordination numbers (CNs) and the geometries of the ligand metal ions also play an important role in investigation processes (Kuppuraj et al. 2011; Dudev et al. 2006).

The noble metals belong to the group of transition elements which have partly filled *d* and *f* subshells in their common oxidation states. The highest oxidation number of a transition element is usually its group number. Hence, they should have a highest oxidation number of VIII, whereas only ruthenium and osmium do so. At the higher oxidation states these metals exhibit covalent and coordinate bonding, whereas ionic bonding is common in lower states.

1.2. Transferrin-metal ion interactions

Transferrins (TFs) are a class of glycoproteins, present in serum at concentrations of 25-50 mM. The function of these metal binding transport proteins is to deliver iron (Fe^{3+}) into the cells. Structurally, transferrins contain two almost identical lobes known as N- and C-lobes, offering two different binding sites for iron. Binding of Fe^{3+} to each lobe causes a significant conformational change

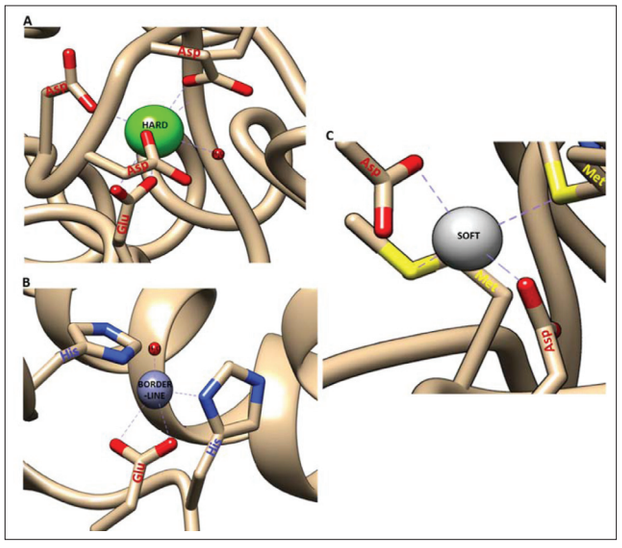

Fig. 1: Binding of hard and soft metal ions to different proteins. **(A)** Calmodulin-hard metal ion (PDB ID: 3CLN), **(B)** carboxypeptidase-soft metal ion (borderline) (PDB ID: 1YME) and **(C)** multicopper oxidase-soft metal ion (PDB ID: 3NSD)

from an open to a closed state, i.e., usually a transition from the open (apoprotein) to a closed (holo-protein) conformation (Sun et al. 1999; Li et al. 2002). In human beings, iron is transported to the cells by Human Serum Transferrin (hTF) through receptor mediated endocytosis. The receptors on the cell surface can recognize only the holo-protein form (diferric), which means that the open form is not able to bind with receptors. Only 30% of transferrins circulating throughout the body is in the holo-protein form (i.e., both the lobes attached with Fe^{3+}) and rest 70% is in the apo- or mono-protein form (Williams and Moreton 1980). As a result, in addition to iron, other metal ions can potentially bind with hTF at empty TF binding sites (many of them with high affinity) and are delivered across the cell membrane. Except the normal amount of iron, these metal ions, when transported into the cells by transferrin can cause a variety of ailments due to long term exposure to the body tissues. In addition to that the normal iron intake can also be significantly affected. The positive aspect is that hTF can serve as a promising delivery vehicle for metal based drugs, mainly cytotoxic metals to tumor cells that overexpress TFR (Sun et al. 1999; Li et al. 2002). The interaction between serum transferrin and two clinically important nonferrous metal ions indium (In^{3+}) and bismuth (Bi^{3+}) have been reported earlier, where both the metals ions were found to have high affinity to hTF (Zhang et al. 2004).

Following pioneering work on protein-metal ion interactions, where we evaluated the binding influence of various metal ions on five globular proteins (Alhazmi et al. 2014; Alhazmi et al. 2015), in the current study we have investigated the interaction behavior of noble metal ions (Ag^{+}, Au^{+}, Au^{3+}, Os^{3+}, Pd^{2+}, Pt^{4+}, Rh^{3+}, Ru^{3+} and Ir^{3+}) with transferrin using Affinity Capillary Electrophoresis (ACE). The noble metal ions were selected for this study because they are medicinally important. The most prominent feature of these metal ions is the ability to form complexes, which govern the development and the use of noble metal-based drugs as medicine. The therapeutic application of noble metals began with the discovery of cisplatin, a well known Pt(II)-based complex, as a drug for ovarian and testicular cancer therapy. Gold complexes are also among the medicinally important metal complexes used for the treatment of rheumatoid arthritis. Although, the noble metal-based drugs are mainly used for the treatment of cancer, a considerable attention has been paid by the researchers to develop therapies for other diseases. As a result, a variety of noble metal complexes have been synthesized and tested for their antibacterial, anti-tubercular, antiviral, antimalarial, antileishnamial, anti-rheumatic and anti-inflammatory activities. Complex formation of different antibiotics with noble metal ions is proved to be very useful to increase their effectiveness as well as to minimize drug resistance (Medici et al. 2015; Brooks 2000). For this study, we selected transferrin as ligand protein because of its high binding constant (for Fe^{3+}, $K_1 = 4.7x10^{20}\ M^{-1}$ and $K_2 = 2.4x10^{19}\ M^{-1}$) and the absence of a metal co-factor which renders it a good test protein for the evaluation of metal ion interactions (Sun et al. 1999).

1.3. Why affinity capillary electrophoresis?

It is well known that the proper understanding of protein interactions is very important because these interactions affect the functions of the proteins, their conformation, aggregation and denaturalization properties (Wang et al. 2010). Protein-ligand interactions can be characterized using a number of modern analytical techniques. These include nuclear magnetic resonance (NMR), fourier transform infrared spectroscopy (FTIR), surface plasmon resonance (SPR), X-ray crystallography, circular dichroism (CD) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), UV–visible absorption spectroscopy (Grasso and Spoto 2013), atomic force microscopy (Demanèche et al. 2009), affinity chromatography (Hage 1999) and ACE (Redweik et al. 2012, 2013; Alhazmi et al. 2014).

There are several factors that should be taken into account in order to select a proper technique for the study of protein-metal ion interactions. One of the most challenging factors is the complexity of the sample preparation because most of the methods are operative on pure samples only. Consequently, the majority of these techniques is difficult to be used under physiological conditions, as most of the biological samples are impure. Other factors include the ability of the techniques to identify strong as well as weaker interactions, moreover, it should be capable to measure not only the change in the protein charges but in the conformation as well. Furthermore, analytical parameters such as sensitivity, precision and accuracy should also be taken into consideration while choosing the method to investigate the protein-metal ion interactions. The preparation of crystals in X-ray crystallography is very difficult, exceptionally time consuming and even impossible in some cases. The application of NMR technique is limited to the evaluation of small and soluble proteins and it operates with highly concentrated samples because often spectral overlaps have been observed with large proteins (Jensen et al. 2007). The technique with good separation capabilities can be utilized for interaction studies when the samples are impure. Two most popular techniques are affinity chromatography and affinity capillary electrophoresis. The high cost of affinity columns makes the affinity chromatography an expensive technique, in addition to the requirement of large quantities of materials including samples (Hage 1999). Recently, affinity electrophoresis has emerged as a valuable tool for the evaluation of protein-metal ion interactions. Its excellent separation efficiency allows investigating the protein interactions in pure as well as in mixtures or impure samples containing a large number of analytes and makes it suitable for the analysis of biological samples. It is a highly sensitive technique, hence very weak interactions can be characterized. Furthermore, ACE is easy to operate, allows very fast method development, rapid analysis, requires small amounts of samples, in addition to excellent sensitivity, precision and accuracy. Among the most attractive features is its ability to investigate the interactions of different isoforms with various metal ions in the same sample. In recent years, many studies on protein interactions have been successfully conducted using ACE in which one of the studies reported the interactions between three ovalbumin isoforms and metal ions (Redweik et al. 2012, 2013; Alhazmi et al. 2014, 2015). A variety of detectors can be attached to ACE, mass spectrometer and UV-spectrophotometer being the most common ones. ACE-UV is more preferred method due to its easy operation and cost effectiveness. Furthermore, low stability and irrelevant behavior of some protein-metal ion complexes in the gas phase make the ACE-MS less favorite (El Deeb et al. 2013; Lehmann et al. 2006).

ACE runs into three main modes, namely dynamic equilibrium, pre-equilibrated and kinetic modes. The current study was conducted on a dynamic mode ACE known as mobility shift-ACE, in which the protein metal ion interactions have a short equilibrium relaxation time with respect to the separation time (Redweik et al. 2012, 2013). The mobility shift-ACE records the change in

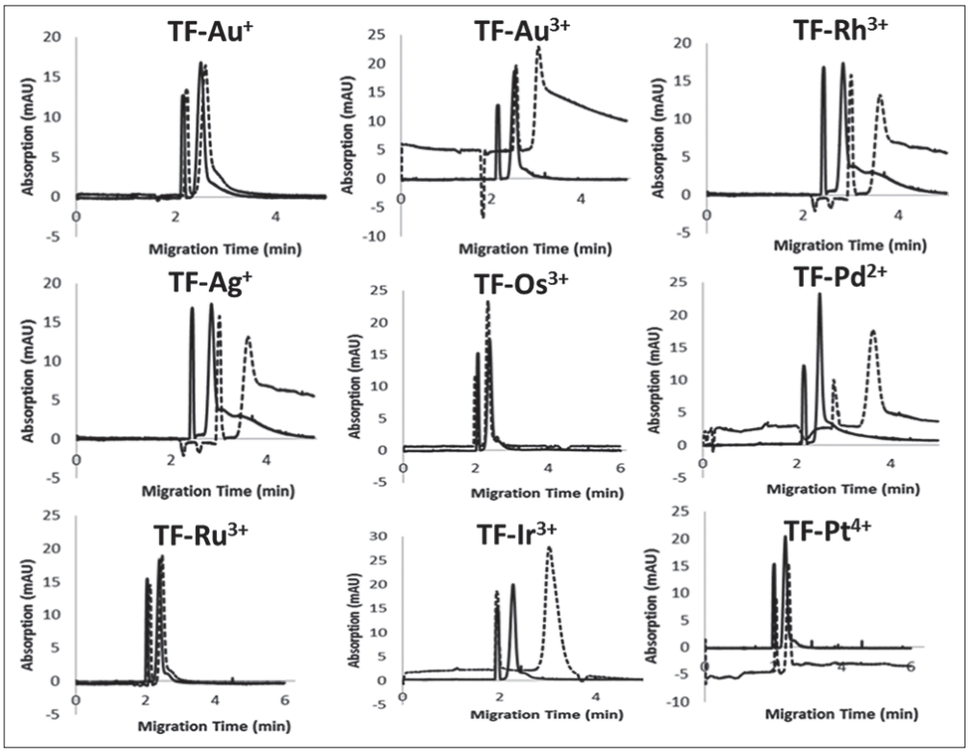

Fig. 2: Electropherograms showing change in the electrophorectic mobility due to metal ion interactions. The first peak is due to acetanilide (EOF marker) and the second peak belongs to transferrin. **Solid line** - transferrin without ligand metal ion; **dotted line** - transferrin with ligand metal ion. *Electrophoretic condition:* tris buffer (20 mM, pH 7.4), sample injected for 4.5s at 0.5 bar then buffer for 2.5s, voltage applied-10kV, 214nm wavelength, capillary: total length 31 cm with effective length 22 cm, 50µm I.D and capillary cartridge temperature 23°C. *Rinsing protocol:* capillary was rinsed with a mixture of 0.1M sodium hydroxide and 0.1M EDTA solutions at 2.5 bar for 2.5 minutes, then water for 1 minute followed by running buffer for 1.5 minutes.

the electrophoretic mobility of proteins under the influence of metal ions (ligands). The metal ions are mixed in running buffer in varying concentrations. Generally, high concentrations are used to achieve the saturation for fast screening (Redweik et al. 2012, 2013). The changes in the electrophoretic mobility occur due to alteration in the overall mass and charge of the test protein after interacting with the metal ions. In addition to the variation in the electrophoretic mobility, some other changes in protein can be interpreted from the change in the peak shape. The change, if any, in the mobility of protein which is not caused by interaction with metal ions can be avoided by the use of an EOF marker. Finally, the interaction results can be calculated by mathematical calculations using the mobility ratios of the EOF marker and the protein with and without metal ion (Redweik et al. 2012; Alhazmi et al. 2015).

2. Investigations, results and discussion

From the last few years the ACE has emerged as a powerful technique to investigate protein-metal ion interactions. Recently some studies have been successfully completed using this technique which reported highly reproducible results (Redweik et al. 2012, 2013; Alhazmi et al. 2014, 2015). Hence in the present work, the interaction behavior between iron transport protein transferrin and noble metal ions has been investigated under physiological pH 7.4 using mobility shift affinity electrophoresis. The biologically significant noble metal ions namely, silver (I), gold (I), gold (III), osmium (III), palladium (II), platinum (IV), rhodium (III), ruthenium (III) and iridium (III) were selected for the study. As most of the proteins possess multiple binding sites for metal ligands (Wang et al. 2010), a higher concentration of metal ions were utilized to achieve the saturation during the screening. A concentration of 250 µM was found to be suitable for all the metal ions under investigation, except rhodium (Rh^{3+}) for which a concentration of 25 µM (ten times lower concentration) was found to be most appropriate. At 250 µM concentration of Rh^{3+}, a strong decrease in EOF was observed in addition to significant baseline drift. Two running sample solutions of test protein, one without and the other with metal ion were injected and for each sample solution, six runs were performed under the same electrophoretic condition.

The protein-metal ion interaction was determined by exploiting the change in the electrophoretic mobility of the test protein after the interaction with ligands in the running buffer (Fig. 2). The change in the electrophoretic mobility was due to the change in the overall charge, mass and size of the protein. The interaction results were expressed in terms of mobility ratio, an excellent precision (% RSD less than 1%, except for Pd^{2+}≈4%) was obtained for repeated runs in the current study. The mobility ratios of the test protein with and without interaction with ligand metal ions, i.e., R_i and R_f respectively with respect to an EOF marker, were calculated using the equation $R = t_{eof}/t_{prot}$, where t_{eof} is the migration time for EOF marker and t_{prot} is the migration time for test protein. The EOF marker used in this study was acetanilide. It was selected for this investigation because it has pKa value of 0.5 and is neutral at pH 7.4. A significant difference in the mobility ratios, R_i and R_f means there is a reliable non-covalent interaction between the protein and ligands (El-Hady et al. 2010; Redweik et al. 2012, 2013). The normalized difference between the mobility ratios $(R_i\text{-}R_f)/R_f$ or $\Delta R/R_f$ were used to represent the interaction results. A confidence interval (*cnf*) of $\Delta R/R_f$ was calculated as per the method reported in the literature and used to detect whether the difference in the mobility ratio is due to the protein-metal ion interactions (Alhazmi et al. 2015). The calculated values of $\Delta R/R_f$ and their confidence intervals (*cnf*) are summarized in the Table. Since, it is difficult to compare the interaction results among the different metal ions with the help of this table, hence to make the comparison easier, a $\Delta R/R_f$ chart (Fig. 3*)* was prepared, which is a comprehensive approach for interpreting protein-metal ions interaction data. Further, the interaction results of transferrin with the metal ions under investigation were compared with the interaction behavior of these metal ions with other two important proteins, BSA and HSA. The data of BSA and HSA-metal ions interactions were taken from the previous reported screening (Alhazmi 2015).

Table: Values of $\Delta R/R_f$ and their confidence intervals (*cnfs*) for BSA, HSA and transferrin-metal ions interactions

Metal ions	BSA*	HSA*	Transferrin
Ag^{+}	0.0053 ± 0.0056	0.0317 ± 0.0090	-0.0062 ± 0.0081
Au^{+}	0.0005 ± 0.0088	0.0088 ± 0.0046	0.0019 ± 0.0106
Au^{3+}	0.0155 ± 0.0086	0.0064 ± 0.0064	-0.0188 ± 0.0017
Os^{3+}	-0.0402 ± 0.0123	-0.0435 ± 0.0164	-0.0148 ± 0.0064
Pd^{2+}	0.0129 ± 0.0117	-0.0147 ± 0.0103	-0.0916 ± 0.0339
Pt^{4+}	-0.0335 ± 0.0105	-0.0519 ± 0.0073	-0.0198 ± 0.0051
Rh^{3+}	-0.0237 ± 0.0048	-0.0200 ± 0.0037	-0.0041 ± 0.0134
Ru^{3+}	-0.0142 ± 0.0053	-0.0050 ± 0.0096	0.0026 ± 0.0086
Ir^{3+}	-0.2511 ± 0.0132	-0.2837 ± 0.0071	-0.2577 ± 0.0124

*Data has been taken from the PhD thesis of Dr. Hassan Alhazmi (Alhazmi, 2015).

not be influenced by the metal ion binding and the value of ΔR comes close to zero, even though there is an interaction. This can give a wrong conclusion of no interaction ($\Delta R \approx 0$) however this is very rare (Ahazmi et al. 2015; Redweik et al. 2012).

In the current study, most of the transferrin-metal ion interaction results showed negative $\Delta R/R_f$ values. The $\Delta R/R_f$ chart shows insignificant interactions between transferrin and few of the investigated metal ions such as Ag^{+}, Au^{+}, Rh^{3+} and Ru^{3+} because their *cnf*($\Delta R/R_f$) values intersect the zero line ($\Delta R/R_f$ = -0.0062 ± 0.0081, 0.0019 ± 0.0106, -0.0041 ± 0.0134 and 0.0026 ± 0.0086 respectively). On the other hand significant interactions have been observed between HSA and Ag^{+} and Rh^{3+}, while Au^{+} showed a weak interaction and Ru^{3+} displayed a non-significant interaction with the same protein. Insignificant interactions were also observed for BSA with monovalent ions Ag^{+} and Au^{+}, while it was significant with trivalent ions

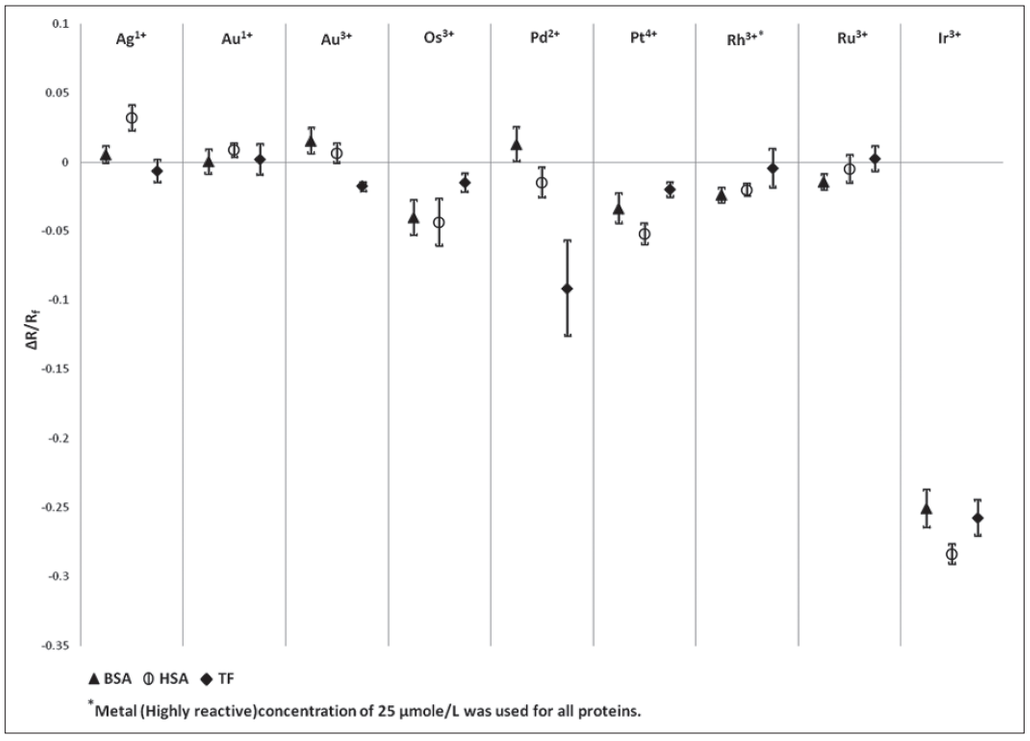

Fig. 3: ΔR/Rf chart showing interaction results of transferrin with all tested noble metal ions. The concentration of metal ions used was 250 µM, except for Rhodium ten times lower concentration was used (25 µM). *Electrophoretic condition:* tris buffer (20 mM, pH 7.4), sample injected for 4.5s at 0.5bar then buffer for 2.5s, voltage applied-10kV, 214nm wavelength, capillary: total length 31cm with effective length 22 cm, 50 µm I.D and capillary cartridge temperature 23°C. *Rinsing protocol:* capillary was rinsed with a mixture of 0.1 mol/L sodium hydroxide and 0.1 mol/L EDTA solutions at 2.5 bar for 2.5 minutes, then water for 1 minute followed by running buffer for 1.5 minutes.

The values of $\Delta R/R_f$ are extremely important to predict the interaction behavior between protein and the tested metal ions and their positive and negative signs indicate the direction of the effect of the interactions. When the overall charge of the tested protein becomes more positive (less negative), the electrophoretic mobility of the test protein will be increased and a positive $\Delta R/R_f$ value will be achieved. In some cases, the bound metal ion on the test protein undergoes further coordination with the surrounding anions in the running buffer; as a result the overall charge of the test protein gets more negative leading to a decreased electrophoretic mobility after the interaction. Consequently, a negative $\Delta R/R_f$ value will be obtained.

A variation of about 0.108s in eletrophoretic mobility corresponds to a change of 0.01 in the ΔR value. When $\Delta R/R_f \geq 0.01$, there is a significant interaction between the protein and tested metal ions, even with an unusually wide confidence interval. More importantly, for a significant interaction, the *cnf* of $\Delta R/R_f$ values should not intersect the zero line (Fig. 3). As a rare exception, the electrophoretic mobility of the protein under investigation might such as Rh^{3+} and Ru^{3+}. Exceptionally weak interactions of Au^{+} with all three proteins may be attributed to the decrease in the effective concentration of Au^{+} due to formation of gold nanoparticles, as the Au^{+} solution turned to blue color during the sonication.

The metal ion Ir^{3+} exhibited exceptionally strong and similar binding interactions towards all three proteins, transferrin ($\Delta R/R_f$ = -0.2577 ± 0.0124), BSA ($\Delta R/R_f$ = -0.2511 ± 0.0132) and HSA ($\Delta R/R_f$ = -0.2837 ± 0.0071). This is possibly attributed to the various selective binding sites present in these proteins for Ir^{3+}. The metal ion Pd^{2+} behaved differently with all three proteins. It showed relatively weaker binding affinities with BSA and HSA, a positive value of $\Delta R/Rf$ was obtained for BSA ($\Delta R/R_f$ = +0.0129 ± 0.0117) whereas a negative value was observed for HSA ($\Delta R/R_f$ = -0.0147 ± 0.0103). On the other hand, a very significant interaction has been observed with transferrin but with wider confidence interval ($\Delta R/R_f$ = -0.0916 ± 0.0339). Different interaction behavior of Pd^{2+} to these three proteins may be due to the difference in the binding sites in all three proteins. From Fig. 3, it can be seen that Os^{3+} and Pt^{4+} interact similarly with all three proteins.

They showed significant interactions with BSA and HSA, whereas relatively weaker interaction with transferrin was observed.

In addition to variation in the electrophoretic mobility, significantly broader peaks of transferrin have been observed after its interaction with Ir^{3+} and Pd^{2+}, moreover the intensity of TF-Ir^{3+} peak was found to be markedly increased, while the TF-Pd^{2+} peak decreased as compared to peaks due to transferrin without Ir^{3+} and Pd^{2+}, respectively. The peaks due to TF-Au^{+}, TF-Os^{3+}, TF-Ru^{3+} and TF-Pt^{4+} were observed to be similar (with respect to sharpness and intensity) to transferrin peaks without interaction with these metal ions in their respective electropherograms. An identical pattern was observed in the electropherograms of TF-Au^{3+}, TF-Ru^{3+} and TF-Ag^{+}, where apart from reduced intensity and sharpness in the peaks due to transferrin after interaction with these metal ions, a significantly elevated baseline was observed after transferrin peak (the right side of the peak did not touch the zero level). In general, the major changes in the intensity and sharpness of the peak due to transferrin was observed mainly with metal ions which showed significant interactions, while no marked differences in the peak shape were observed with those metal ions which displayed weak or insignificant interactions with transferrin (Fig. 2). This is because strong interactions may bring significant alterations in the properties of proteins including conformational changes.

In conclusion, the interaction of transferrin with noble metal ions was successfully screened using mobility shift-affinity capillary electrophoresis. Variations in the electrophoretic mobility of transferrin after interaction with ligand metal ions were recorded. Along with the change in the electrophoretic mobility, alteration in the peak shape were also observed in some cases which have been interpreted as the conformational change in protein after metal binding (*Fig. 2*). The normalized difference in the mobility ratios ($\Delta R/R_f$) of test protein with and without interaction of metal ions and its confidence interval (*cnf* of $\Delta R/R_f$) were calculated to represent the interaction results. Among all the tested metal ions Ir^{3+} showed very strong affinity towards all three proteins discussed above, this is possibly due to numerous selective binding sites for this metal ion in these proteins. The metal ion Pd^{2+} has exhibited a very significant interaction with transferrin, whereas weaker interactions have been observed with BSA and HSA. Overall, in most of the interactions negative $\Delta R/R_f$ values were obtained. This could be due to the further coordination of the bound metal ion with the surrounding anions inside the capillary tube. Overall, these interaction results will be helpful to understand the selectivity of the noble metal ions towards the investigated protein. In addition to protein-metal ion interactions, this study may provide valuable preliminary information to investigate other interactions such as, enzyme-metal ions, protein-protein, drug-metal ions and drug-protein interactions. Moreover, this technique can be helpful in the development of organometallic pharmaceutical agents.

3. Experimental

3.1. Chemicals and reagents

Transferrin Human (≥98%, 76-81kDa), tris powder, gold(I) chloride (AuCl), gold(III) chloride ($AuCl_3$), osmium(III) chloride hydrate ($OsCl_3$), palladium(II) nitrate dehydrate ($Pd(NO_3)_2.2H_2O$), platinum(IV) chloride ($PtCl_4$), rhodium(III) nitrate hydrate ($Rh(NO_3)_3$), ruthenium(III) chloride hydrate ($RuCl_3$) and iridium(III) chloride ($IrCl_3$) were obtained from Sigma-Aldrich (Steinheim, Germany). Silver(I) nitrate ($AgNO_3$) was purchased from Grüssing (Filsum, Germany). Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA-$Na_2.2H_2O$) and sodium chloride (NaCl) were obtained from Riedel de Haën (Hannover, Germany). Acetanilide was procured from Fluka (Steinheim, Germany). Concentrated hydrochloric acid (Conc. HCl) was obtained from Merck (Darmstadt, Germany). Double distilled water was prepared in our laboratory.

3.2. Apparatus and instruments

Investigations of all transferrin-metal ion interactions were carried out on an Agilent CE system model G1600A (Agilent Technologies, Germany) equipped with a capillary cooling system, an autosampler and a diode array detector (at 214 nm). A pressure of 2.5 bar was applied externally by using the normal air plug within the laboratory. Bare fused silica capillaries were procured from Polymicro Technologies (Phoenix, AZ, USA) which have 50 µm I.D. and used in a total length of 31 cm, corresponding to an effective length of 22 cm to the detector window. The electropherograms were monitored by Agilent ChemStation software installed with instrument. The resulted electropherograms are integrated by homemade (self made) integration software CISS (Andreini et al. 2008). Mettler Toledo FE20/EL20 pH-meter (Carl Roth, Karlsruhe, Germany) was used to measure the pH of the buffer solutions used throughout the analysis. Rotilabos-syringe filters (CME 022 mm) purchased from Carl Roth (Karlsruhe, Germany) were used to filter the sample solutions. The calculation of mobility ratio ($\Delta R/R_f$), its confidence interval and statistical analysis are performed by using Microsoft EXCEL™ (Microsoft Corporation, Version 2007).

3.3. Rinsing protocol and separation modes

Before starting any analysis, the new capillaries were conditioned by flushing at 1 bar for 20 min with 1M sodium hydroxide solution followed by water for 10 min. At the start of each analysis, the capillary was again flushed with 0.1M sodium hydroxide solution for 10 minutes at 2.5 bar and with water for 5 min, the same procedure was repeated at the end of each analysis day. The capillary was flushed with a mixture of 0.1 M sodium hydroxide and 0.1M EDTA solutions at 2.5 bar for 2.5 min followed by water for 1 min and running buffer for 1.5 min before the start of each run (Alhazmi et al. 2014). Finally, at the end of each screening, capillary was rinsed at 2.5 bar with 0.1M sodium hydroxide solution for 10 min followed by water for 5 min to get the reproducible results in next analysis, this also helps in minimizing the background noise.

The protein samples were injected hydrodynamically into the capillaries at 0.05 bar for 4.5 s, the samples were further pushed by the injection of running buffer at 0.05 bar for 2.5 s. The electrophoretic separations were executed at 23°C by applying a voltage of 10 kV, setting a maximum current at 20 µA, which means if the maximum current is achieved; the instrument will shut down automatically. Although throughout the investigation a stable current (≤ 13 µA) was observed, as a result the effect of the joule heating was insignificant (the joule heating is directly proportional to produced current). All separations were performed using normal mode i.e., capillary inlet at anode and outlet at cathode. Each sample was repeated for six times.

3.4. Preparation of solutions

3.4.1. Tris buffer (20 mM, pH 7.4)

An amount of 2.42 g tris was weighed in duplicate and dissolved in 200 mL double distilled water in separate volumetric flasks. The pH of the first solution was adjusted to 7.4 with hydrochloric acid and the volume was made upto 1000 mL with double distilled water. The pH of the second solution was adjusted to 7.4 with acetic acid and made upto 1000 mL with double distilled water. The second solution was used for the dissolution of metal salts of Ag^{+}, Pd^{2+} and Rh^{3+} which are precipitated with Cl^{-}, if the buffer solution would have hydrochloric acid.

3.4.2. Acetanilide stock solution (750 µg/mL)

37.5 g of acetanilide was weighed and dissolved in 50 mL tris buffer. The solution was sonicated until complete dissolution.

3.4.3. Protein solutions (20 µM and 6.66 µM)

The protein solution was prepared by dissolving 39.25 mg of transferrin in tris buffer into a 25 mL volumetric flask. Then 5 mL of acetanilide stock solution was added and the volume was made up with tris buffer. This protein solution was diluted threefold to obtain 6.66 µM before injection to minimize the band broadening and protein adsorption.

3.4.4. Metal ion solutions

The stock solutions for each metal ion were prepared in the tris buffer pH 7.4 to obtain the concentrations of 25 and 250 µM.

Each protein and metal ion solutions were freshly prepared in the same tris buffer before everyday's analysis. Moreover, all the solutions were filtered through 0.22 µm filter and degassed by sonicating for 5 min before injecting into the capillary.

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