Research Article

Spectroscopic Studies for Rhodium (III) Binding to Apo-Transferrin

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Transferrin belongs to a class of monomeric glycoproteins, which sequestrate and transport iron inside the body. Apart from iron, transferrin binds with various other metal ions and is assumed to deliver medicinally important metal ions to cells. Hence, the investigation of binding characteristics may provide crucial information for new drug developments. To study the biological impacts of medicinally important metal ions, in this work, we explored the binding behavior of Rh(III) ion with serum apo-transferrin (ApoHST) using FT-IR and UV-Vis spectroscopy. In FT-IR, interaction of Rh(III) with ApoHST was studied at three concentrations (0.25, 0.5, and 1 mM) of metal ion at different time intervals (15, 30, and 60 min). The IR spectra of Rh(III)-ApoHST coordinates revealed a marked reduction in amide I and II band intensities with alterations in band positions. The α-helical part of protein secondary structure reduced considerably (from 53% to 49%, 42%, and 39%), followed by an increment in β-sheet and β-turn components with the increasing concentrations of metal ion. Saturation level reached at 1 mM concentration of Rh(III) ion. In the UV-Vis spectroscopic study, absorption of metal ion-protein coordinates successively raised as concentration of Rh(III) ion increased. The binding constant (K) was calculated as $K = 1.6 \times 10^4 \, \text{M}^{-1}$, which showed a strong binding of the test metal ion with the protein. Upon coordination with a metal ion, the microenvironment of aromatic protein residues changed, which was detected by these spectroscopic techniques. The results revealed the existence of a significant interaction between Rh(III) ion and ApoHST. These research outcome may present new insight into the possible utilization of Rh(III) ion-based compounds in biomedicine. However, more investigations are needed to interpret the exact cellular mechanism.

1. Introduction

In the last few decades, metal ion-based drugs have proven their efficacy as potential antitumor agents [1, 2]. They are now extensively utilized in the diagnosis and clinical therapeutics for other conditions as well. Avci et al. [3] prepared new complexes of Cu(II), Co(II), and Zn(II) containing 6-methylpyridine-2-carboxylic acid and 2,2′-pyridylamine and their structures were characterized using FT-IR, LC-MS/MS, XRD, and UV-Vis-spectroscopic techniques. The compounds exhibited α-glucosidase inhibitory activity and IC$_{50}$ values in the range of 513.10–600 μM were recorded. Molecular docking study was performed to establish interaction between the synthesized complexes and target protein. A newly synthesized dinuclear Cu(II) complex of 2,5-furandicarboxylic acid with 4(5)-methylimidazole was evaluated for α-glucosidase inhibitory and cytotoxic activities through IC$_{50}$ values. The structure of the new compound was characterized by using the above mentioned techniques and Density Functional Theory (DFT) was used to find out the optimized geometry and vibrational harmonic frequencies for the synthesized complex. The interaction of new Cu(II)
complex with the target protein, which was also established through molecular docking studies [4]. In another similar study, a number of mixed-ligand M(II) complexes containing 2,2′-bipyridyl as α-glucosidase inhibitor possessing anti-diabetic activity were synthesized. The IC\textsubscript{50} values were determined in the range of 0.184 to >600 μM. The structural characterizations of the newly synthesized complexes were achieved by using LC-MS/MS, XRD, FT-IR, and UV-Vis spectroscopy. The complexes were subjected to molecular docking studies to investigate the interaction with target protein [5].

Rh(III) ion-based compounds are very much appreciated in the scientific literature because of their significant biological properties [6, 7]. Mollin et al. developed Rh(III) complexes exhibiting selective inhibition of proteinase activity [8]. Recently, Rh(III) complex was reported to be effective as epigenetic modulator in prostate cancer cells through inhibition of lysine-specific histone demethylase 1 [9], and in another study, Rh (III) complex was found to downregulate 8-activating enzymes through inhibition of neural precursor cell and effective against inflammatory bowel disease [10]. Kim et al. reported Rh (III) complexes that interacted with calf-thymus DNA and caused photo-induced cleavage of pBR322 [11]. The above observation supported the medicinal use of Rh(III)-based compounds as phototherapeutic agents in chemotherapy.

The serum apo-transferrin (ApoHST) is a glycoprotein containing two glycan components with 680-690 amino acid residues. Its molecular weight is 80 kDa. ApoHST transports Fe (III) ion throughout the human body in 25-50 μM concentration [12, 13]. It carries two closely related lobes (C and N) present in the protein C and N terminals. These lobes hold similar amino acids residues; however, their iron uptake and release behavior are distinct. At physiological pH 7.4, the stoichiometric binding constant of the C-terminal site is five times higher than the N-terminal site. It is also reported that the binding site of transferrin comprises two histidine residues, two tyrosine residues, and hydroxide ion and carbonate (CO\textsubscript{3}\textsuperscript{2−}/bicarbonate (HCO\textsubscript{3}−) ions, making six-coordinate complexes with Fe (III) [14]. Figure 1 shows the 3-D structure of ApoHST displaying its binding sites.

Approximately 30% of the human serum transferrin is saturated with iron. In comparison, the rest 70% exists as apo-transferrin (apoHST, iron-free transferrin), indicating the binding capability of apoHST with metal ions available in the blood [15, 16]. Transferrin plays a role of carrier protein and transports different metal ions including In(III), Ga (III), Zn(II), Al(III), and Ru(III) from one compartment to another in the biological system [17, 18]. Therefore, ApoHST is considered an important protein that mediates the transportation of diagnostically and therapeutically useful metal ions through blood plasma. Interaction studies of some noble metal ions such as Ru(III), Pt(IV), Au(III), Au (I), Os(III), and Ir(III) with ApoHST have been performed earlier by our research group using affinity electrophoresis [19], where we recorded noticeable metal-protein interactions through alteration in the electrophoretic migration of proteins along with the changes in the shape and intensity of ApoHST peaks. Several studies related to interaction of metal ions with transferrin and ApoHST have been performed in the past using different analytical techniques like ESI-mass spectrometry [20], UV-Vis-spectroscopy [21], and capillary electrophoresis [19]. Apart from the availability of numerous analytical techniques to study the binding behavior of protein-metal ions, UV-Vis and FT-IR spectroscopy are preferable because of their easy handling, fast and economical methods, in addition to accurate binding results. In the present work, we investigated the interaction of biologically active metal ion Rh(III) with ApoHST using UV-Vis and FTIR techniques. We selected this work based on the vital roles of ApoHST in the transportation of metal ions inside the body and the unavailability of much information of interaction with Rh(III) ion. The study will provide crucial information regarding the Rh(III)-ApoHST interaction, which may be useful to interpret the mechanism of Rh(III) ion-based compounds with ApoHST protein.

2. Materials and Methods

2.1. Chemicals and Instruments. ApoHST (lyophilized powder, >99% pure), rhodium (III) nitrate (>99% pure), and tris(hydroxymethyl) aminomethane (Tris) were procured from Sigma Aldrich (Saint Louis, USA). Double distilled grade water was used in this study to wash and prepare the solutions. A UV-Vis spectrophotometer (Shimadzu, Japan) was used for determining the binding constant, while a FT-IR spectrophotometer (Nicolet iS10, Thermo Fischer Scientific, USA) was used for recording IR spectrum in this investigation.

2.2. Preparation of Buffer, Metal, and Protein Stock Solutions. Accurately weighed 1.21 g of tris dissolved in 1 L of double distilled water to get a 10 mM tris buffer solution, and the pH of the solution was adjusted to 7.4 with diluted acetic acid. 40 mg of Apo HST was dissolved in 10 mM tris buffer
(1 mL, pH 7.4) to obtain a protein stock solution of 1 mM concentration. A stock solution of rhodium (III) nitrate (2 mM) was prepared in tris buffer and diluted to obtain 1 mM and 0.5 mM concentrations. The above solutions were filtered and degassed with the help of a sonicator.

2.3. FT-IR Analysis. Target metal ion concentrations (1, 0.5, and 0.25 mM) and the protein concentration of 0.5 mM were prepared by mixing equal volumes of rhodium (III) nitrate and Apo HST solutions. Solutions were correctly mixed and incubated for 15, 30 min, and 1 h at 25 ± 3°C; the spectra were recorded using FT-IR spectrophotometer by making hydrated films on silicon discs. Spectra were taken at 4 cm⁻¹ resolution and 400-4000 cm⁻¹ range at ambient room temperature (25 ± 2°C) [22]. The protein secondary structure was determined from the band in 1660-1650 cm⁻¹ range by using second derivative analysis [23, 24] and self-deconvolution-curve fitting methods [25]. These analyses were performed using the OriginPro 2021b (Origin Lab Corporation, Massachusetts, USA) software.

2.4. UV-Spectroscopic Analysis. Binding interactions of ApoHST with Rh(III) were studied through a UV-Vis spectrophotometer by recording the UV absorption spectra at 200-400 nm wavelength range. A deuterium lamp was used as an ultraviolet light source in the spectrophotometer. For the titration experiment, a constant concentration (15 μM) of ApoHST was mixed with sequentially increasing levels of Rh(III) nitrate-alone spectrum from the absorbance at 221 nm. Spectra were taken at 4 cm⁻¹ resolution and 400-4000 cm⁻¹ range at ambient room temperature (25 ± 3°C) [22]. The protein secondary structure was determined from the band in 1660-1650 cm⁻¹ range by using second derivative analysis [23, 24] and self-deconvolution-curve fitting methods [25]. These analyses were performed using the OriginPro 2021b (Origin Lab Corporation, Massachusetts, USA) software.

2.5. Determination of Drug-Protein Binding Constant. The binding constant (K) was determined using UV absorbance titration methods [22, 26], equations (1) and (2) can be derived:

\[
\text{Apo HST} + \text{Rh(III)} \leftrightarrow \text{Rh(III) \cdot Apo HST}, \quad (1)
\]

\[
K = \frac{[\text{Rh(III) \cdot Apo HST}]}{[\text{Apo HST}] [\text{Rh(III)}]]. \quad (2)
\]

Considering \([\text{Rh (III) \cdot Apo HST}] = C_B\),

\[
K = \frac{C_B}{(C_{\text{Apo HST}} - C_B)(C_{\text{Rh(III)}} - C_B)}. \quad (3)
\]

where \(C_{\text{Rh(III)}}\) and \(C_{\text{Apo HST}}\) represent the concentrations of Rh(III) and Apo HST, respectively. According to the Beer-Lambert law,

\[
C_{\text{Apo HST}} = \frac{A_0}{\epsilon_{\text{Apo HST}} \ell}, \quad (4)
\]

\[
C_B = \frac{(A_0 - A)}{\epsilon_B \ell}, \quad (5)
\]

where \(A_0\) is the absorbance of ApoHST without Rh(III) at 221 nm. \(A\) is the absorbance of ApoHST with Rh(III) at the same wavelength. \(\epsilon_{\text{ApoHST}}\) is the molar extinction coefficient of ApoHST. \(\epsilon_B\) is the molar extinction coefficient for bound Rh(III), whereas \(\ell\) is the 1 cm path length.

Further deduction can be carried out by substituting the values of \(C_{\text{Apo HST}}\) and \(C_B\) from equations (4) and (5) to obtain the following equation:

\[
\frac{A_0}{A_0 - A} = \frac{\epsilon_{\text{Apo HST}}}{\epsilon_B + \epsilon_{\text{Apo HST}} K C_{\text{Rh(III)}}}. \quad (6)
\]

By using equation (6), the variables of \(1/(A_0 - A)\) (y-axis), and \(1/C_{\text{Rh(III)}}\) (x-axis), a plot (double reciprocal) was drawn, which exhibited a linear relationship. The binding constant (K) was obtained from the ratio of intercept to slope of the double reciprocal plot.

\[
K = \frac{\text{Intercept}}{\text{Slope}}. \quad (7)
\]

3. Results and Discussion

3.1. FT-IR Analysis. FT-IR spectroscopy is applied to detect the actual conformation of ApoHST with and without the interaction of foreign materials. ApoHST displays four typical absorption peaks at 3299, 1654, 1549, and 1288 cm⁻¹ [27, 28], mirroring the amide A N-H stretching, amide I C=O stretching, amide II N-H bending coupled with CN-stretching, and amide III NH bending and CN-stretching frequencies, respectively. After adding Rh(III) ion at 0.25, 0.5, and 1 mM concentrations and developing them by incubating for 15, 30, and 60 min, the positions and intensities of these four absorption peaks were changed. The band position of amide I was shifted to 1645 cm⁻¹, 1644 cm⁻¹, and 1631 cm⁻¹ after 15, 30, and 60 min incubation times, respectively, in Rh(III)-ApoHST coordinate at 0.25 mM Rh(III) ion concentration (Figure 2). In Rh(III)-ApoHST coordinate at 0.5 mM Rh(III) ion concentration, the peak position of amide I band was shifted to 1644 cm⁻¹ (at 15 min) and 1634 cm⁻¹ (at 30 and 60 min) (Figure 3). While at 1 mM Rh(III) ion concentration, the peak position of amide I was changed to 1644 cm⁻¹, 1627 cm⁻¹, and 1625 cm⁻¹ after 15, 30, and 60 min incubation (Figure 4). Furthermore, the amide II band position was shifted to 1529 cm⁻¹, 1515 cm⁻¹, and 1512 cm⁻¹ in Rh(III)-ApoHST coordinate at 15, 30, and 60 min time points, respectively, and 0.25 mM Rh(III) concentration (Figure 2). In Rh(III)-ApoHST coordinate at 0.5 mM ion concentration, the amide II band position was changed to 1515 cm⁻¹, 1514 cm⁻¹, and 1512 cm⁻¹ at 15, 30,
and 60 min times, respectively (Figure 3), whereas, at 1 mM Rh(III) ion concentration, the position of amide II band was shifted to 1514 cm\(^{-1}\), 1507 cm\(^{-1}\), and 1506 cm\(^{-1}\) at 15-, 30-, and 60-min incubation times (Figure 4). No significant variation in intensity and position was observed in these bands when the solutions were incubated for more than 60 min. These findings indicated that binding interactions of ApoHST with Rh(III) at three different concentrations (0.25, 0.5, and 1 mM) induced alteration in its native conformational structure. As the shifting of four characteristic bands are more substantial in Rh(III)-ApoHST coordinate at 1 mM concentration than in the other two lower concentrations, the native conformation of ApoHST varied the most. A significant decrease in intensities of amide I (1654 cm\(^{-1}\) for unbound ApoHST) and amide II band (1549 cm\(^{-1}\) for unbound ApoHST) were noticed in the spectra of Rh(III)-ApoHST coordinates (Figures 2–4).

The shift in the intensity and shape of the peaks is likely attributed to the changes in the conformation of the protein molecule after interaction with metal ion. The hydrogen bonding interactions between Rh(III) and the functional groups of ApoHST (C-N, C=O, and N-H) caused these remarkable alterations in the spectra. Rh(III) ion interaction with the C-N group in ApoHST was apparent, as demonstrated by the displacement of the amide A band to a higher wave number from 3299 cm\(^{-1}\) (NH stretching, unbound ApoHST). The interaction (hydrogen bonding) of Rh(III) with the C-N and C=O functional groups of ApoHST caused
Figure 4: FT-IR spectra of free ApoHST and ApoHST-Rh (III) ion coordinates, at different time intervals (15, 30, and 60 min) at room temperature and physiological pH with higher metal concentrations (1 mM) and fixed ApoHST concentration (0.5 mM; 40 mg/mL) in 4000-1000 cm⁻¹ range.

Figure 5: Curve fitting analysis of amide I (1700-1600 cm⁻¹) of (a) ApoHST with no Rh(III) - α-helix 53%, β-sheet 23%, β-turns 10%, and β-antiparallel 14%; (b) Rh (III)-ApoHST 0.25 mM, ApoHST:Rh (III) = 1:0.5 - α-helix 49%, β-sheet 32%, β-turns 13%, and β-antiparallel 6%; (c) Rh (III)-ApoHST 0.5 mM, ApoHST:Rh (III) = 1:1 - α-helix 42%, β-sheet 41%, β-turns 11%, and β-antiparallel 6%; (d) Rh (III)-ApoHST 1 mM, ApoHST:Rh (III) = 1:2 - α-helix 39%, β-sheet 43%, β-turns 12%, and β-antiparallel 6%. The bonding interaction was performed in tris buffer at physiological pH.
<table>
<thead>
<tr>
<th>Components amide I (cm$^{-1}$)</th>
<th>Free ApoHST (%) (0.5 mM)</th>
<th>Rh(III) - ApoHST 0.25 mM coordinate ApoHST:Rh(III) =1 : 0.5(%)</th>
<th>Rh(III) - ApoHST 0.5 mM coordinate ApoHST:Rh(III) =1 : 1(%)</th>
<th>Rh(III) - ApoHST 1 mM coordinate ApoHST:Rh(III) =1 : 2(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β–Sheet; 1637-1614 (±2)</td>
<td>23</td>
<td>32</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td>α–Helix; 1660-1650 (±4)</td>
<td>53</td>
<td>49 (-7.5%) *</td>
<td>42 (-20.7%) *</td>
<td>39 (-26.4%) *</td>
</tr>
<tr>
<td>β–Turn; 1678-1670 (±2)</td>
<td>10</td>
<td>13</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>β–Antiparallel; 1692-1680 (±2)</td>
<td>14</td>
<td>06</td>
<td>06</td>
<td>06</td>
</tr>
</tbody>
</table>

*Percentage variations in ApoHST α-helix content upon coordination with Rh(II) ion at physiological pH and room temperature.
the amide I and amide II bands to shift, in FT-IR analysis. The results of the current FT-IR investigation were in agreement with the report available in the literature. In our previous investigation, we observed similar results when binding interaction of Pt(IV), Ir(III), and Fe(II) with bovine serum albumin (BSA) was studied. Spectral shifting as well as reduction in the intensities of the amide I and amide II bands of the protein was observed [29]. Belatik et al. reported significant interaction between Pb(II) and human serum albumin (HSA) and bovine serum albumin (BSA). They observed remarkable reduction in the intensity of amide I and amide II bands of the proteins, which they concluded was due to hydrophilic contacts of Pb(II) with C=O, C-N, and N-H groups of the proteins [30].

3.2. Secondary Structure Analyses. FT-IR measurements were further employed to track the amide bands for better understanding the influence of Rh(III) binding on ApoHST conformation. The amide I band of proteins, which falls between 1600 and 1700 cm⁻¹, may be attributed to C=O stretching vibrations and displays a greater sensitivity to protein structural changes than the amide II band [31]. In this study, we used self-deconvolution and multipeak-fitting methods to process the amide I band of ApoHST with and without the Rh(III) ion, and the observed results are shown in Figure 5. For free ApoHST, there were six absorption bands that indicated various secondary structures: α-helix (1650 and 1660 cm⁻¹), β-sheet (1614 and 1625 cm⁻¹), β-turn (1678 cm⁻¹), and β-antiparallel (1691 cm⁻¹) (Figure 5(a)). Figure 5 and Table 1 showed the quantitative assessments of ApoHST secondary structure. According to the literature [32, 33], the amide I band of free ApoHST secondary structure consists of β-sheet (23%), β-turn (10%), α-helix (53%), and β-antiparallel (14%). The α-helix components were the largest which inferred the fact that ApoHST primarily occurred in the α-helix conformation. As demonstrated in Figures 5(b), 5(c), and 5(d) and Table 1 as the Rh(III) concentration increased, the content of β-sheet and β-turn enhanced, while the content of α-helix decreased, implying partial protein unfolding [34]. The secondary structure analysis implied a noteworthy reduction in the α-helix content from 53% (free ApoHST protein concentration) to 49% (at 0.25 mM metal ion concentration), 42% (at 0.5 mM metal ion), and 39% (at 1 mM metal ion concentration). At 1 mM metal ion concentration, a saturation threshold of interaction was obtained. The components of other secondary structures were also altered to some degrees. The results revealed that the interaction of Rh(III) with ApoHST influenced the protein secondary structure. These findings corroborated our hypothesis that when iron is removed from the structure of transferrin, it becomes more “open” or “flexible.” The results of the secondary structure analysis in the present investigation were in agreement with those reported by previous studies in the literature. Belatik et al. reported a remarkable reduction in the α-helix content followed by increment in the β-turn and β-antiparallel structure of HSA and BSA after interaction with Pb(II) at physiological pH [30]. Similarly, in our previous study, we observed a marked decrease in the α-helix structure of BSA upon interaction with the metal ions, Pt(IV), Ir(III), and Fe(II). On the other hand, an increase in the β-turn and β-antiparallel structure of the protein was noticed [29].

3.3. UV-Spectroscopic Analysis. UV-Vis spectroscopy is a simple and efficient tool for investigating the protein-ligand binding and detecting the state of protein conformational changes [35]. It was used to evaluate the binding interaction of the Rh(III) metal ion with ApoHST. Figures 6 and 7 depict the effect of Rh(III) ion on the ApoHST absorption spectra, while the experiments were performed at physiological pH. The spectra of ApoHST showed a significant absorption band at 221 nm, corresponding to $\pi \longrightarrow \pi^*$ transition of Trp, Tyr, and Phe amino acid residues [36]. As illustrated in Figures 6, the band at 221 nm expanded as the metal ion concentration was increased. A little red shift was also noticed in addition to the hyperchromic impact. These alterations indicated an interaction between ApoHST and Rh(III) ion via static quenching. Unfolding and unraveling of the protein backbone induced a change in $\lambda_{max}$. As a result of secondary structural changes of ApoHST and, thus, alteration in hydrophobicity around the above mentioned aromatic amino acid residues, further change in polarity of the Trp and/or Tyr microenvironment may have occurred [37, 38]. The existence of an interaction between the ApoHST and the Rh(III) ion was clearly demonstrated by these findings.

A reciprocal plot (Figure 7) was plotted between $1/(A_0 - A)$ and $1/C_{Rh(III)}$, where $A_0$ and $A$ represented the absorbance of unbound protein and Rh(III) ion-ApoHST coordinates, respectively, with varying metal ion concentrations at 221 nm and $C_{Rh(III)}$ represented the molar concentrations of Rh(III) ion in different Rh(III)-ApoHST solutions. The plot
was observed to be linear ($R^2 > 0.99$), and the intercept to slope ratio was utilized to calculate the binding constant ($K$) of the interaction. The binding constant was determined to be $1.16 \times 10^4 \, M^{-1}$, which suggested a strong binding interaction between metal ion and protein. Binding constant, also known as its association constant, is a measurement of interaction of a ligand with a protein and indicates how rapidly the ligand-protein interaction takes place. The binding constant needs to be high enough for a significant amount of ligand to be distributed throughout the body, while being low enough for the ligand to be released once reached the binding sites.

The optimal binding constant range falls between $10^4$ and $10^6 \, M^{-1}$ [29], and the ligands with the binding constants values in this range are considered to be suitable for drug development. In the present research, the binding constants of metal ions demonstrated that Rh(III) ions bind to ApoHST protein effectively and will be distributed easily throughout the biological system.

4. Conclusions

The capability of metal-based compounds to bind serum protein is critical in drug development, since it is involved in the transportation, distribution, metabolism, and excretion of these pharmaceuticals. Binding of Rh (III) ion with ApoHST was efficiently examined and quantified by using FT-IR and UV spectroscopy. These strategies have a plethora of virtues over other techniques, including the fact that they are simple and cost-effective. When ApoHST interacted with Rh(III) ion, significant alterations in the amide I band intensity were noticed, this alteration was exploited to determine the portion of their interaction. The variations in the position of amide I, amide II, amide III, and amide A bands in FT-IR spectra were recorded, which indicated a significant interaction of test metal ion with the ApoHST. Partial unfolding and change in the protein secondary structure was identified by a substantial reduction in the $\alpha$-helical component and its conversion to $\beta$-turn and $\beta$-sheet. The results indicated that the binding of metal ion has taken place on the protein surface through electrostatic forces and also interacted to the aromatic amino acid residues leading to modification in the microenvironment around them. The estimated binding constants revealed that the selected metal ion bound to ApoHST protein effectively. These findings may help in understanding the binding dynamics of the chosen metal ion with ApoHST in vivo, as well as the transport and biotransformation of Rh(III) ion-ApoHST coordinates within the body system.

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 is no conflict of interest regarding the publication of this article.

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