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New Insights into the Redox Chemistry of Ruthenium Metallopharmaceuticals: The Electrochemical Behaviour of [LH][*trans*-$Ru^{III}Cl_4L_2$] (L = imidazole or indazole) Complexes

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Here we report the findings from a study on the electrochemical behaviour of two Ru^{III} complexes [LH][*trans*-$Ru^{III}Cl_4L_2$] (L = imidazole, ICR, or indazole, IndCR) in aqueous solution at different pH values. An electrochemically reversible and chemically quasi-reversible one-electron reduction is observed for both compounds. Despite the similarity of the structures, the fate of the electrogenerated Ru^{II} species is different; in the case of ICR, imidazole, followed by a chloride ion, is released as the consequence of the reduction, while for IndCR two subsequent water for chloride substitutions take place. Our measurements suggests that the Ru^{III} complexes under study may serve as pro-drugs, being activated by reduction in a suitable aqueous environment and, therefore, binding biomolecules more rapidly.

Introduction

Three Pt^{II} compounds, commercially known as cisplatin, carboplatin, and oxaliplatin, are currently the most widely used anticancer agents. Notwithstanding the widespread applications of platinum anticancer drugs, there is still a great need for the development of novel metal-based compounds with additional features.

The search for "nonclassical" metal antitumor drugs has long stimulated investigations into the field of non-platinum metal drugs. Non-platinum active compounds are likely to have different mechanisms of action, biodistribution and toxicity from those of platinum drugs, and might therefore be active against human malignancies that are resistant, or have acquired resistance, to the latter agents. They may also show reduced host toxicity. Ruthenium seems to be the most promising among the several metals investigated.[1,2]

Among the different metal complexes, [ImH][*trans*-$RuCl_4$(dmso-S)(Im)] (NAMI-A, where Im = imidazole) was selected because of its very good antimetastatic activity; from 1999 to 2002 it was introduced into clinical trials.[3] Two similar complexes, containing imidazole (Im) and indazole (Ind) as ligands, namely [ImH][*trans*-$RuCl_4Im_2$], ICR, and [IndH][*trans*-$RuCl_4Ind_2$], IndCR, showed excellent antitumour activity in various animal models.[4] In particular, IndCR entered phase I clinical trials in 2003 as an anticancer drug which is very active against colon carcinomas and in metastases (Figure 1).[2]

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Despite the large number of works published, the mechanism of action of ruthenium complexes is still largely unknown. Given that Ru^{III} complexes are kinetically more inert than the corresponding Ru^{II} derivatives, an "activation by reduction" mechanism has been proposed to explain the biological activity of some ruthenium derivatives.[5,6] According to this hypothesis, NAMI-A is considered to be a *pro-drug*, a compound that is converted in the body through reductive metabolism to the Ru^{II} pharmacologically active species. This reactive intermediate should link the biological target after rapid dissociation of some of its ligands. Solid tumours create hypoxic (reducing) regions because they grow faster than the tissue can generate new blood vessels to supply enough oxygen.[7] Nontoxic drugs which can only be "activated" in hypoxic regions (*hypoxia-activated prodrugs*) therefore offer the promise of a specific systemic treatment against solid tumours, including metastases. For this reason, the Ru^{III}/Ru^{II} electron transfer reaction is particularly interesting.

Aquation of the imidazole (NAMI-A[8] and ICR[9]) and indazole (IndCR[10]) complexes leads mainly to mono- and diaqua complexes, at different time scales. The initial transformation into a monoaqua complex seems to play a crucial role for further biological activity, since reactions with biological substrates are much faster in "aged" solutions of ICR than in "fresh" solutions. Further hydrolysis products are unknown, but could be μ-oxo complexes. Formation of such di- or polynuclear Ru complexes is pH-dependent, as hydrolysis proceeds faster at higher pH, leading to precipitation. Recently, we reported a detailed electrochemical study on NAMI-A indicating the structural and chemical consequences stemming from the easy Ru^{III}/Ru^{II} electron

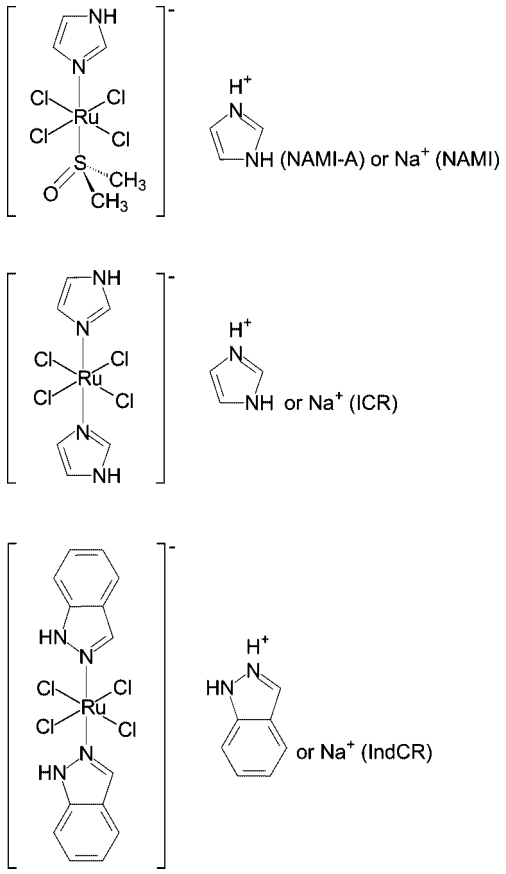

Figure 1. Sketch of compounds NAMI-A, ICR and IndCR.

Results and Discussion

Electrochemical Behaviour of ICR [ImH][*trans*-$Ru^{III}Cl_4Im_2$] (Im = imidazole)

As illustrated in Figure 2, the cyclic voltammetric (CV) response of an aqueous solution of $[Ru^{III}Cl_4Im_2]^-$ (1.0 mM Ru complex in 0.1 M $NaClO_4$, pH 4.0) at a glassy carbon (GC) electrode reveals a coulometrically checked (see below) one-electron reduction (peak A) coupled to chemical complications, as testified by the appearance of peak C in the back scan at ΔE_p = 260 mV.

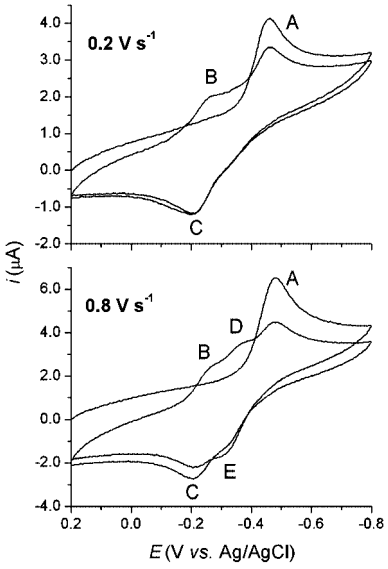

Figure 2. CV response of a 1.0 mM aqueous solution of $[Ru^{III}Cl_4Im_2]^-$ (0.1 M $NaClO_4$, pH 4.0), at a GC electrode, scan rate = 0.2 (top) and 0.8 (bottom) V s^{-1}.

transfer in aqueous solution.[11] Surprisingly, the reduction in acidic solutions (pH 6.0) generates the transient Ru^{II} homologue, which quickly undergoes substitution of Im by a water molecule affording $[Ru^{II}(H_2O)Cl_4(DMSO)]^{2-}$, followed after a longer period by the substitution of one chloride ligand by a water molecule affording the ultimate product $[Ru^{II}(H_2O)_2Cl_3(DMSO)]^-$. At physiological pH (7.4), only the substitution of two chlorides by water is observed, just in the longer period of the electrolysis time scale. The hypoxic and acidic environment present in a tumoral tissue may favour the Ru^{III}/Ru^{II} reduction and the subsequent Im hydrolysis, thereby promoting a unique means of activation of such a metallic drug in tumour tissue.

A thorough study of the electrochemical behaviour of the title compounds in aqueous solution has not yet been published. A few remarks were made by Sadler et al.[9] and, very recently, a seminal electrochemical study in organic solvents (DMF and DMSO) was published by Keppler et al.[12] In order to shed light on the redox properties of antitumour ruthenium complexes and their possible activation by reduction in experimental conditions nearer to the physiological ones, we show here a voltammetric, coulometric, and NMR study on ICR and IndCR in water at different pH values.

When a double cycle CV experiment is performed, the cathodic counterpart of the oxidation C (peak B) is observed, indicating that the peak system B/C is related to a stable species produced after the reduction occurring at peak A. In the scan rate (v) range 0.05–5.00 V s^{-1}, no return peak associated to peak A is observed. At a $v > 0.8$ V s^{-1} a third peak system (D/E) appears between A and B/C. This new couple is chemically quasi-reversible and electrochemically reversible. For peak systems A and B/C, the current functions i_p vs. $v^{1/2}$ and i_p vs. concentration plots are linear through the origin, supporting the absence of adsorption phenomena. Table 1 summarizes the CV data.

All these observations are consistent with an ECE mechanism: the chemical reaction following reduction A produces further electroactive species.[13,14]

In order to substantiate the identity of such intermediates, the CV response of $[Ru^{III}Cl_4Im_2]^-$ was compared with that of two possible candidates, namely $[Ru^{III}(H_2O)Cl_3Im_2]$ and $[Ru^{III}(H_2O)Cl_4Im]^-$ (obtained on standing $[Ru^{III}Cl_5Im]^{2-}$ in water, see Experimental Section). Since $[Ru^{III}(H_2O)Cl_3Im_2]$ is stable only under acidic conditions (pH < 4.0),[15] an accurate comparison was feasible only with such pH values. Figure 3 shows the overlap of the three CVs. All three complexes display a similar ECE behaviour, with the final

Table 1. Relevant electrochemical data for $[Ru^{III}Cl_4Im_2]^-$ and its aquated compounds.

	E_p(A) (V vs. Ag/AgCl at 0.2 V s^{-1})	$E°'$(B/C) (V vs. Ag/AgCl)	$E°'$(D/E) (V vs. Ag/AgCl)
$[Ru^{III}Cl_4Im_2]^-$	–0.46	–0.24	–0.34 (at v>0.8 V s^{-1})
$[Ru^{III}(H_2O)Cl_3Im_2]$	–	–0.24	–0.34
$[Ru^{III}(H_2O)Cl_4Im]^-$	–0.44	–0.23	–

redox couple (peaks B/C) located at the same potential, while the first reduction (A) is obviously located at different values.

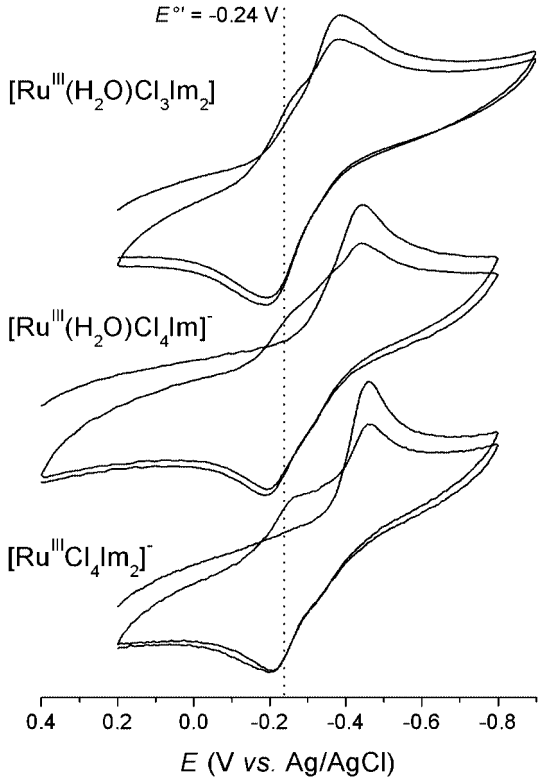

Figure 3. CV response of 1.0 mM aqueous solutions of $[Ru^{III}(H_2O)Cl_3Im_2]$ (top), $[Ru^{III}(H_2O)Cl_4Im]^-$ (middle), and $[Ru^{III}Cl_4Im_2]^-$ (bottom) (0.1 M $NaClO_4$, pH 4.0), at a GC electrode, scan rate = 0.2 V s^{-1}.

We propose the diaqua $[Ru^{III}(H_2O)_2Cl_3Im]$ as the final product of the ECE sequence, as it is the only intermediate that could reasonably be obtained from all three complexes. In the case of $[Ru^{III}Cl_4Im_2]^-$ the intermediate $[Ru^{III}(H_2O)Cl_3Im_2]$ is clearly present at $v > 0.8$ V s^{-1} (Figure 2). The same behaviour and the same CVs are also observed in phosphate buffer (PB) at pH 6.0; for this reason, we can extend the mechanism of reaction (Figure 4) to this pH value as well.

Bulk electrolysis of an aqueous, acidic solution (pH 4.0 and 6.0) of $[Ru^{III}Cl_4Im_2]^-$ (E_w = –0.60 V) consumes 1 mol of electrons per mol of complex, producing a change in colour from deep orange to lemon yellow, indicative of the formation of a Ru^{II} species. In the longer electrolysis time scale, the Im/water and Cl^-/water exchanges occur completely so that the $E°'$ of the final species correspond to that of the B/C peak couple. The overall bulk electrolysis

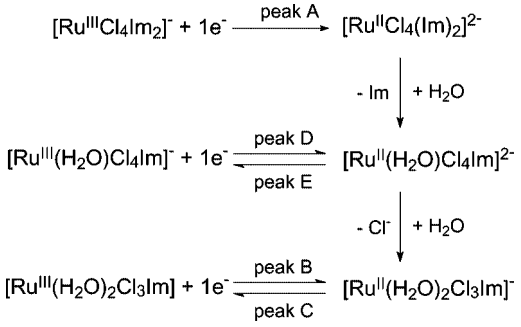

Figure 4. Proposed reaction scheme for the reduction of ICR.

behaviour is confirmed by the chemical reduction with reduced glutathione (GSH) followed by CV tests; the results show that the diaqua complex is obtained during the reaction (30 min), although some decomposition occurs (ca. 20%).

In neutral solution (0.1 M PB, pH 7.4) the general trend is maintained. However, there is a moderate shift in E_p(A) and a strong shift in $E°'$(B/C). Indeed, E_p(A) changes from –0.46 to –0.49 V by passing from pH 4.0 to 7.4, while $E°'$(B/C) shifts from –0.24 to –0.37 V in the same pH range. This may be due to the less efficient protonation of the leaving ImH^+ (peak A), and to the partial formation of hydroxylated aqua species (peaks B/C). Given that the A and B/C signals partially overlap, the peak couple D/E is also completely obscured at high v. The similarity of the behaviour at pH 7.4 of the two Ru^{III} complexes with that at acidic pH values confirms the stepwise loss of Im and chloride with replacement by the water after the initial reduction step in the pH range 4.0–7.4.

Chemical Reduction of ICR Followed by NMR Spectroscopy

In order to confirm the assignment of the species produced at different pH values, chemical reductions with GSH were followed by ^1H NMR spectroscopy. To avoid the interference of the imidazolium countercation, all experiments were performed with ICR sodium salt. The oxidation of glutathione to glutathione disulfide (GSSG) is a two-electron reaction (2 GSH → GSSG + $2e^-$ + 2 H^+). In order to increase the reaction speed, an ICR sodium salt/GSH ratio of 1:2 was used (37 °C, NMR measured after 15 min); the resulting mixture exhibited no broadening of the internal standard peak [referenced to the water-soluble silane 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt, DSS] due to the presence of paramagnetic species as residual ICR (Ru^{III} d^5) or glutathyil radical intermediate.

The reduction carried out on a 10 mM solution of ICR in 0.1 M acetate buffer (pH 4.0), and PB (pH 6.0 and 7.4), parallels that reported by Keppler et al.[16] (Figure 5). Two sets of signals are observed; the first corresponds to the free imidazole (A), while the second is typical of a coordinated imidazole (B). Pattern A increases with time, while pattern B decreases as the result of the reduction of the complex to the corresponding Ru^{II} analogue and the following Im/H_2O exchange. All these observations are consistent with the electrochemical data in which the substitution of Im by a water molecule in the reduced dianion $[Ru^{II}Cl_4Im_2]^{2-}$ (not observed in NMR) produces $[Ru^{II}(H_2O)Cl_4Im]^{2-}$ and/or $[Ru^{II}(H_2O)_2Cl_3Im]^-$ (pattern B). At longer reaction times, the reduced species is completely destroyed (only pattern A is observed).

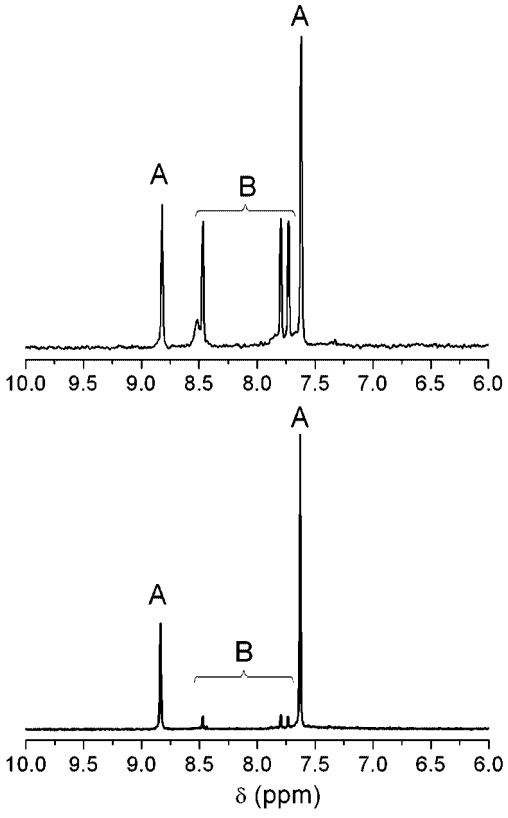

Figure 5. Expanded 6.0–10.0 ppm resonance region of the ^1H NMR spectrum of ICR (10 mM in 0.1 M phosphate buffer, pH 6.0) recorded 15 min (top) and 1 h (bottom) after the addition of glutathione (20 mM) at 37 °C.

Electrochemical Behaviour of IndCR Sodium Salt [Na][*trans*-$Ru^{III}Cl_4Ind_2$] (Ind = indazole)

Since the indazolium salt of IndCR is barely soluble in water, all experiments were performed with the sodium salt.

Figure 6 shows the CV response of an aqueous solution of $[Ru^{III}Cl_4Ind_2]^-$ (1.0 mM Ru complex in 0.1 M $NaClO_4$, pH 4.0, water solution) at a GC electrode. The coulometrically checked one-electron reduction (peak-system F/G) is followed by chemical complications, as testified by the appearance of peak J in the back scan. In the scan rate range 0.05–5.00 V s^{-1}, the diagnostic electrochemical features are:

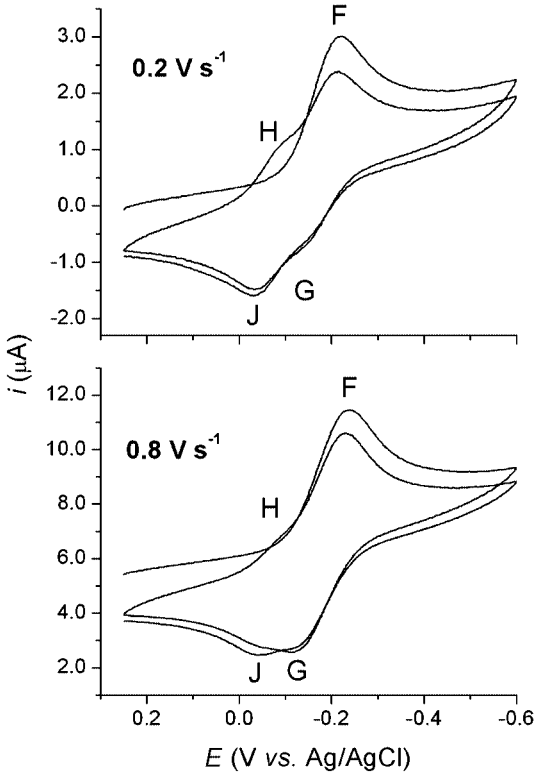

Figure 6. CV response of a 1.0 mM aqueous solution of $[Ru^{III}Cl_4Ind_2]^-$ (0.1 M $NaClO_4$, pH 4.0), at a GC electrode, scan rate: 0.2 (top) and 0.8 (bottom) V s^{-1}.

i. The current ratio $i_{p,a}(G)/i_{p,c}(F)$ is 0.3 at 0.05 V s^{-1} and progressively increases with v until it reaches unity at 1.5 V s^{-1};
ii. The peak-to-peak separation $\Delta E_p = E_{p,a} - E_{p,c}$ remains practically constant (80 mV) by increasing v in the range of v where peak G is present ($v > 0.8$ V s^{-1});
iii. The formal electrode potential $E°' = (E_{p,c} + E_{p,a})/2$ is equal to –0.160 V vs. Ag/AgCl ($v > 0.8$ V s^{-1});
iv. The plots of i_p vs. $v^{1/2}$ and i_p vs. concentration are linear through the origin, indicating the absence of any adsorption process.

Also in this case, when a double cycle CV experiment is performed, the cathodic counterpart of the oxidation J (peak H) is observed. This couple is located at –0.072 V vs. Ag/AgCl, and shows electrochemical parameters similar to that of the F/G system.

After increasing the pH to 6.0 and 7.4, all the peaks remain at the same potential except peak H, which slightly shifts under peak F. The overall electrochemical behaviour is compatible with an ECE mechanism.

Bulk electrolysis of an aqueous, acidic solution (pH 4.0) of $[Ru^{III}Cl_4Ind_2]^-$ (E_w = –0.35 V) consumes 1 mol of electrons per mol of complex, yielding the usual Ru^{II} yellow lemon solution. Despite strong decomposition, after 0.5 Faradays, the two peak couples F/G and H/J are still present. After 1 Faraday, only a new species at $E°'$ = +0.065 V vs. Ag/AgCl is present (peaks K/L, Figure 7). At

pH 6.0 only a species at $E°' = -0.065$ V, compatible with the H/J system, is present, while at pH 7.4 the decomposition is so high that after 45 min of electrolysis the complex is fully decomposed. The chemical reduction with GSH parallels the above-reported results.

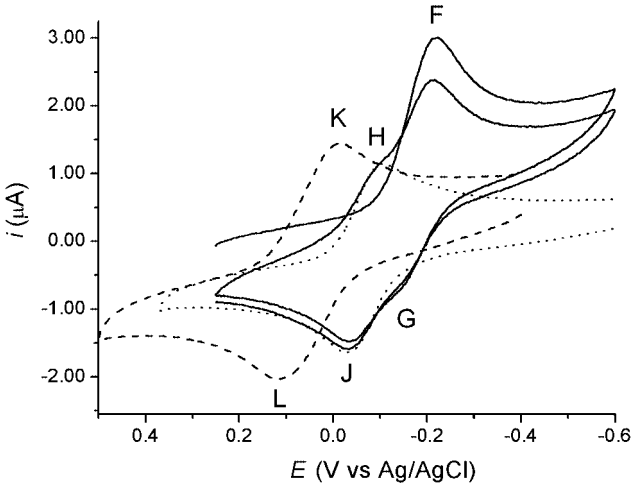

Figure 7. CVs performed before electrolysis (solid line), after 1 F in 0.1 M acetate buffer (pH 4.0, dashed line) and after 1 F in 0.1 M phosphate buffer (pH 6.0, dotted line).

Chemical Reduction of IndCR Followed by NMR Spectroscopy

The reduction carried out with GSH ([IndCR] = 10 mM, IndCR/GSH ratio 1:2, 37 °C, NMR measured after 15 min) in 0.1 M acetate buffer (pH 4.0), and PB (pH 6.0, Figure 8) shows a similar pattern of peaks. Free indazole is not present throughout the experiment. If we consider only the set of signals at ca. 9 ppm (in particular, the singlet due to the proton in position 3; this signal is the most easily characterized), the fate of the chemically generated Ru^{II} species is unambiguous: the singlet at $\delta = 9.06$ ppm (peak C) decreases until it disappears after 1 h, while another singlet at $\delta = 8.97$ ppm (peak D) increases as well. After 30 min, a new singlet at $\delta = 8.92$ ppm (peak E) starts to increase. At pH 6.0, peak C′ ($\delta = 8.96$ ppm) represents the only species present at the beginning of the experiment. After 30 min, peak D′ ($\delta = 8.92$ ppm) begins to increase. After 2 h a 1:1 ratio is reached, and after 4 h peak D′ is about twice as high.

By combining the experimental results obtained in CV and NMR, we can propose the reaction pattern reported in Figure 9. The reduction of IndCR produces the dianion $[Ru^{II}Cl_4Ind_2]^{2-}$. This species is more stable than the corresponding $[Ru^{II}Cl_4Im_2]^{2-}$, and slowly exchanges stepwise two Cl^- for water. At pH 6.0 this exchange is inhibited, so that only the first step is observed both in NMR and bulk electrolysis. Surprisingly, in this case, the "traditional" Cl^-,Cl^-/$2H_2O$ substitution takes place, while in ICR the Im,Cl^-/$2H_2O$ substitution, as observed also for NAMI-A in acidic conditions, is the predominant reaction.

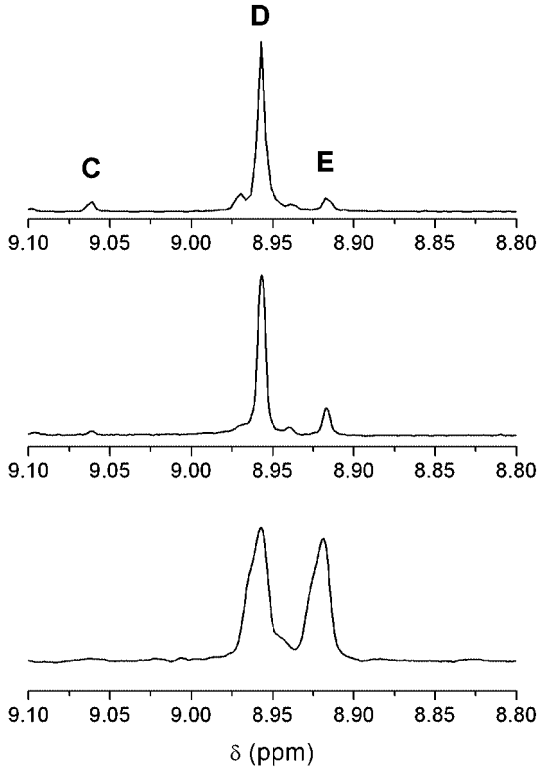

Figure 8. Expanded 8.80–9.10 ppm resonance region of the ^1H NMR spectrum of IndCR (10 mM in 0.1 M acetate buffer, pH 4.0) recorded 15 min (top), 1 h (middle), and 2 h (bottom) after the addition of glutathione (20 mM).

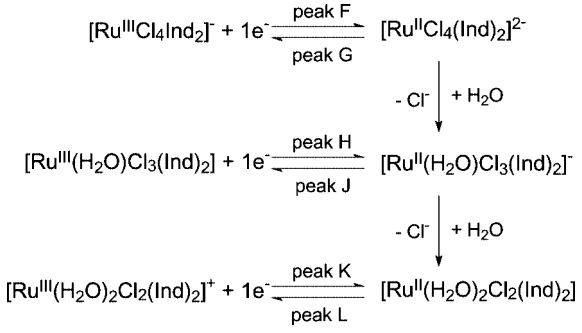

Figure 9. Proposed reaction scheme for the reduction of IndCR.

Conclusions

We have reported on a study of the electrochemical behaviour of the two Ru^{III} complexes [LH][*trans*-$RuCl_4L_2$] (L = imidazole, ICR, or indazole, IndCR) in aqueous solution at different pH values. An electrochemically reversible and partially chemically reversible one-electron reduction is observed for both compounds. Despite the similarity of the structure, the fate of the electrogenerated Ru^{II} species is different; in the case of ICR, imidazole, followed by a chloride ion, is released as the consequence of the reduction, while in IndCR Cl^-,Cl^-/$2H_2O$ substitution takes place. The chemical complications following the electron transfer in IndCR are also independent of pH, and act only on the kinetic aspect of the substitutions. It is noteworthy that, while the acti-

vation of the Ru^{III} homologues occurs in a time interval of hours,[9] that of the reduced species takes place in less than one hour. The differences in the chemical behaviour of the electrogenerated Ru^{II} species may be imputable to the different basicity of Im (pK_a = 7.11) and Ind (pK_a = 1.25).[17] In fact, the propensity toward protonation of the axial ligand L (where L is Im or Ind) influences the formation of $[LH]^+$ and, hence, its detachment from the complex. As reported in the Introduction, we found precedents for this in a recent study on the electrochemical behaviour of NAMI-A:[11] Furthermore, as reduction of Ru^{III} to Ru^{II} fills the dπ orbitals, an axial ligand such as Ind, having a higher electronic delocalization than Im by virtue of annulation, could partially act as a π-acceptor and, thus, increase the Ru–L bond stability.

There are many redox couples in a cell that maintain the redox environment, with the glutathione disulfide/reduced glutathione (GSSG/2GSH) being the most abundant.[18] In proliferating cells, the reduction potential is about –0.24 V vs. NHE (i.e. –0.44 V vs. Ag/AgCl).[19] According to the $E°'(Ru^{III}/Ru^{II})$ measured, the biological reduction process of ICR and IndCR is thus possible. The Ru^{II} species show a lower stability with respect to the Ru^{III} counterpart and thus the chemical reactions following the electron transfer may produce more reactive intermediates. The combination of electrochemical and NMR measurements shed light on the overall mechanism, suggesting that Ru^{III} complexes may serve as prodrugs, which may be activated by reduction in vivo to coordinate more rapidly to biomolecules. The low O_2 content in tumour cells (hypoxic environment) should favour the reduction to Ru^{II}, which is generally more active than Ru^{III} enforcing the "activation by reduction" hypothesis.

Experimental Section

Complexes $Na[RuCl_4Im_2]$[16] and $[ImH][RuCl_4Im_2]$,[20] (Im = imidazole), and $Na[RuCl_4Ind_2]$ (Ind = indazole)[21] were obtained following published procedures. Their aquated products ($[Ru(H_2O)Cl_4Im]^-$[22] and $[Ru(H_2O)Cl_3Im_2]$[15]) were obtained directly in solution according to data in the literature. The identity and the purity of all species were checked using the usual techniques. All salts were analytical grade and used as received. Deionized water was obtained through a Milli-Q (18 MΩ) deionizing system and was used for the preparation of all solutions. ^1H NMR spectra were measured in D_2O with a JEOL Eclipse Plus spectrometer operating at 400 MHz. Proton chemical shifts were reported in parts per million (ppm) referenced to residual solvent proton resonance (i.e. HDO 4.8 ppm) or silane standard 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS).

Electrochemical experiments were performed using an EG&G PAR 273 electrochemical analyser interfaced to a personal computer, employing PAR M270 electrochemical software. A standard three-electrode cell was designed to allow the tip of the reference electrode to closely approach the working electrode. The reference electrode was silver/silver chloride (Ag/AgCl), containing saturated, aqueous KCl. Its stability was checked using the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple. The Ag/AgCl reference electrode was stable with a precision of ±5 mV. The working electrode was a glassy carbon disk (diameter 0.1 cm) sealed in epoxy resin. The solid electrodes were polished with alumina followed by diamond paste, then washed with distilled water and dried. This process yielded a reproducible surface for all experiments. Positive-feedback iR compensation was applied routinely. All electrochemical measurements were carried out under nitrogen in water; solutions were 1.0 mM with respect to the compounds under study and 0.1 M with respect to the supporting electrolyte. The temperature of the solution was kept constant (20 ± 1 °C), by circulation of a thermostatted water/ethanol mixture through a jacketed cell.

The reduced species were produced in quantitative yield by bulk electrolysis. This was carried out in a conventional two-compartment glass cell with porous glass separation. A Pt basket was used as the cathode at a potential ca. 100–150 mV more negative than the formal potential $E°'$, a Pt wire was used as the anode, and Ag/AgCl was the reference electrode. Nitrogen was continuously bubbled through the solution during electrolysis. In all cases, transformation of Ru^{III} to Ru^{II} was checked by current integration and by in situ CV.

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