Luminiscent Metal Complexes for Diagnostic Applications
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Chapter 4

Multimetallic Ruthenium(II) Complexes as ECL Labels

Two homometallic complexes [Ru2-Lysf] and [Ru3-LysLysf] containing ruthenium polypyridyl units bound to lysine (Lys) amino acid or the related dipeptide (LysLys), as bridging ligand, were synthesized and their electrochemical, spectroscopic and electrochemiluminescence (ECL) properties investigated. The electrochemical and spectroscopic results prove that the two metal complexes mainly retain the electronic properties of [4-carboxypropyl-4'-methyl-2,2'-bipyridine]bis(2,2''-bipyridine)ruthenium(II) complex, [Ru-Reff], isostructural with the ruthenium moieties in the two complexes.

The ECL studies show that an improvement by 30% of the ECL intensity can be achieved for the dinuclear and trinuclear complexes as compared to [Ru-Reff]. A larger improvement is prevented by their slower diffusion rate. Heterogeneous ECL studies, performed on larger dendritic complexes containing up to 8 ruthenium units, have shown that limitations due to slow diffusion can be easily overcome by means of nanoparticle technology, leading to ECL intensities proportional to the number of ruthenium moieties in the complex. However, care must be taken when considering large multimetallic systems as hydrophobic interactions with nanoparticles and biological moieties, present in the assay buffer, may dramatically increase the background signal.
4.1 Introduction.

Since the discovery\(^1\) of the photoluminescence of \([\text{Ru(bpy)}_3]^{2+}\) (bpy = 2,2'-bipyridine) several studies have been performed on this metal complex. The strong interest in \([\text{Ru(bpy)}_3]^{2+}\) arises from its specific features, such as the emission wavelength, the long excited state lifetime, the high emission quantum yield, the numerous redox states suitable for a number of electron transfer reactions, the good solubility in various organic solvents and aqueous media, the photochemical- and thermal stability and, importantly, the possibility to populate the excited state by a redox reaction.\(^2\)-\(^7\) In particular, the electrogenerated chemiluminescence (ECL) of \([\text{Ru(bpy)}_3]^{2+}\) and its diverse applications have been subject of numerous studies.\(^8\)-\(^13\)

ECL for \([\text{Ru(bpy)}_3]^{2+}\) can be obtained upon charge recombination between the electrogenerated \([\text{Ru(bpy)}_3]^{3+}\) and \([\text{Ru(bpy)}_3]^{-}\) (ion annihilation mechanism), that leads to population of the emitting triplet metal-to-ligand charge-transfer (\(^3\)MLCT) excited state.\(^14\) A more detailed description of the mechanism can be found in Chapter 1 (Section 1.8). Alternatively, ECL can be generated upon reaction between \([\text{Ru(bpy)}_3]^{3+}\) (or \([\text{Ru(bpy)}_3]^+\)) and a reductant (or oxidant) species.\(^9\),\(^10\),\(^14\),\(^15\)

One of the most important applications for the ECL of \([\text{Ru(bpy)}_3]^{2+}\) lies in diagnostics, e.g. for immunoassays and DNA-probing assays.\(^9\),\(^10\),\(^16\) The ruthenium complex, labelling a biological molecule, undergoes an ECL reaction by oxidative-reduction mechanism with tri-n-propylamine (TPrA) as co-reactant (see Section 1.8). The possibility to avoid the well known radioactive assays, the facile triggering of the electrochemical reaction, the low detection limit (200 fmol dm\(^{-3}\)) and the large dynamic range (six orders of magnitude) are among the most important advantages of the ECL technique over isotope or fluorescence labelling techniques.\(^16\)-\(^18\) Despite the good performance of the ECL assays, higher sensitivity (signal-to-noise ratio) is required, due to the increasing demands for accuracy in diagnostics. The enhancement of the ECL intensity therefore becomes a crucial point. Several studies have recently dealt with the role of numerous parameters involved in the ECL process, such as the electrode surface,\(^19\),\(^20\) different coreactants,\(^21\) pH dependence.\(^22\) Less successful were attempts to increase the ECL quantum yield by using complexes of different metals.\(^23\)-\(^26\) Only recently an osmium complex has been reported to exhibit ECL signal higher than \([\text{Ru(bpy)}_3]^{2+}\).\(^22\) A possible approach to improve the performance of the ruthenium ECL label is to modify the chelating ligands with suitable substituents in order to increase the emission quantum yield. For instance diphenyl-substituted bipyridine and phenanthroline ruthenium complexes, known to have emission quantum yield higher than \([\text{Ru(bpy)}_3]^{2+}\), exhibit an increased ECL intensity.\(^27\) The emission efficiency of \([\text{Ru(bpy)}_3]^{2+}\) can also be enhanced by shielding the metal complex from dioxygen quenching by means of large dendritic branches bound to the
bipyridyl ligands. However, this approach, very promising for labelling purposes where photoexcitation is employed (see Chapter 5), is less interesting for the ECL assay, since it has the disadvantage that ruthenium core is more difficult to oxidize and the interaction between the ruthenium core and the active TPrA species can be hindered.


A more promising approach towards the enhancement of luminescence of the label, and hence the ECL signal, is to build multinuclear systems. Such strategy has the advantage of providing multiple redox centres, increasing the probability of oxidation of at least one them and of charge recombination event. Fundamental requirements for the above mentioned improvement are (i) the accessibility of the ruthenium centres to the electrode surface and active TPrA species and (ii) the electronic equivalence of the chromophores, to avoid intramolecular energy transfer from the excited chromophores to the lowest-lying unoccupied molecular orbital (LUMO) of an acceptor moiety. A proper choice of the bridging ligand is then of key importance in designing the multinuclear systems for the ECL reaction. Amino
acids and peptides, bearing various functional groups, are versatile tools to design bridging ligands with specific structure. Furthermore, their hydrophilic character will favour the solubility of the multimetallic complexes in water, in addition, their facile terminal functionalization will constitute a good linkage to the ruthenium bipyridine moieties bearing appropriate substituents.32

Scheme 4.1 Schematic representation of the multimetallic complexes [Ru4-Dend]8+ and [Ru8-Dend]16+ conjugated to a progesterone molecule.

We have synthesized a dinuclear and a trinuclear homometallic complex containing ruthenium polypyridyl units bound to a bridging ligand by a propoxycarbonyl linker (Scheme 4.1). The bridging ligands are the amino acid lysine (Lys) and the related dipeptide (LysLys), respectively, with amino functional groups suitable for the anchoring of the ruthenium units by a peptidic
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bond, and a carboxylic group, available for conjugation to biological molecules. In this work we present the syntheses and electrochemical, spectroscopic and electrochemiluminescence investigations of the two complexes, hereafter indicated as [Ru2-Lys] and [Ru3-LysLys] (Scheme 4.1) The influence of surfactant on the spectroscopy and electrochemiluminescence of the two complexes is also discussed. The (4-carboxylpropyl-4'-methyl-2,2'-bipyridine)bis(2,2'-bipyridine)ruthenium(II) complex, [Ru-Refl] and [Ru4-Dend] (Scheme 4.1), isostructural with the ruthenium moieties in the two complexes, was investigated as reference compound.

Furthermore, we present the electrochemiluminescence properties of three larger dendritic complexes containing up to 8 ruthenium units, indicated as [Ru2-Dend] and [Ru4-Dend] and [Ru8-Dend] (see Scheme 4.2 for [Ru4-Dend] and [Ru8-Dend]). The ECL behaviour was also investigated in progesterone immunoassay, where nanoparticle technology (heterogeneous immunoassay, see Chapter 2, Section 2.4) was applied.

4.2 Results and discussion.

4.2.1 Electrochemistry.

The electrochemical data for the investigated complexes [Ru2-Lys] and [Ru3-LysLys] and the reference compounds [Ru-Refl] and [Ru(bpy)3] in acetonitrile solution, are summarized in Table 4.1. The redox behaviour of the bi- and trinuclear compounds is consistent with metal-based oxidation and ligand-based reductions, in agreement with the behaviour of several multinuclear complexes reported in the literature.

The oxidation of [Ru2-Lys] and [Ru3-LysLys] occurs as one reversible multielectron (two- and tri-electron respectively, see below) step at $E_{1/2} = +0.83$ and +0.86 V, respectively, which is very close to [Ru-Refl] oxidized at $E_{1/2} = +0.84$ V (Table 4.1). The anodic potential of the three complexes is slightly less positive than that of the reference compound [Ru(bpy)3] (Table 4.1) in agreement with the presence of electron donor groups on the bipyridines of the bridging ligand that better stabilize the oxidized metal Ru(III) centre.

In the cathodic region, [Ru2-Lys] and [Ru3-LysLys] exhibit the first multielectron (two and tri-electron, respectively) reversible wave at the same potential as [Ru-Refl], $E_{1/2} = -1.74$ V, close to the value found for [Ru(bpy)3] (E$E_{1/2} = -1.72$ V). This step is assigned to the unresolved reduction of one ancillary 2,2'-bipyridine at each Ru(II) centre, consistent with their electron acceptor character. At more negative potentials, the reduced bi- and trinuclear complexes [Ru2-Lys] and [Ru3-LysLys] undergo second multielectron reduction process, with a sharp peak developed along the corresponding reoxidation step due to adsorption of the neutral species [Ru2-Lys] and [Ru3-LysLys] on the electrode. The reduction potentials are $E_{1/2} = -1.88$ and -1.81 V, respectively. The second cathodic process is assigned to the reduction of the remaining neutral ancillary 2,2'-bipyridine ligands. Further reductions could not be readily observed due to
strong adsorption of the neutral products. Application of glassy carbon disk electrode did not improve the voltammetric record. The peak currents of the first and second cathodic steps are similar to that of the Ru$^{III}$ anodic peak, consistent with the identical number of electrons exchanged. Furthermore, the poor resolution of the multielectron anodic and cathodic waves proves negligible electronic communication between, respectively, the metal centres and the remote ancillary 2,2'-bipyridine ligands.

| Table 4.1. Electrochemical data of the investigated complexes and reference compounds.$^a$ |
|-----------------|-------|--------|---------|--------|
|                 | Ru$^{II/III}$ | bpy$^{0/-1}$ | $n_{app}^b$ | $D^c$ $(10^2 \text{ cm}^2 \text{s}^{-1})$ | Ref.   |
| [Ru-Ref]$^{2+}$ | + 0.84 | - 1.74 | 1.1 ± 0.1 | 1.10 ± 0.06 | this work |
| [Ru2-Lys]$^{4+}$ | + 0.83 | - 1.74 | 1.8 ± 0.1 | 0.64 ± 0.03 | this work |
| [Ru3-LysLys]$^{6+}$ | + 0.86 | - 1.74 | 3.4 ± 0.3 | 0.34 ± 0.03 | this work |
| [Ru(bpy)$_3$]$^{2+}$ | + 0.89 | - 1.72 | 1.75 | 1.93 | this work, $^{37}$ |
| [Ru(bpy)$_2$(4-octoxy-bpy)]$^{2+}$ | + 0.75$^d$ | - 1.82$^d$ | 1.05 | $^{31}$ |

$^a$Redox potentials ($E_{1/2}$) in Volt vs Fe/Fe$^+$, in acetonitrile at 293 K. $^b$Number of electrons transferred during the oxidation. $^c$Diffusion coefficient. $^dE_{1/2}(\text{Fe/Fe}^+)=0.421$ V vs Ag/AgCl in acetonitrile.

For [Ru2-Lys]$^{4+}$, [Ru3-LysLys]$^{6+}$ and the reference complex [Ru-Ref]$^{2+}$, the number of electrons exchanged at the electrode surface during the oxidation step ($n_{app}$) and the diffusion coefficient ($D$) were separately determined, following the convenient literature procedure reported by Amatore. $^{39,40}$ In particular, transient (chronoamperometry) and steady-state (cyclic voltammetry at ultramicroelectrode, UME) techniques are combined to provide two independent equations for the faradaic current, $i$, as function of the two independent variables $n_{app}$ and $D$.

For chronoamperometry, the current response to the potential step at a planar disk electrode is given by Eq. 4.1:$^{41}$

$$i = n_{app} F A D^{1/2} c_0 (\pi t)^{1/2}$$

(4.1)

where $t$ is the duration time of the applied potential step, $F$ the Faraday constant, $A$ the apparent surface area of the working electrode and $c_0$ the bulk concentration of the analyte.
For CV at a disk UME, performed at low scan rates (ν < 100 mV), the current response reaches a limiting value, \( i_{\text{lim}} \), given by Eq. 4.2:

\[
i_{\text{lim}} = 4 n_{\text{app}} F r_0 D c_0
\]

where \( r_0 \) is the radius of the disk UME.

A standard compound, ferrocene, with known \( n_{\text{app}}(\text{Fc}) = 1 \) and \( D(\text{Fc}) = 1.9 \times 10^{-5} \, \text{cm}^2\,\text{s}^{-1} \) (in acetonitrile) was employed to avoid errors arising from the determination of \( A \), in Eq. 4.1.

For the ferrocene / analyte system the following Eqs. 4.3-4.6 apply:

\[
R_{\text{chrono}} = \frac{[i(\text{Fc})]_0}{[i(\text{Fc})]_0} = \frac{n_{\text{app}}}{n_{\text{app}}(\text{Fc})} \left[ \frac{D}{D(\text{Fc})} \right]^{1/2}
\]  

(4.3)

\[
R_{\text{UME}} = \frac{[i_{\text{lim}}(\text{Fc})]_0}{[i_{\text{lim}}(\text{Fc})]_0} = \frac{n_{\text{app}}}{n_{\text{app}}(\text{Fc})} \left[ \frac{D}{D(\text{Fc})} \right]
\]  

(4.4)

Then, \( D \) and \( n_{\text{app}} \) can be calculated as:

\[
n_{\text{app}} = n_{\text{app}}(\text{Fc}) \left[ \frac{R_{\text{chrono}}^2}{R_{\text{UME}}} \right]
\]  

(4.5)

\[
D = D(\text{Fc}) \left[ \frac{R_{\text{chrono}}}{R_{\text{UME}}} \right]^2
\]  

(4.6)

The values of \( n_{\text{app}} \) and \( D \) determined for \([\text{Ru-Ref}]^{2+}\), \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\) in acetonitrile are reported in Table 4.1. As expected, the number of electrons transferred (1.1±0.1, 1.8±0.1 and 3.4±0.3, respectively) in the oxidation process of the three complexes is proportional to the number of the poorly communicating metal centres that oxidize at the same potential. The diffusion coefficients \( (D) \) are 1.10±0.06 \times 10^{-5}, 0.64±0.03 \times 10^{-5}, and 0.34±0.03 \times 10^{-5} \, \text{cm}^2\,\text{s}^{-1} \) for \([\text{Ru-Ref}]^{2+}\), \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\), respectively. The D value for \([\text{Ru-Ref}]^{2+}\) is in good agreement with that reported in the literature for a similar ruthenium complex, \([\text{Ru(bpy)}_2(4\text{-octoxy-2,2'-bipyridine})\text{(PF}_6)_2]\) in deuterated acetonitrile \((D = 1.05 \times 10^{-5} \, \text{cm}^2\,\text{s}^{-1})\). The values of D determined for \([\text{Ru-Ref}]^{2+}\), \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\) decrease with increasing number of metal units and size of the complexes, as one would expect.

4.2.2 UV/Vis Absorption and Emission.

The spectroscopic data for the complexes \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\) are summarized in Table 4.2. The reference compound \([\text{Ru-Ref}]^{2+}\) is also reported for comparison. In all cases the data refer to aqueous phosphate buffer solutions (pH 6.8). The absorption spectra of \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\) and the emission spectrum of \([\text{Ru3-LysLys}]^{6+}\) in phosphate buffer solutions are depicted in Figure 4.1.
The UV/Vis spectra of [Ru2-Lys]$^{4+}$ and [Ru3-LysLys]$^{6+}$ are similar to that of [Ru-Ref]$^{2+}$, characterized by an intense band in the UV region due to intraligand (IL) $\pi$-$\pi^*$ transitions within the bipyridine ligands and by a broad band in the visible region due to metal-to-ligand charge-transfer (MLCT) transitions (Figure 4.1 and Table 4.2). The absorption bands do not shift in the series [Ru-Ref]$^{2+}$, [Ru2-Lys]$^{4+}$ and [Ru3-LysLys]$^{6+}$ and the molar absorbance of the di- and trinuclear complexes is proportional to the number of chromophores, about two- and three-fold, respectively, that of the mononuclear compound (Table 4.2). This result is again consistent with the absence of strong electronic interaction between the chromophores in the multinuclear complexes.

Room temperature emission spectra of [Ru-Ref]$^{2+}$, [Ru2-Lys]$^{4+}$ and [Ru3-LysLys]$^{6+}$, recorded in phosphate buffer solution, show nearly identical emission maxima, centred at 616, 617 and 619 nm, respectively (Figure 4.1 and Table 4.2). The emitting $^3$MLCT excited state is therefore the same for all the three complexes and lies at the same energy, suggesting that the linkage of several chromophores by a peptidic bridge has negligible effect on the electronic properties, as already observed from the redox data. The determined luminescence quantum yield is very similar for [Ru2-Lys]$^{4+}$ and [Ru3-LysLys]$^{6+}$, viz. 0.027 and 0.029, respectively (Table 4.2).
Table 4.2. UV/Vis absorption, luminescence and ECL data of the investigated complexes and reference compound.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Absorption</th>
<th>Luminescence</th>
<th>ECL(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\lambda_{\text{max}}), nm ((\varepsilon \times 10^4, \text{M}^{-1} \text{cm}^{-1}))</td>
<td>(\lambda_{\text{max}}), nm</td>
<td>(\phi_{\text{em}})</td>
</tr>
<tr>
<td>[Ru-Ref](^{2+})</td>
<td>456 (1.40)</td>
<td>616</td>
<td>617</td>
</tr>
<tr>
<td>[Ru2-Lys](^{4+})</td>
<td>456 (2.61)</td>
<td>617</td>
<td>616</td>
</tr>
<tr>
<td></td>
<td>286 (14.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Ru3-LysLys](^{6+})</td>
<td>456 (4.29)</td>
<td>619</td>
<td>618</td>
</tr>
<tr>
<td></td>
<td>286 (24.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)In phosphate buffer solution \((3 \times 10^{-1} \text{ M, pH 6.8})\). \(^b\)In the presence of 1.8 \times 10^{-1} \text{ M TPrA.}\(^c\)In the presence of non-ionic surfactant.

The spectroscopic properties were also investigated for [Ru-Ref]\(^{2+}\), [Ru2-Lys]\(^{4+}\) and [Ru3-LysLys]\(^{6+}\) in phosphate buffer solutions containing a non-ionic surfactant above its critical micellar concentration (cmc).

Upon addition of surfactant, UV/Vis and emission spectra reveal only minor changes, with emission maxima at 617, 616 and 618 nm, for [Ru-Ref]\(^{2+}\), [Ru2-Lys]\(^{4+}\) and [Ru3-LysLys]\(^{6+}\) respectively, and quantum yields of 0.030, for both [Ru2-Lys]\(^{4+}\) and [Ru3-LysLys]\(^{6+}\) (Table 4.2). Formation of complex-micelle aggregations in the presence of non-ionic surfactants was observed for some methyl- and phenyl-substituted phenanthroline ruthenium complexes, resulting in strong emission changes: red-shift of the emission maxima, higher emission quantum yields and longer excited-state lifetimes.\(^{42,43}\) However, [Ru(bpy)\(_3\)]\(^{2+}\) does not exhibit pronounced changes of the emission, due to the weak hydrophobic interactions between the 2,2'-bipyridine ligands and the hydrophobic cavity of the micelles.\(^{42,43}\) The behaviour of [Ru2-Lys]\(^{4+}\) and [Ru3-LysLys]\(^{6+}\) closely resembles that of [Ru(bpy)\(_3\)]\(^{2+}\), with the bipyridine ligands only weakly interacting with the surfactant.

### 4.2.3 Electrochemiluminescence

The electrochemical and spectroscopic studies prove that the metal moieties of [Ru2-Lys]\(^{4+}\) and [Ru3-LysLys]\(^{6+}\) do not strongly interact and retain to a large extent the electronic properties of the mononuclear compound [Ru-Ref]\(^{2+}\). This is an important requirement when more metal centres are linked by a bridging ligand in order to increase a specific output (current, emission) as the sum of the contributions of the single units.
Our interest was then to investigate whether the multinuclear complexes do exhibit ECL signals stronger than that of the reference compound [Ru-Ref]$^{2+}$.

The ECL reaction was performed by oxidative-reduction mechanism using tri-\textit{n}-propylamine, TPrA, as coreactant. The mechanism is described in Chapter 1 (Section 1.8).

Solutions of [Ru2-Lys]$^{4+}$, [Ru3-LysLys]$^{6+}$ and the reference [Ru-Ref]$^{2+}$ for the ECL experiments were in phosphate buffer and contained TPrA in large excess (> 10$^6$ fold the concentration of the complexes). The solutions contained equivalent concentration of ruthenium moieties. A non-ionic surfactant was also added in order to investigate the ECL behaviour of the multinuclear complexes under the same experimental conditions applied for routine-immunoassays. Surfactant is indeed employed in automatized instruments,\textsuperscript{44} in order to have a good liquid flow, avoid bubbles formation and better remove the analytes from the ECL cell after each measurement. Furthermore, it increases the solubility of the ruthenium complexes in aqueous solutions.

![Figure 4.2](image)

**Figure 4.2.** ECL of [Ru-Ref]$^{2+}$ (---), [Ru2-Lys]$^{4+}$ (-----) and [Ru3-LysLys]$^{6+}$ (-----), in phosphate buffer solution containing TPrA and surfactant.

The ECL signals for [Ru-Ref]$^{2+}$, [Ru2-Lys]$^{4+}$ and [Ru3-LysLys]$^{6+}$ were recorded against time, over a range of 700 ms after triggering the reaction, and are depicted in Figure 4.2. The ECL results are given as intensity integrals relative to [Ru-Ref]$^{2+}$ ($I_{ecl,rel} = 1$) calculated per ruthenium unit, and are summarized in Table 4.2. The data are an average of six trials.

The signal of [Ru-Ref]$^{2+}$, [Ru2-Lys]$^{4+}$ and [Ru3-LysLys]$^{6+}$ increases sharply within a few milliseconds after triggering the reaction, then it slowly decays (Figure 4.2). The initial sharp peak is due to the presence of the ruthenium complexes and TPrA molecules that are close to the
electrode surface and readily oxidize. The consumption of the active species in the proximity of the anode and their slow diffusion from the bulk solution result in decreased signal intensity. Passivation of the electrode surface due to platinum oxides formation also contributes to the signal decrease. Surprisingly, the intensity monitored at the maximum for [Ru-Ref]^{2+}, [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+} decreases in the series. This behaviour is not totally clear, since one would expect it to be similar for the three complexes, the concentration of the active species at the anodic surface being initially the same. These differences are probably due to adsorption of the complexes at the electrode. The relative intensities per ruthenium unit of [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+} are 0.66 and 0.44, respectively (Table 4.2). Some decrease of ECL intensity is expected in the series [Ru-Ref]^{2+}, [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+}, as the diffusion coefficients decrease (1.1, 0.65 and 0.33 cm^{2} s^{-1}, respectively, Table 4.1). On the base of the diffusion coefficients, the relative ECL intensities per ruthenium unit are estimated to be ca. 0.8 and 0.5 for [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+}, respectively, as the faradaic current is proportional to D^{1/2}. The experimental values are then lower than expected, in particular for the dinuclear complex.

In order to investigate whether this discrepancy is due to a less efficient reaction between the multinuclear complexes and the radical species generated from TPrA or whether it is due to some effects caused by the surfactant, ECL measurements were performed in phosphate buffer solution without surfactant.

In the absence of detergent almost no signal could be detected. This result suggests a crucial role played by the surfactant in the ECL measurements. Recent studies have shown that protection of the electrode surface from passivation, for instance upon addition of halides (e.g. I, Br) to the assay buffer solution, greatly enhances the current due to TPrA oxidation and the ECL signal of the TPrA/[Ru(bpy)]^{2+} system. Enhancement of anodic current and ECL output was also observed upon increasing the hydrophobicity of the electrode surface, e.g. upon formation of a layer of thiols or surfactant. It was suggested that the hydrophobic interactions between electrode surface and TPrA molecules promote their closer approach to the electrode, facilitating the electron transfer reaction. Hence, the surfactant is likely important to protect the electrode from passivation and to facilitate the oxidation of the active species.

ECL measurements were then performed for [Ru-Ref]^{2+}, [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+}, in phosphate buffer, after washing the cell with a buffer solution containing surfactant. This allowed a hydrophobic layer to form on the electrode surface prior to the measurement. The solutions of the complexes were in equivalent concentration of ruthenium units and contained an excess of TPrA. The ECL intensities are reported in Table 4.2, and the signals are depicted against time in Figure 4.3.

The presence of surfactant in the washing buffer solution resulted in a dramatic increase of the ECL signal, compared to the measurements performed without surfactant. The ECL signal reaches a maximum within few milliseconds after the potential step, decaying slowly afterwards.
(Figure 4.3). The peak maximum is similar for the three complexes. Importantly, the relative ECL intensities per ruthenium unit (0.80 and 0.60 for [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+}, respectively, Table 4.2) are in good agreement with the values estimated on the base of the diffusion coefficients (0.8 and 0.5, respectively), suggesting that the differences in ECL intensities of [Ru-Ref]^{2+}, [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+} in the absence of surfactant in the assay buffer can be easily explained on the base of the diffusional rates of the complexes. No evidence for different reactivity of the three complexes with the active TPrA species could be found.

![Graph](image)

**Figure 4.3.** ECL of [Ru-Ref]^{2+} (----), [Ru2-Lys]^{4+} (-----) and [Ru3-LysLys]^{6+} (-----), in the phosphate buffer solution containing TPrA, in the absence of surfactant.

The presence of a layer of detergent on the electrode surface is then important for the ECL intensity to be largely enhanced. However, a high concentration of surfactant in the assay buffer slightly decreases the ECL signal, the absolute ECL intensity of [Ru-Ref]^{2+} being 80% of the intensity measured when surfactant was only added to the cleaning solution and used prior to the measurements. Furthermore, the relative intensities for the complexes [Ru2-Lys]^{4+} and [Ru3-LysLys]^{6+} where lower (0.66 and 0.44, respectively) when surfactant was used in the assay buffer solution, see above.

The spectroscopic properties of the ruthenium complexes have already shown no dependence on the surfactant, its influence on reducing the ECL response must therefore depend on other factors. It was reported that, in spite of the weak interaction between [Ru(bpy)_{3}]^{2+} and micelles of non-ionic surfactant, a different diffusion coefficient is found for [Ru(bpy)_{3}]^{2+} in water ($D =$
7.33 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}) and in aqueous solution containing Triton X100 surfactant \((D = 4.70 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1})\). A similar effect can be expected for the complexes \([\text{Ru-Ref}]^{2+}\), \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\), resulting in smaller diffusion coefficients when surfactant is added to the assay buffer solutions, thereby explaining the lower ECL intensity observed.

The diffusion rates are then a limiting factor for the improvement of the ECL intensity by the investigated complexes, \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\). However, as the ECL intensities considered so far are calculated per ruthenium unit (0.66 and 0.44, in the presence of surfactant in the assay buffer), solutions of equimolar concentrations of the multimetallic complexes yield relative intensities of 1.32 for both \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\), hence resulting in an increase of ca. 30% with respect to mononuclear reference \([\text{Ru-Ref}]^{2+}\).

4.2.4 ECL Immunoassay.

In the research laboratories of Roche Diagnostics GmbH, large dendritic peptidic structures have been developed containing two, four and eight \([\text{Ru(bpy)}_3]^{2+}\) moieties, viz. \([\text{Ru2-Dend}]^{4+}\), \([\text{Ru4-Dend}]^{8+}\) and \([\text{Ru8-Dend}]^{16+}\), respectively. The molecular structures of \([\text{Ru4-Dend}]^{8+}\) and \([\text{Ru8-Dend}]^{16+}\) are depicted in Scheme 4.2. The multinuclear complexes were bound to a progesterone molecule to test them in competitive progesterone immunoassay (see also Chapter 2, Sectio 2.4).

![ECL curve](image)

**Figure 4.4** ECL of \([\text{Ru2-Dend}]^{4+}\) (---), \([\text{Ru4-Dend}]^{8+}\) (----) and \([\text{Ru8-Dend}]^{16+}\) (-----), in phosphate buffer solution containing TPrA and non-ionic surfactant
The ECL behaviour was first investigated in homogeneous assay, in phosphate buffer containing large excess of TPrA. Non-ionic surfactant was also added to perform the measurements in the same experimental conditions as those used for routine progesterone immunoassays. The ECL signal, recorded for equimolar solutions of [Ru2-Dend]4+, [Ru4-Dend]6+ and [Ru8-Dend]16+, sharply increases after few milliseconds after triggering the reaction, and then slowly decreases (Figure 4.4). Its intensity integral does not much differ for the three complexes, the absolute values being 43400, 45500 and 36422 counts, respectively. As for [Ru2-Lys]4+ and [Ru3-LysLys]6+ (see above) different diffusional rates probably play an important role in limiting the enhancement of ECL intensity, expected upon increasing the number of ruthenium units in the complexes.

The compounds [Ru2-Dend]4+, [Ru4-Dend]6+ and [Ru8-Dend]16+ were then tested in progesterone immunoassay, employing magnetic nanoparticle technology (heterogeneous assay), see Chapter 2 for description. The analyte (progesterone) was added in increasing concentrations to a solution where labeled progesterone molecules, biotin-antibodies conjugates and streptavidin-coated nanoparticles were let incubate for a few minutes (see Experimental Section). ECL intensities, given as absolute integrals, are reported in Table 4.3.

<table>
<thead>
<tr>
<th>Progesterone (10⁹ mol dm⁻³)</th>
<th>[Ru2-Dend]⁴⁺</th>
<th>[Ru4-Dend]⁶⁺</th>
<th>[Ru8-Dend]¹⁶⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>43111</td>
<td>99583</td>
<td>78021</td>
</tr>
<tr>
<td>0.175</td>
<td>40053</td>
<td>90049</td>
<td>64555</td>
</tr>
<tr>
<td>1.75</td>
<td>26212</td>
<td>65779</td>
<td>55863</td>
</tr>
<tr>
<td>60·10⁻³</td>
<td>2361</td>
<td>14791</td>
<td>40130</td>
</tr>
</tbody>
</table>

*Data are given as absolute intensity integrals for solutions in phosphate buffer (3 x 10⁻¹ M, pH 6.8) containing (0.32 x 10⁻⁹ M) multimetallic complex, (0.32 x 10⁻⁹ M) biotin-antibodies and streptavidin-coated nanoparticles with 0.24 x 10⁻³ M biotin-binding capacity. Solutions also contained 1.8 x 10⁻¹ M TPrA and non-ionic surfactant. In 0.16 x 10⁻⁹ mol dm⁻³ with (0.16 x 10⁻⁹ M) biotin-antibodies.

In the absence of analyte, the ECL intensity of the three complexes (43100, 99600 and 78000 counts, respectively) is proportional to the number of ruthenium units (considering that [Ru8-Dend]¹⁶⁺ was half concentrated than [Ru2-Dend]⁴⁺ and [Ru4-Dend]⁶⁺), see Table 4.3. Upon addition of progesterone the ECL intensity decreases, as the analyte competes with the labeled progesterone for the binding site of the antibody. For a large excess of the analyte (> 100 times the concentration of the complex), the ECL of [Ru2-Dend]⁴⁺ drops to 5% of the initial signal, consistent with the large competition of the analyte present in solution (Table 4.3). This residual
ECL (background signal) increases moderately for $[\text{Ru4-Dend}]^{8+}$ (10%) and dramatically for $[\text{Ru8-Dend}]^{16+}$ (46%), Table 4.3. The high ECL background found for $[\text{Ru4-Dend}]^{8+}$ and, in particular, for $[\text{Ru8-Dend}]^{16+}$ is probably due to non-specific binding, via strong hydrophobic interactions, of the large ruthenium complexes to the surface of the nanoparticles or to the antibodies.

The immunoassay results show that upon increasing the number of ruthenium units bound to the peptidic dendritic structure, the ECL signal is proportionally enhanced. Since in the heterogeneous assay, where nanoparticle technology is employed, the mass transport by diffusion to the electrode does not occur and limitations due to the reduced diffusion coefficients of the large complexes are overcome.

### 4.3 Conclusions

We have synthesised two homonuclear complexes, $[\text{Ru2-Lys}]^{4+}$ and $[\text{Ru3-LysLys}]^{6+}$, containing two and three, respectively, modified ruthenium tris(bipyridine) units linked by an amino acid or peptidic bridging ligand. The redox and spectroscopic properties of the complexes show that these ruthenium moieties remain independent and retain the electronic properties of the isostructural mononuclear compound $[\text{Ru-Ref}]^{2+}$. The ECL behaviour was investigated in phosphate buffer solution (homogenous assay) containing a surfactant and TPrA. An increase of ECL intensity by 30% can be achieved for equimolar solutions of $[\text{Ru2-Lys}]^{4+}$ and $[\text{Ru3-LysLys}]^{6+}$ with respect to the reference mononuclear compound. The slow diffusion of the two oligonuclear systems, prevents stronger enhancement of the ECL signal. We have also shown that while the surfactant does not affect the spectroscopic properties of $[\text{Ru2-Lys}]^{4+}$ and $[\text{Ru3-LysLys}]^{6+}$, it increases significantly the ECL output.

ECL experiments were then performed for larger dendritic complexes, $[\text{Ru2-Dend}]^{4+}$, $[\text{Ru4-Dend}]^{8+}$ and $[\text{Ru8-Dend}]^{16+}$, in homogeneous and heterogeneous assays. The results show that stronger enhancement of ECL intensity can be achieved in heterogeneous assay, where nanoparticle technology is employed. The ECL process becomes in fact independent of the diffusion of the complexes, and the ECL signal intensity is proportional to the number of ruthenium units of the multinuclear complexes. ECL intensity can be then strongly enhanced by increasing the number of ruthenium moieties in the complex. However, large systems, such as $[\text{Ru8-Dend}]^{16+}$, show high background signal due to hydrophobic interactions with biological molecules present in the assay buffer and with the nanoparticles coated with streptavidin. Further studies are currently in progress in order to reduce the ECL background.
4.5 Experimental Section.

**Materials.** [4-(N-succimidylxocarbonylpropyl)-4'-methyl-2,2'-bipyridine]bis(2,2'-bipyridine)ruthenium(II) dihexafluorophosphate, \[^{16,18,33,34}\] [Ru(bpy)]\(_2\) (bpyOSu)](PF\(_6\))\(_2\), [4-carboxypropyl-4'-methyl-2,2'-bipyridine]bis(2,2'-bipyridine)ruthenium(II) dihexafluorophosphate ([RuRef](PF\(_6\))\(_2\)) and the multinuclear complexes [Ru2-Dend](PF\(_6\))\(_4\), [Ru4-Dend](PF\(_6\))\(_8\) and [Ru8-Dend](PF\(_6\))\(_i66\) were obtained by Roche Diagnostics GmbH. L- (+)-2,6-diamino-7N-caproic acid (Lys, Bachem), L-a-L-Lysyl-L-Lysine-trihydrochloride (Bachem), ammoniumhexafluorophosphate (Alrich), trifluoroacetic acid (TFA, Merck), tri-n-propylamine (Aldrich), dimethylformamide (Acros, synthesis grade) and acetonitrile (Merck, HPLC grade) were used as received. For electrochemistry, acetonitrile (Acros, synthesis grade) was dried over Ca\(_2\)H\(_2\) and freshly distilled under nitrogen prior to use. Tetrabutylammonium hexafluorophosphate (Bu\(_4\)NPF\(_6\), Aldrich) was recrystallized twice from ethanol and dried overnight under reduced pressure at 60 °C. Ferrocene (Alrich) was used as supplied.

**Syntheses.** [Ru2-Lys](PF\(_6\))\(_4\). L- (+)-2,6-diamino-7N-hexanoic acid (Lys, 30 mg, 0.108 mmol) in phosphate buffer solution (10 ml, pH 7.4) was added dropwise to [Ru(bpy)]\(_2\) (bpyOSu)](PF\(_6\))\(_2\) (500 mg, 0.473 mmol) previously dissolved in dimethylformamide (20 ml). After stirring at room temperature overnight, the solvents were removed under reduced pressure at 40 °C. Purification was performed by preparative HPLC with Millipore water and acetonitrile (both containing TFA, 0.1%) as eluent, following a gradient method (0 to 40% acetonitrile in 120 min. The collected fractions containing the product were regrouped according to the analytical HPLC retention time, and stored, after addition of a saturated aqueous NH\(_2\)PF\(_6\) solution, overnight at 4 °C. The precipitate was filtered off, washed with water and dried under vacuum at 60 °C, to give the product as pure orange powder.

Yield: 311 mg (75%). In the NMR assignments the traditional numbering scheme for bpy ligands is used. The substituted bpy ligands are denoted by an added "a" after the number, and for this ligand the two different rings are indicated by nothing and a prime, respectively. \(^1\)H NMR (CD\(_2\)Cl\(_2\)): 8.41 (m, 8H, 3), 8.35/8.34 (2s, 4H, 3a+3a'), 8.03 (m, 8H, 4), 7.64-7.88 (m, 8H, 6), 7.36-7.52 (m, 12H, 5+6a+6a'), 7.25/7.21 (2d, J = 3.3 Hz, 4H, 5a+5a'), 6.90 (s, 1H, CH(COOH)-NH), 6.24 (s, 1H, CH(COOOH)-(CH\(_2\))\(_2\)-NH), 4.23 (s, 1H, COOH), 3.65 (m, 1H, CH(COOH)), 3.14/3.06 (2 m, 2H, CH(COOH)-(CH\(_2\))\(_2\)-CH\(_2\)), 2.78 (m, 4H, CH(COOH)-(CH\(_2\))\(_2\)-CH\(_2\)), 2.83 (m, 4H, CH(COOH)-(CH\(_2\))\(_2\)), 2.39 (m, 4H, CH(COOH)-(CH\(_2\))\(_2\)), 1.55 (br m, 12H, CH(COOH)-CH\(_2\)-CH\(_2\)-CH\(_2\)), 1.28 (br m, 2H, CH(COOH)-CH\(_2\)-CH\(_2\)), ppm. ESI-MS: m/z 483.7 [M\(^+\) - 4PF\(_6\) - H], 362.3 [M\(^+\) - 4PF\(_6\)].

[Ru3-LysLys](PF\(_6\))\(_6\). This compound was synthesized following the procedure described for [Ru2-Lys](PF\(_6\))\(_4\). N\(^2\)-L-Lysine-trihydrochloride (29 mg, 0.075 mmol) in phosphate buffer solution (10 ml, pH 8) was added dropwise to [Ru(bpy)]\(_2\) (bpyOSu)](PF\(_6\))\(_2\) (350 mg, 0.302 mmol) in dimethylformamide (50 ml). The product was obtained as an orange powder.

Yield: 105 mg (45%). NMR numbering as for the previous compound. \(^1\)H NMR (CD\(_3\)CN): 8.48 (m, 12H, 3), 8.39 (m, 6H, 3a+3a'), 8.04 (m, 12H, 4), 7.71 (m, 12H, 6), 7.53 (m, 6H, 6a+6a'), 7.39 (m, 12H, 5), 7.23 (m, 6H, 5a+5a'), 6.97/6.69 (2 t, 1H, NH), 6.44 (m, 2H, NH), 4.24 (br s, 1H, COOH), 3.04 (m, 2H, CH(COOH)-(CH\(_2\))\(_2\)-CH\(_2\)), 2.78 (m, 4H, CH(COOH)-(CH\(_2\))\(_2\)-CH\(_2\)), 2.52 (s, 6H, CH(COOH)-(CH\(_2\))\(_2\)), 1.5-2.3 (br m, 12H, CH\(_2\)-CH\(_2\)-CH\(_2\)+ CH(COOH)-(CH\(_2\))\(_2\)-CH\(_2\)-CH\(_2\)), 1.40/1.32 (2 m, 2H, CH(COOH)-CH\(_2\)-CH\(_2\)-CH\(_2\)), ppm. ESI-MS: m/z 556.7 [M\(^+\) - 6PF\(_6\) - 2H], 445.7 [M\(^+\) - 6PF\(_6\) - H].
General Techniques. Analytical HPLC was performed on a Merck Hitachi apparatus equipped with L 7100 HPLC pump, L 7200 autosampler, L 7400 UV-detector, 3612 ERC Erma degasser and Vyda C18 (300 Å, 5 µm) column. The set-up for preparative HPLC was equipped with a Gynkotek pump (M480P), a Soma detector (S-3710), a Abimed automatized fraction collector (M202), a Vyda C18 (300 Å, 15-20 µm, 50-250 mm) column. Electron Spray Ionisation (ESI) mass spectra were measured on a Platform II (Micromass) spectrometer. Cyclic voltammetry (CV), chronoamperometry and voltammetry at ultramicroelectrode (UME) were performed with a gastight single-compartment cell under an atmosphere of dry nitrogen or argon. For conventional CV and chronoamperometry, the cell was equipped with Pt disk working (apparent surface area of 0.42 mm²), Pt wire auxiliary, and Ag wire pseudoreference electrodes. The working electrode was carefully polished with a 0.25 µm-grain diamond paste between scans. The UME working electrode was a home-made d = 10 µm Pt disk. The potential control was achieved with a PAR Model 283 potentiostat. For chronoamperometry, the potential was stepped from a value typically 100-300 mV less positive than the oxidation potential of the complex to a value 100-300 mV more positive. All redox potentials are reported against the ferrocene-ferrocenium (Fc/Fc⁺) redox couple used as an internal standard⁴⁶ \(E_{1/2}^{i} \approx +0.63\ \text{V vs NHE}\). Ferrocene also served as standard for \(D\) and \(n_{app}\) determination. In this procedure, \(R_{chrono}\) was determined as average between different potential step durations \(t\), within the range of 15-300 ms around the value of \(T_s = 53\ \text{ms}\), calculated for ferrocene \(D_{(Fc)} = 1.9 \times 10^{-2}\)cm²s⁻¹ and the UME employed \(r_0 = 5\ \mu m\). For each experiment it was checked that \(T_s\), calculated using the value of \(D\) determined experimentally for the investigated complexes, was falling within the range of time \(t\) applied in the chronoamperometric measurements. The acetonitrile solutions of \(ca. 4 \times 10^4\ \text{M}\) complex were prepared under nitrogen. \(10^1\ \text{M} \text{Bu}_4\text{NPF}_6\) was used as supporting electrolyte.

UV/Vis spectra were recorded on a Hewlett Packard 8453 diode-array spectrophotometer. Emission spectra were recorded on a Spex 1681 spectrophotometer. All emission spectra were corrected for the photomultiplier response.⁴⁴

ECL studies were performed using an Elecsys® instrument (Roche Diagnostics GmbH).⁴⁴ It consists of an automatized system for handling the solutions, a flow-through chamber cell, a potentiostat and a red-sensitive photomultiplier tube, placed above an optically transparent window of the cell. The cell was equipped with a sheet platinum working electrode (4.8 mm x 5.0 mm) and a platinum auxiliary electrode made of two wires symmetrically placed above the working electrode. As reference, an Ag/AgCl (KCl saturated) electrode was employed. Solutions for ECL homogeneous assays were prepared in phosphate buffer (\(3 \times 10^{-1}\) M phosphate salt in deionised water) containing \(1.8 \times 10^{-1}\) M tri-\(n\)-propylamine. The pH value was adjusted to 6.8 with NaOH or H₃PO₄ aqueous solutions. Non-ionic surfactant was added, when required, in concentration above its critical micellar concentration (cmc). Solutions were \(10^8\) mol dm⁻³ in ruthenium units for \([\text{Ru}-\text{Ref}]^{2+}\), \([\text{Ru2-Lys}]^{4+}\) and \([\text{Ru3-LysLys}]^{6+}\), and \(10^8\) mol dm⁻³ of the complex for \([\text{Ru2-Dend}]^{4+}\), \([\text{Ru4-Dend}]^{6+}\) and \([\text{Ru8-Dend}]^{16+}\). Solutions for ECL heterogenous progesterone immunoassays contained \((0.32 \times 10^{-9})\) M multimetallic complex, \((0.32 \times 10^{-9})\) M biotin-antibodies conjugates, streptavidin-coated nanoparticles with biotin binding capacity of \(0.24 \times 10^{-9}\) M, \((1.8 \times 10^{-1})\) M TPrA and non-ionic surfactant. For \([\text{Ru8-Dend}]^{16+}\) solutions were \(0.16 \times 10^{-9}\) M in the multimetallic complex and \(0.16 \times 10^{-9}\) M in biotin-
antibodies conjugates. Solutions for heterogeneous assays were measured after a few minutes of incubation time. Estimated experimental error for the reported ECL intensities is 5%.
References

21) Knight, A. W.; Greenway, G. M. Analyst 1996, 121, 101R-106R.
31) Zhou, J.; Roovers, J. Macromolecules 2001, 34, 244-252.