Luminiscent Metal Complexes for Diagnostic Applications
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Chapter 2

Experimental Techniques.
2.1 Electronic Absorption and Emission Measurements.

Electronic absorption spectra of the compounds under study were recorded in quartz cuvettes (Hellma), using a Hewlett-Packard 8453 diode array spectrophotometer (range 190-1100 nm). Continuous-wave (CW) emission spectra were obtained on a Spex 1681 fluorimeter, equipped with an Xe arc light source, a Hamamatsu R928 photomultiplier tube detector and double excitation and emission monochromators. Emission spectra were corrected for source intensity and detector response by standard correction curves. Excitation spectra were referenced to an internal rhodamine-B solution. Luminescence quantum yields ($\Phi_{em}$) were measured in optically dilute solutions, using [Ru(bpy)$_3$]Cl$_2$ in aerated H$_2$O ($\Phi_{em} = 0.028$) as reference emitter, according to Eq.2.1

$$\Phi_{em}^s = \Phi_{em}^r \left( \frac{I_s}{I_r} \right) \left( \frac{A_r}{A_s} \right) \left( \frac{\eta_s}{\eta_r} \right)^2$$

where the subscripts s and r denote respectively the sample and the reference, $I$ is the integrated emission intensity, $A$ is the absorption at the excitation wavelength and $\eta$ is the refractive index of the solvent. Where required, deaerated solutions were prepared by freeze-pump-thaw technique on a vacuum line.

Time-resolved emission measurements were performed in quartz cuvettes using optically dilute ($A < 0.3$) solutions of the compounds. The excitation source was a continuously tunable ($\lambda = 400-700$ nm) Coherent Infinity XPO laser, with a pulse duration of 2 ns FWHM. Full spectra were recorded using a Hamamatsu C5680-21 streak camera, equipped with a M 5677 sweep unit. Kinetics were determined by single emission wavelength measurements, using an Oriel monochromator, a Hamamatsu P28 photomultiplier tube, and recording the signal on Tektronix TDS3052 (500 MHz) oscilloscope. A photodiode was used as external trigger source.

2.2 Nanosecond to (Sub)Picosecond Transient Absorption Spectroscopy.

2.2.1 Principles

Transient absorption spectroscopy is a powerful tool to investigate electronic processes and their dynamics occurring in molecules upon photoexcitation. It consists of recording the electronic absorption spectra of transient species, which can be excited states or intermediate photoproducts.

The transient absorption signal can be recorded in two ways, either as full-range spectra or at single wavelengths. In the former case the exciting laser pulse is followed by white light pulse as probe light. In a nanosecond transient absorption setup this is typically a Xe flash.
lamp, while in (sub)picosecond transient absorption spectroscopy white light can be generated by focusing a laser pulse into a cuvette with solvent, e.g. with water. The light that is transmitted by the solution is recorded by a spectrographic detection system such as an optical multi-channel analyser (OMA) or streak camera.

Since the absorption spectrum of the non-irradiated solution is generally set to zero absorption, the transient absorption spectrum shows both negative and positive absorption bands. The negative absorption bands (bleaching) are due to depopulation of the ground state after the exciting laser pulse. The positive features are instead due to the absorption bands of the transient species.

In the case of a (sub)picosecond setup care must be taken, since due to the extreme short time scale the influence of the speed of light becomes of importance, blue light travelling faster than red light. For accurate determination of rate constants of excited state processes, single wavelength measurements can be performed. In the case of a nanosecond setup, these can be obtained by replacing the spectrographic detection system with a monochromator-photomultiplier-oscilloscope combination, as described for time-resolved emission spectroscopy above. In contrast, for a (sub)picosecond setup, a second laser line is used as probe light. The time delay between exciting and probing laser pulses is obtained by guiding the probe light over a so-called delay line, which is a set of mirrors that can be moved with great accuracy over the distance of about a meter. In that way the probe light has to travel a precisely determined longer distance than the excitation light, which results in the desired time delay. By repeating the measurement at different time delays and using different probe wavelengths, accurate kinetic results are obtained.

2.2.2 Nanosecond Time-Resolved Transient Absorption Spectroscopy: Setup

Nanosecond time-resolved absorption spectra were obtained using a setup (Figure 2.1) described previously. The irradiation source was a continuously tunable (400-700 nm) Coherent Infinity XPO laser working at 10 Hz (2 ns FWHM). Excitation laser output was typically less than 5 mJ/pulse. Samples were prepared to have optical density, at the excitation wavelength, of ca. 0.3 in a 1 cm cuvette. For each sample, spectra were measured at no less than 25 different time delays.
2.2.3 (Sub)Picosecond Time-Resolved Transient Absorption Spectroscopy: Setup

The laser system, employed for the ultrafast transient absorption experiments, was based on a Spectra-Physics Hurricane Titanium Sapphire regenerative amplifier system. The optical bench assembly of the Hurricane included a seeding laser (Maï Tai), a pulse stretcher, a Ti:Sapphire regenerative amplifier, a Q-switched pumped laser (Evolution) and a pulse compressor. The output of the laser is typically 1 mJ/pulse (130 fs FWHM) at a repetition rate of 1 kHz. Two different pump-probe setups were used (see Figure 2.2): (i) a full spectrum setup based on an optical parametric amplifier (Spectra-Physics OPA 800) as pump, with the fundamental light (2 µJ/pulse) being used for white light generation, which was detected with a CCD spectrograph; (ii) single-wavelength kinetics measurements based on two OPAs, one of them being used as pump and the other as probe, and an amplified Si-photodiode for detection. For both setups the OPA(1) was used to generate excitation pulses at 350 nm (fourth harmonic of the 1400 nm OPA signal beam) and OPA(2) 460/550 nm (fourth harmonic of the 1840/2200 nm OPA idler beam). The output power was typically 4 µJ/pulse.

The pump light was passed over a delay line (Physik Instrumente, M-531DD) that provides an experimental time window of 1.8 ns with a maximal resolution of 0.6 fs/step. The white light generation was accomplished by focusing the fundamental (800 nm) into a H2O flow-through cell (10 mm, Hellma). For the single-wavelength measurements, the polarization of probe light was controlled by a Berek Polarization Compensator (New Focus). The energy of the probe pulses was ca. $5 \times 10^{-3}$ µJ / pulse at the sample. The angle between the pump and probe beams was typically 5-7°. The circular cuvette ($d = 1.8$ cm; 1 mm, Hellma), with a solution of the sample, was placed in a homemade rotating ball bearing (1000 rpm), avoiding local heating by the laser beams.

For the white light / CCD setup, the probe beam was coupled into 400 µm optical fiber after passing the sample, and detected by a CCD spectrometer (Ocean Optics, PC2000). The
chopper (Rofin Ltd., $f = 20 - 10$ Hz), placed in the excitation beam, provided $I$ and $I_0$ depending on the status of the chopper (open or closed). The excited state spectra were obtained by $\Delta A = \log (I / I_0)$. Typically, 2000 excitation pulses were averaged to obtain the transient at a particular time. A chirp of $< 1$ ps was observed between 425 and 700 nm. For the single wavelength kinetic measurement, an amplified Si photodiode (New-Port, 818 UV / 4832-C) was used. The output of the Si photodiode was connected to an AD-converter (National Instruments, PCI 4451, 205 kS/s), enabling the intensity measurement of each separate pulse. Also here, analogous to the white light / CCD setup, the chopper ($f = 50$ Hz), placed in the excitation beam provided $I$ and $I_0$ and $\Delta A$, respectively. Typically, 500 excitation pulses were averaged to obtain the transient at a particular time.

![Figure 2.2 Schematic representation of the (sub)picosecond transient absorption setup: 1, Hurricane; 2, OPA-800 (pump); 3, OPA-800 (probe); 4, delay line; 5, white light generator; 6, Berek polarizer; 7, chopper; 8, sample; 9, CCD camera; 10, Si-photodiode.](image)

The CCD spectrograph, chopper, Si-photodiodes, AD-converter and delay line were controlled by a computer. In-house developed LabVIEW (National Instruments) software routines were used for spectral acquisition, and single line measurements over a series of different delay settings. The total instrument rise time of the ultrafast spectrometer was ca. 300 fs. The solutions of the samples were prepared to have an optical density of ca. 0.8 at the excitation wavelength in a 1 mm cell. The absorbance spectra of the solution were measured before and after the experiments. No photodecomposition was observed.
2.3 (Spectro)electrochemistry.

Cyclic and differential pulse voltammetric scans were performed with a gastight single-compartment cell under an atmosphere of dry nitrogen or argon. The cell was equipped with a Pt disk working (apparent surface area of 0.42 mm$^2$), 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 potential control was achieved with a PAR Model 283 potentiostat. All redox potentials are reported against the ferrocene-ferrocenium (Fc/Fc$^+$) redox couple used as an internal standard$^4$ ($E_{1/2} = 0.38$ V vs SCE in acetonitrile)$^5$. Tetrabutylammonium hexafluorophosphate (Bu$_4$NPF$_6$) was used as supporting electrolyte.

By performing redox reactions in situ inside a spectrometer, using an electrochemical cell that is transparent at the wavelength range of interest, the redox reaction can be followed in time, and the products can be characterised spectroscopically. In this Thesis UV/Vis spectroelectrochemical experiments were performed with an air-tight optically transparent thin-layer electrochemical (OTTLE) cell,$^6$ equipped with a Pt minigrid working electrode and quartz optical windows. The controlled-potential electrolyses were carried out with a PA4 potentiostat (EKOM, Czech Republic). All electrochemical samples were $5 \times 10^{-4}$ M in the studied complex and contained $3 \times 10^{-1}$ M Bu$_4$NPF$_6$.

2.4 ECL Immunoassays

2.4.1 Principles of immunoassay

In immuno- and DNA-probe assays based on ECL detection, a modified [Ru(bpy)$_3$$^{2+}$ moiety (such as [4-N-succinimidoyloxycarbonylpropyl]-4'-methyl-2,2'-bipyridine]bis(2,2'-bipyridine)ruthenium(II) dihexafluorophosphate, Figure 2.3)$^7$ is used to label proteins, haptens and nucleic acids.
Several types of immunoassays and DNA-probe assays can be performed. For example, for haptens (small molecules foreign to the organism) of low molecular weight a competitive assay is usually carried out. The haptens labeled with ruthenium complexes compete with the free analyte for the binding to the recognition site of the antibodies (Figure 2.4).

Increasing the free analyte concentration, a smaller number of labeled haptens bind to the antibody. A separation step is then performed to remove the unbound species and avoid their interference with the detection of the bound molecules.
2.4.2 Heterogeneous ECL immunoassay: nanoparticles technology

One of the most widely used methods to separate unbound analytes from the bound ones is based on paramagnetic nanoparticle technology. As it involves the binding of the species to a solid substrate, the immunoassay is called "heterogeneous".

Boehringer Mannheim GmbH / Roche Diagnostics GmbH have developed heterogeneous immunoassays (Elecsys®) where the binding of the antibodies to the nanoparticles is performed by means of streptavidin / biotin system (Figure 2.4). Streptavidin is a protein with molecular weight of 60000 Dalton and four binding sites for biotin, a ligand known to exhibit a strong non-covalent binding with streptavidin, with an association constant ($K_D \sim 10^{15}$ mol/l) comparable to covalent bonds (Figure 2.5). The magnetic particles (made of polystyrene and containing iron oxides) are covered with streptavidin, adsorbed on their surfaces, while the antibodies are modified and bound to a biotin molecule.

![Streptavidin + 4 Biotin ↔ Streptavidin*4 Biotin](image)

\[ K_D : 10^{-15} \text{mol/L} \]

**Figure 2.5** Representation of the biotin-streptavidin equilibrium.

In heterogeneous immunoassays, the solutions containing nanoparticles, antibodies and haptens (free and labeled) are mixed prior to the measurement and let incubate for a certain time. The assay buffer is then drawn into a flow-through chamber measuring cell (Figure 2.6). Upon lifting a magnet below the working electrode, the paramagnetic nanoparticles are forced to lie on its surface, and a buffer solution is drawn into the cell to remove all the unbound species. Finally, the ECL reaction is triggered upon application of a positive voltage at the working electrode and the emitted light is recorded by a photomultiplier placed opposite to the working electrode.

Besides the possibility to separate bound and unbound species, the nanoparticle technology has the advantage to collect the bound species on the electrode surface, thus ensuring that a large proportion of ruthenium labels is accessible for the ECL reaction.

The sensitivity of the immunoassays based on the ECL reaction of $[\text{Ru(bpy)}_3]^{2+}$ and tri-$n$-propylamine is very high, with low detection limit in the sub-picomolar range and linear dynamic range extended over six or more orders of magnitude.
Figure 2.6 Scheme of the flow-through cell for the ECL measurements performed by means of magnetic nanoparticles.

2.4.3 Instrumentation.

The ECL measurements were performed using an Elecsys® instrument (Roche Diagnostics GmbH). It consisted of an automated system for handling the solutions, a flow-through chamber measuring cell, a potentiostat and a red-sensitive photomultiplier tube, placed above an optically transparent window of the cell. The cell was equipped with a platinum sheet working electrode (4.8 mm x 5.0 mm) and a platinum auxiliary electrode made of two wires symmetrically placed above the working electrode. A Ag/AgCl (KCl saturated) electrode was employed as reference. After each measurement, the cell was washed with a cleaning solution. The surface of the working electrode was regenerated upon application of a potential stepped at positive and negative values.

2.5 Synthesis

The synthesis procedures of the complexes investigated in Chapters 4 and 5 are reported in the respective Chapters. The compounds described in Chapters 3 were prepared by the group of Prof. Fritz Vögtle (University of Bonn, Germany) following procedures described elsewhere. The complexes described in Chapters 6 and 7 were prepared by the group of Prof. Peter Belser (University of Fribourg, Switzerland).
References.