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Light-induced tryptophan radical generation in a click modular assembly of a sensitiser-tryptophan residue†

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Click chemistry was used as an efficient method to covalently attach a chromophore to an amino acid. Such easily prepared model systems allow for time-resolved studies of one-electron oxidation reactions by the excitation of the chromophore by a laser flash. The model complex ruthenium–tryptophan (**Ru–Trp**) has been synthesised and studied for its photophysical and electrochemical properties. Despite a small driving force of less than 100 meV, excitation with a laser flash results in fast internal electron transfer leading to the formation of the protonated radical ($Trp^{\cdot}H^{+}$). At neutral pH electron transfer is followed by deprotonation to form the neutral $Trp^{\cdot}$ radical with the rate depending on the concentration of water acting as the proton acceptor. The formation of the tryptophan radical was confirmed by EPR.

Introduction

Electron transfer reactions are crucial processes in biology.[1] Most significantly, the energy transduction pathways of respiration and photosynthesis rely heavily on electron transfer reactions between well-organised cofactors in the protein matrices. Examples of such cofactors include chlorophylls, cytochromes, quinones, and metal centres, as well as redox active amino acid residues such as tyrosine and tryptophan.[2–4] The study of radical formation on amino acids is a field of growing interest.[5,6] In general, electron transfer chain reactions involve the participation of a series of redox active units where tuning of their relative position, and redox properties, is crucial in order to optimise and control charge flow over large distances.[7] Further complications arise from the coupling of redox events to protonation/deprotonation reactions. Studies concerning the mechanistic aspects of proton-coupled electron transfer, amino acid radical formation and its consequent migration in simpler model systems are of great interest for the elucidation of structure–function relationships in more complex biological systems.[8]

The simplest approach is to study amino acid oxidation by intermolecular interaction with a photosensitiser.[9–11] Model complexes with tryptophan or tyrosine residues covalently linked to an Ru–bipyridyl sensitiser *via* an amide bond have been previously reported.[12] Such assemblies offer, among other advantages, (i) to fix the constitutive units at a given distance, (ii) exclude the energy needed for the encounter complex for electron transfer processes, (iii) minimise deleterious intermolecular pathways, and (iv) permit intramolecular electron transfer. However, the synthetic tactics for realising these assemblies is quite restrictive and a more versatile approach leading to the synthesis of a library of molecular assemblies is still needed.

We have recently shown that the use of copper catalysed azide–alkyne cycloadditions (CuAAC), commonly termed as click chemistry,[13] is a relatively easy synthetic method to form modular assemblies composed of ruthenium–polypyridyl type chromophores and functional ligands.[14,15] Additionally, we have shown that the resulting triazole functional group formed between the modules provides the right electronic coupling to allow for efficient electron transfer, while preserving the redox and optical properties of the different modules. The components used for the click reaction are an alkyne-derived Ru-complex and a ligand bearing an azido group.[16] Herein, we present the synthesis and photophysical characterisation of a dyad composed of a chromophore and a tryptophan amino acid covalently linked *via* a triazole bridge.

Results and discussion

The azide modified tryptophan derivative was synthesised following the method of Todd *et al.*[17] Nucleophilic substitution

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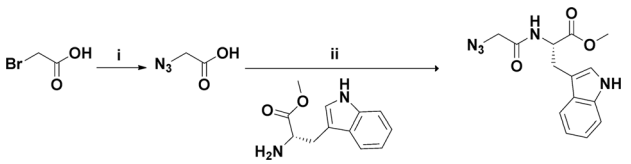
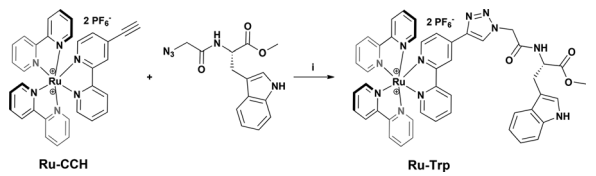


Scheme 1 *Reagents and conditions*: (i) NaN_3, H_2O (93%),[17,18] (ii) HBTU, DMAP, DIPEA, CH_3CN (99%).[17,18] DMAP = 4-(dimethyl-amino)-pyridine; HBTU = *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; DIPEA = *N*,*N*-diisopropylethylamine.



Scheme 2 *Reagents and conditions*: (i) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2–H_2O (90%).

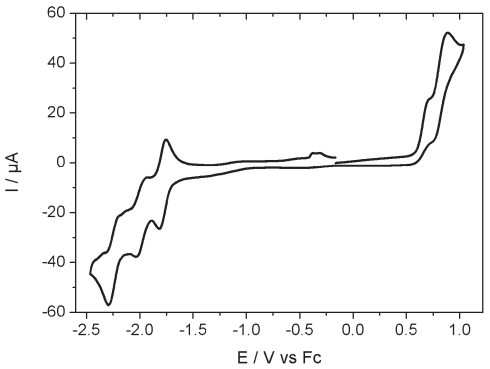


Fig. 1 Cyclic voltammetry of 1 mM **Ru–Trp** *vs.* ferrocene in argon-saturated acetonitrile with tetrabutyl ammonium hexafluoro phosphate as the supporting electrolyte. Scan rate: 100 mV s^{-1}.

of 2-bromo acetic acid in the presence of sodium azide (NaN_3) gave 2-azido acetic acid in high yield. The peptide linkage with tryptophan methyl ester yielded the azido tryptophan derivative quantitatively (Scheme 1). The complex Ru–triazole–tryptophan (**Ru–Trp**) was obtained after performing a copper catalysed azide–alkyne cycloaddition reaction between the alkyne-derived complex, **Ru–CCH**, and the azido-derived tryptophan (Scheme 2).

Ru–Trp shows ground state absorption and emission properties very similar to those of the **Ru–CCH** and $Ru(bpy)_3$ parent compounds. Its absorption spectrum is dominated by strong π–π^* (bpy) transitions at 290 nm, and a metal to ligand charge transfer (MLCT) absorption with a maximum at 458 nm (Fig. S1 in the ESI†). The emission maximum is found at 620 nm and emission kinetics shows monoexponential decay with a lifetime of 1040 ns (Fig. S2 in the ESI†). This value is in agreement with the emission quantum yield of 0.074 which was determined from steady state emission using $Ru(bpy)_3$ as a reference.[19] These data show that the photophysical properties of the chromophore are unaltered in the Ru–triazole–tryptophan assembly, which is in agreement with previous results obtained on other triazole-connected chromophore–ligand assemblies.[14,15] The fact that the emission is not quenched shows the absence of either excited state energy transfer, or electron transfer between the ruthenium chromophore and the ligand.

The electrochemical properties of **Ru–Trp** in acetonitrile were studied by cyclic and differential pulse voltammetry using ferrocene as the reference. Besides the typical features of ruthenium trisbipyridyl complexes, *i.e.* a quasi-reversible Ru(III)/Ru(II) oxidation wave at 0.82 V (ΔE_p = 110 mV) and three quasi-reversible reduction waves for the bipyridine ligands (−2.25, −1.99, −1.78 V; ΔE_p = 90, 80, 60 mV), we observed an additional irreversible wave at 0.69 V (Fig. 1) which is attributed to the oxidation of the tryptophan. No redox activity of the triazole unit was detected in the potential window investigated in these experiments. The pH dependence of the oxidation potential of **Ru–Trp** was studied using differential pulse voltammetry in water while varying the pH of the solution by the addition of diluted solutions of HCl and NaOH. The potential was independent of pH below 3 and displayed a slope of −30 mV per pH unit in the range pH 3–8, corresponding to a two-electron, one-proton process. These results are in agreement with those reported in similar systems.[11,20] A pK_a of about 2.7 ± 0.5 can be estimated for the oxidised tryptophan which is at least one unit lower than typical values for an isolated tryptophan (pK_a ∼ 4.3–4.7).[21,22] No significant change of the Ru(III)/Ru(II) potential with pH was observed.

Flash photolysis experiments were performed in order to investigate the light-induced oxidation of the tryptophan residue. Excitation of **Ru–Trp** in a MeCN–H_2O (50 : 50) mixture with 460 nm laser flashes in the presence of 10 mM methyl viologen acting as a reversible electron acceptor showed depletion of the Ru(II) signal as evidenced by a bleach in its 450 nm absorption band, and the concomitant formation of reduced methyl viologen ($MV^{+\bullet}$) observed as an increase in the absorption at 605 nm (Fig. 2). The recovery of the 450 nm Ru(II) signal is very fast (350 ns) whereas the recovery of the oxidised methyl viologen occurs on a much longer time scale (≈300 µs, inset in Fig. 2). These results clearly indicate that the Ru(II) ground state absorption is recovered by intramolecular electron transfer from the tryptophan ligand to the oxidised chromophore rather than by intermolecular charge recombination between Ru(III) and the reduced form of the electron acceptor. From a thermodynamic point of view, this process is in accord with the electrochemical data where the oxidation of Trp by the Ru(III) is expected to be exergonic by about 130 mV. A comparison of the emission lifetime in the presence of the electron acceptor and the kinetics of recovery of Ru(II) reveals apparent rates which are identical within the error range. This indicates that, under the MV^{2+} concentrations used (10 mM), the formation of the oxidised state of the chromophore by inter-molecular reduction of MV^{2+} is rate-limiting for the subsequent ligand oxidation, and that the intrinsic rate of internal electron

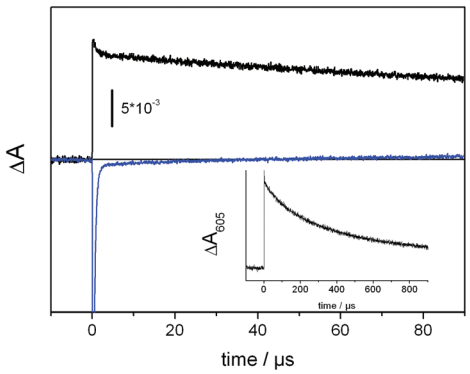


Fig. 2 Transient absorption traces for **Ru–Trp** in argon-saturated MeCN–H_2O (50 : 50) solutions at 605 nm (black) and 450 nm (blue) upon excitation with nanosecond laser flashes at 460 nm. 10 mM MV^{2+} was used as an external electron acceptor. Inset: decay of 605 nm transient at a longer time scale.

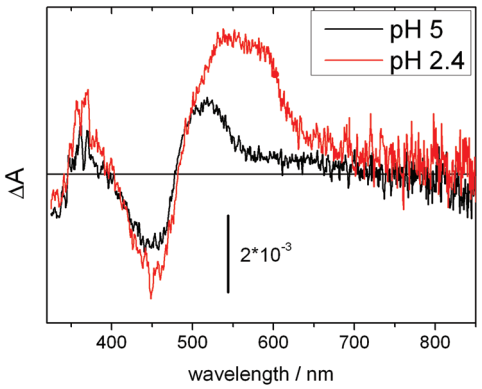


Fig. 4 Transient absorption spectra recorded 3 μs after the laser flash for **Ru–Trp** in MeCN–H_2O (50 : 50) with 20 mM $[Ru(NH_3)_6]^{3+}$ as an electron acceptor and 5 mM citric acid as a buffer.

transfer is significantly faster. Using higher MV^{2+} concentrations allows to accelerate the formation of the Ru(III) state to about 20 ns, as detected by the emission decay kinetics. Under these conditions the rate of internal electron transfer could be determined as 100 ns (10^7 s^{-1}) by kinetic analysis of the 450 nm kinetics. This value confirms the hypothesis of the triazole ring providing a link which allows for efficient electron transfer.[14,15]

Laser flash photolysis results suggest that light excitation of **Ru–Trp** in the presence of an external electron acceptor leads to oxidation of the ligand, *i.e.* the formation of a tryptophan radical. We performed EPR control experiments on a sample frozen during continuous illumination for 10 seconds in the presence of 4-nitrobenzenediazonium tetrafluoroborate as a sacrificial, irreversible electron acceptor. As depicted in Fig. 3, we observed the formation of a narrow signal at $g \sim 2.00$ (width 14 G) typical of an organic radical. This signal is attributed to the formation of the tryptophanyl radical. The broad signal at $g \sim 2.72$ is attributed to Ru^{III} species formed after additional oxidation of the ruthenium–tryptophanyl species.

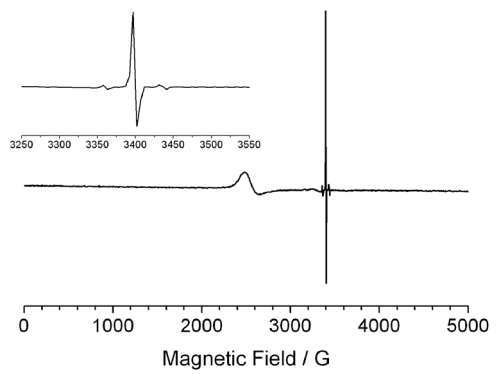


Fig. 3 X-band EPR spectrum (10 scans) of a mixture of 500 μM **Ru–Trp** with 25 mM 4-nitrobenzenediazonium tetrafluoroborate in acetonitrile after 10 second illumination with 450 nm light. Experimental conditions: microwave frequency 9.494 GHz, microwave power 0.100 mW, modulation amplitude 10 G, temperature 50 K, time constant 1.28 ms, scan width 5000 G, modulation frequency 100 kHz. Inset: zoom on the $g \sim 2.00$ radical signal.

The use of MV^{2+} as an external electron acceptor has the advantage that its redox state can easily be followed due to the strong and characteristic absorption of its reduced form at 390 and 605 nm. However, the same property represents a drawback when monitoring the changes in the visible range that might arise from the oxidation of the ligand. Therefore, hexaminerutheniumIII chloride, which shows negligible absorption changes in the visible region upon reduction, was used as an alternative electron acceptor. Fig. 4 shows the transient absorption spectra at 3 μs after the excitation flash of a mixture of the complex **Ru–Trp** and 20 mM hexaminerutheniumIII chloride upon light excitation at 460 nm light in an acetonitrile–water mixture. Citric acid buffer was used to adjust the pH of the solution at values above and below the pK_a of the oxidised Trp. The two spectra are characteristic of the protonated (pH 2.4) and deprotonated (pH 5) forms of the tryptophan radical which show a broad absorption maximum at 530–600 nm for the former, and a sharper maximum at 500 nm for the latter.[23] At time = 3 μs after the excitation flash the two species are fully formed and decay in some hundred microseconds due to charge recombination with the reduced form of the electron acceptor.

At pH > pK_a ($Trp^{\bullet}H^+$) oxidation of Trp is expected to be accompanied by deprotonation. Time-resolved spectra with a sample in water (neutral pH) show indeed transition from the protonated form of the Trp radical to the deprotonated form on a time scale of 1 μs (Fig. 5).

From the spectra of the two forms (Fig. 4 and 5) it appears that the deprotonation reaction could be followed in the 500–600 nm spectral region. Absorption transients at 570 nm and 510 nm are shown in Fig. 6. At both wavelengths the prompt rise in absorption is due to the flash-induced formation of the Ru excited state which decays quickly in about 25 ns due to quenching by the electron acceptor. At 570 nm a slower decay phase is detected which is well described by a single exponential with a time constant of 410 ns. This transient is attributed to the fast (<100 ns) formation of the $Trp^{\bullet}H^+$ radical by internal electron transfer and its subsequent disappearance due to deprotonation to form $Trp^{\bullet}$ which has

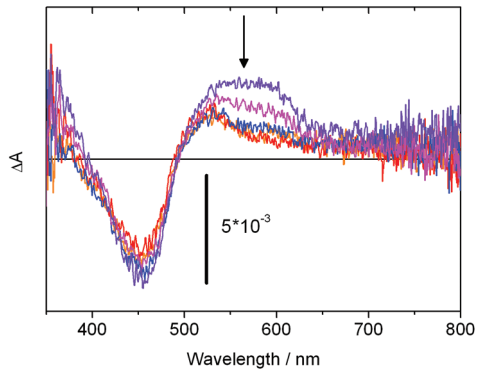


Fig. 5 Transient absorption spectra of **Ru–Trp** in water with 40 mM $[Ru(NH_3)_6]^{3+}$ as an electron acceptor measured at 200 (purple), 300, 500, 700, 1000 ns (red) after laser flash.

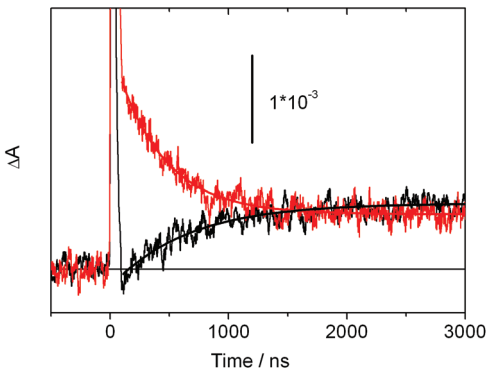


Fig. 6 Kinetic traces at 510 nm (black) and 570 nm (red) with 100 mM $[Ru(NH_3)_6]^{3+}$ as an electron acceptor in MeCN–H_2O (50 : 50). The smooth lines represent best fits with monoexponential functions for $t > 100$ ns.

lower absorption at 570 nm. From the initial signal amplitude (2.6×10^{-3}) and using an extinction coefficient[23] for $Trp^{\bullet}H^{+}$ of 3000 M^{-1} cm^{-1}, an initial concentration of 0.9 μM could be estimated. This value compares well with the concentration of the reduced electron acceptor (which equals the concentration of Ru(III)) formed under our experimental conditions (compare Fig. 2) and shows that oxidation of the Trp is quantitative. At 510 nm an exponential rise in absorption is apparent, described by a rate of 645 ns^{-1}. Similar increases in absorption are observed at shorter wavelengths down to 495 nm and we assign this absorption rise to the formation of $Trp^{\bullet}$, the deprotonated form of the Trp radical, which has a slightly higher extinction coefficient than the protonated form in this region. The similar kinetics observed at 570 nm and 510 nm show that under these conditions oxidation of the tryptophan residue is followed by deprotonation with a rate of 2×10^6 s^{-1}.

The deprotonation of the tryptophan residue at neutral pH is thermodynamically driven by the low pK_a of its oxidised form. However, from a kinetic point of view proton dissociation is controlled by preformation of a favorable configuration between the acid and a suitable base acting as the proton acceptor.[24] With water as the proton acceptor successful propagation of the dissociating proton requires a

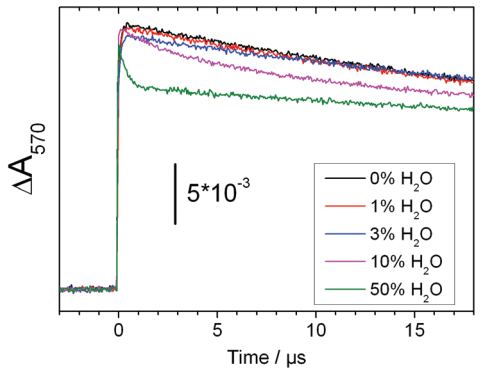


Fig. 7 Kinetic traces at 570 nm in MeCN with different concentrations of H_2O; 20 mM MV^{2+} was present as an electron acceptor.

percolating network of hydrogen-bonded molecules. Addition of organic solvents has been reported to break this structure and lead to a decrease in the rate of deprotonation reactions.[25] This effect is also observed in our complex as demonstrated by the results shown in Fig. 7 where the concentration of water is varied. Methyl viologen was used in these experiments because of the insufficient solubility of hexaammineruthenium(III) chloride in acetonitrile. The deprotonation of the $Trp^{\bullet}H^{+}$ radical can be followed by the distinct decay of absorption on top of the larger absorption due to the $MV^{+\bullet}$ radical, which decays on the hundreds of microseconds time scale. With decreasing concentration of water, the rate of deprotonation is drastically slowed down. For 10 vol% the corresponding time constant is 5 μs and for 3% it is 74 μs. For concentrations below 3%, deprotonation can no longer be observed as its rate approaches the rate of charge recombination between $MV^{+\bullet}$ and $Trp^{\bullet}H^{+}$.

Conclusions

In summary, we have used click chemistry as an efficient tool to perform the modular assembly of a chromophore and an amino acid. Such easily prepared model systems allow for time-resolved studies of one-electron oxidation reactions by creating a strongly oxidising state on the chromophore by excitation with a laser flash in the presence of external electron acceptors.

Kinetic transient absorption studies in aqueous solution upon excitation with a nanosecond laser flash revealed fast intramolecular electron transfer leading to the formation of a protonated tryptophanyl ($Trp^{\bullet}H^{+}$) species detected by its characteristic absorption at 570 nm. At pH > pK_a ($Trp^{\bullet}H^{+}$), the absorption at 570 nm disappears in less than one microsecond with a concomitant increase in absorption at 510 nm, typical of the deprotonated form of Trp radicals. This observation demonstrates that electron transfer from Trp to Ru(III) is followed by deprotonation to yield the neutral $Trp^{\bullet}$ radical species. The kinetics of the deprotonation reaction is strongly dependent on the water concentration.

From a thermodynamic point of view, the system studied in this work is characterised by a moderate driving force for internal electron transfer from the ligand to Ru(III) resulting in the formation of a weak acid. The value of $\Delta G = -130$ meV deduced from the difference of redox potentials of the chromophore and the ligand (below its pK_a) has to be considered as the maximum possible value for the electron transfer step. Corrections for electrostatic interactions would bring this value close to, or even below, 100 meV. Despite this low driving force, the rate of internal electron transfer is fast ($>10^7$ s^{-1}). This fast rate is taken to indicate good electronic coupling between the chromophore and the tryptophan *via* the triazole spacer, but the relative close proximity between the tryptophan and the sensitiser together with the flexibility of the amide link might also allow through-space electron transfer to occur. The estimation of the electron transfer rate in the framework of Marcus theory shows furthermore that the reorganization energy of the reaction must be relatively small. The latter fact is in agreement with a stepwise mechanism where electron transfer is independent of deprotonation of the Trp radical.

The covalently linked chromophore–amino acid system described in this work can be looked at as a first step in an effort to bind a chromophore to a peptidic motif. The preparation of azide-functionalised proteins by different methods has been described.[26–28] Combining the high reaction yields, mild reaction conditions of click chemistry, and the stability of the triazole link, this approach seems to be an optimal alternative for site-specific and selective post modification of proteins. Importantly, our results show that coupling of the ruthenium complex does not lead to significant modifications of the redox and acid–base properties of the addressed amino acid. We will use this synthetic strategy to address electron transfer studies in more sophisticated polypeptide and protein systems where visible light activation by a covalently linked chromophore can lead to photoactivation of complex units in both oxidation and reduction reactions.

Acknowledgements

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