# Photoinduced reduction of catalytically and biologically active Ru(II)bisterpyridine–cytochrome *c* bioconjugates

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**Ruthenium(II)bisterpyridine chromophores were covalently linked to iso-1 cytochrome *c* from yeast to create light-activated donor–acceptor bioconjugates.**

The emerging field of bioelectronics has relied heavily upon redox proteins and enzymes[1] due to their efficient participation in electron transfer chains. Recent advances have seen the development of stable, multiple enzyme systems that generate electric current in the presence of substrate.[2] However, control over enzyme activity remains an ongoing challenge, usually requiring site-specific protein modification[3] with an appropriate synthetic ligand, as demonstrated in magneto-controlled bioelectronic devices,[1,4] which require linking of the protein to a ferromagnetic particle. Light activated systems, which require the ligand to function as either an $e^-$ donor or acceptor, have been investigated extensively with regard to electron transfer pathways,[1,5,6] but surprisingly few studies are aimed at practical applications (*e.g.*, converting light- to chemical-energy). In addition, the few systems that have been reported make use of reconstituted myoglobin or similar proteins with little catalytic or biological activity.[7] To address this issue, we have focused on modifying the native heme protein iso-1 cytochrome *c* from *Saccharomyces cerevisiae* (**cyt**, $e^-$ acceptor) with Ru(II)(tpy)$_2$ (**1**, tpy = 2,2′:6′,2″-terpyridine, $e^-$ donor) to form bioconjugates of the type **1–spacer–cyt** (Fig. 1). We have chosen ruthenium tpy chromophores due to their stability and interesting photophysical properties.[8] Furthermore, the symmetry of **1** and the ease of functionalising tpy provide straightforward routes toward surface attachment and other potential applications of **1–spacer–cyt**. We investigated two different spacers – one a long, flexible alkyl chain (**LC**), the other a short, rigid aromatic chain (**SC**). We show here that the resulting bioconjugates are both catalytically and biologically active. We also show that the nature of the spacer has surprisingly little effect on the photophysical properties of these bioconjugates, and that upon prolonged irradiation of the donor **1**, the heme group of **cyt** is reduced from Fe(III) to Fe(II).

The long chain tpy **2** was synthesised as previously reported[9] from *N*-hydroxysuccinimide-6-maleimidehexyl ester and 5-(2,2′:6′,2″-terpyridin-4′-yloxy)-pentylamine.[10] The previously reported tpy **3**[11] was synthesised using a solvent-free Green Chemistry method[12] followed by conversion to the corresponding maleimide-tpy **4** by reduction of the nitro-group and

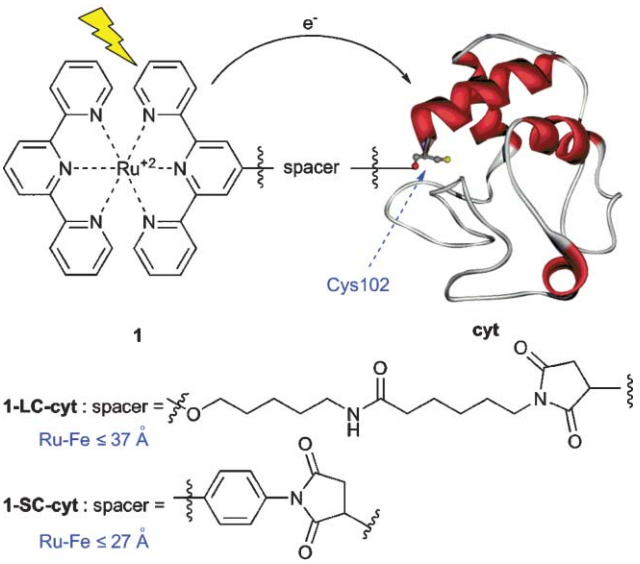
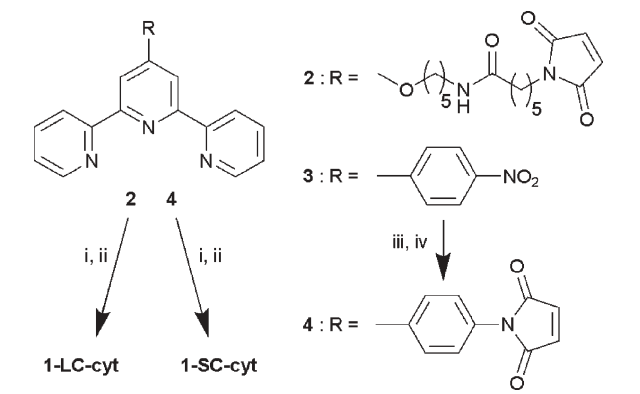



**Fig. 1** Ru(II)bisterpyridine–cytochrome *c* bioconjugates with either a long, flexible spacer (**1–LC–cyt**) or a short rigid spacer (**1–SC–cyt**) (with estimated Ru to Fe distances) for photoinduced electron transfer.

reaction with maleic anhydride (Scheme 1).† The maleimide groups in **2** and **4** were incorporated to allow selective attachment to the single free cysteine (Cys102) of **cyt**. Terpyridines **2** and **4** were then complexed with Ru(III)tpy followed by precipitation with aqueous $NH_4PF_6$. After washing away unreacted starting material, NMR and mass spectrometry indicate the presence of ring opened malonic acid Ru(II)(tpy)$_2$ as well as the desired




**Scheme 1** Synthetic route to bioconjugates with two different spacers – (i) EtOH, Ru(tpy)$Cl_3$, not isolated, (ii) **cyt**, 50 mM $NaH_2PO_4$, 15 mM EDTA, pH 7, 5% $CH_3CN$, 35 °C, 40–50%, (iii) EtOH, $H_2NNH_2{\cdot}H_2O$, Pd/C, Δ, 87%, (iv) glacial AcOH, maleic anhydride, Δ, 89%.

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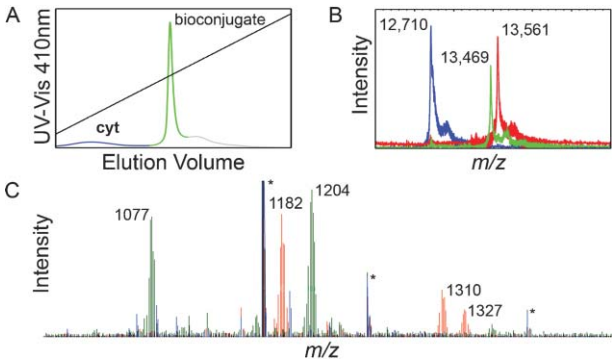


**Fig. 2** (A) Representative IMAC chromatogram showing the imidazole gradient elution (black) which affords separation of **cyt** (blue), **1–SC–cyt** (green), and unknown side products (grey). (B) MALDI data for purified **cyt** (blue, expected 12707), **1–LC–cyt** (red, expected 13569) and **1–SC–cyt** (green, expected 13463). (C) Peptide map (trypsin digest) data for **cyt** (blue), fragments common to all samples (*), **1–LC–cyt** (red, expected 1183 $[R]^+$, 1311 $[R{\cdot}PF_6 - OH]^+$ and 1328 $[R{\cdot}PF_6]^+$) and **1–SC–cyt** (green, expected 1077 $[R{\cdot}OH]^+$, 1205 $[R{\cdot}PF_6]^+$, and 1350 $[R{\cdot}(PF_6)_2]^+$). R denotes the **1**–spacer–ACE fragment.

$Ru(II)(tpy)_2$ maleimides. The resulting crude mixtures were subsequently used (in *ca.* 10–40 fold excess based on UV–Vis absorbance from **1**) to functionalise **cyt** in a phosphate buffered EDTA solution at neutral pH. The bioconjugate reaction mixtures were then purified by immobilised metal affinity chromatography (IMAC, Fig. 2A) to afford either pure long chain bioconjugate **1–LC–cyt** or short chain bioconjugate **1–SC–cyt** in 40–50% yield.‡

Bioconjugates **1–LC–cyt** and **1–SC–cyt** have been characterised by mass spectrometry (MALDI and ESI), peptide mapping, and gel electrophoresis. In each case, MALDI data (Fig. 2B) show detection of only a single peak within 10 amu of the expected mass, indicating that the bioconjugates contain a single attached chromophore and that the products are of the desired purity. This is confirmed by gel electrophoresis (data not shown). Peptide mapping (Fig. 2C) was performed to identify the location of chromophore attachment. After trypsin digest, peptide fragments containing Cys102 (the ACE fragment in native **cyt**) show an increase in mass corresponding to the mass of the chromophore, as expected. The combined results of MALDI, gel electrophoresis and peptide mapping confirm the attachment of a single chromophore to the free cysteine (Cys102) of **cyt**.

UV–Vis spectroscopy (Fig. 3) shows that spectra for bioconjugates **1–LC–cyt** and **1–SC–cyt** can be closely approximated as the linear sum of the spectra for oxidized **cyt** and **1**, which indicates that there is negligible ground state communication between the chromophores. Additionally, there is no apparent change in the

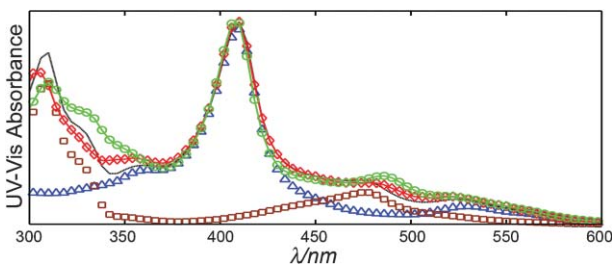


**Fig. 3** UV–Vis spectra for oxidized **cyt** (△), **1** (□), the linear sum of **cyt** and **1** spectra (—), **1–LC–cyt** (◇) and **1–SC–cyt** (○).

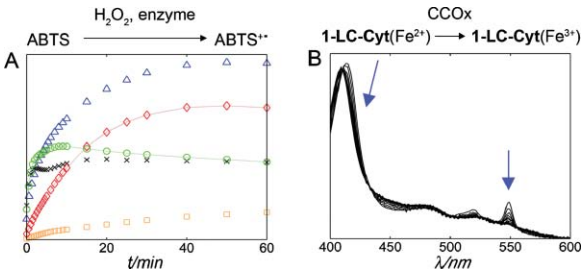


**Fig. 4** (A) ABTS assay results comparing catalytic activity of **cyt** (△) to that of **1** (□), a non-covalent mixture of **cyt** and **1** (×), **1–LC–cyt** (◇), and **1–SC–cyt** (○). (B) CCOx assay results showing decreasing absorbance of heme peaks at 410 and 550 nm which is indicative of **cyt** oxidation.

Soret- and Q-bands for **cyt**, signifying that protein secondary and tertiary structure and the heme group are intact.

The catalytic and *in vitro* biological activity of bioconjugates **1–LC–cyt** and **1–SC–cyt** was measured by the oxidation of 2,2′-azino-bis[ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS)[13,14] and oxidation by cytochrome *c* oxidase (CCOx),[15] respectively. Results of the ABTS and CCOx measurements (Fig. 4) indicate that attachment of the ruthenium chromophore has not significantly affected the structure or activity of either bioconjugate. It is interesting to note from the ABTS assay that while native **cyt** and **1–SC–cyt** have the same initial rate of reaction, the catalytic activity of **1–SC–cyt**, and a non-covalent mixture of **cyt** and **1**, decreases noticeably after only a few minutes. Interestingly, this behaviour is not seen for **1–LC–cyt**, which appears to have a catalytic lifetime not unlike that of unmodified **cyt**, although a slower initial rate of reaction may indicate lower activity. Loss of peroxidase-like activity has been attributed to radical generation and propagation mechanisms, the effect of which is sometimes referred to as "suicide inactivation".[13,16] It may be that, although the heme active site in **1–SC–cyt** is preserved in a state similar to that in the native protein, the chromophore has forced the protein into a conformation that is more susceptible to inactivation by radicals generated in the active site. **1–LC–cyt**, on the other hand, may have more conformational degrees of freedom, allowing it to more closely mimic the activity of native **cyt**.

Time-resolved emission experiments were undertaken to measure energy/electron transfer rates in bioconjugates **1–LC–cyt** and **1–SC–cyt**. Due to the short lifetimes of $Ru(II)(tpy)_2$ complexes at room temperature,[17] emission lifetime measurements were made in degassed 1 : 1 water–glycerol (v/v) glass at 77 K (Fig. 5A). While emission lifetime data for **1** and a non-covalent mixture of **1** and **cyt** can be fitted as single exponential decays (7.2 and 8.4 μs, respectively), bioconjugates **1–LC–cyt** and **1–SC–cyt** show double exponential decay behaviour with components having lifetimes matching those above and second, significantly shorter lifetimes (2.4 and 1.4 μs, respectively). If we attribute this double exponential behaviour to the presence of two environments in each of the bioconjugates, one in which the $Ru(II)(tpy)_2$ groups are essentially isolated, and one in which the $Ru(II)(tpy)_2$ groups undergo electron transfer, electron transfer rates of $2.78 \times 10^5$ and $5.95 \times 10^5\ s^{-1}$ result for **1–LC–cyt** and **1–SC–Cyt**, respectively. This is in line with expected electron transfer rates for metal–metal distances of 25–35 Å in ruthenium modified proteins.[1]

Attempts at measuring nanosecond transient absorbance changes have proven ineffective with difficulties arising from what

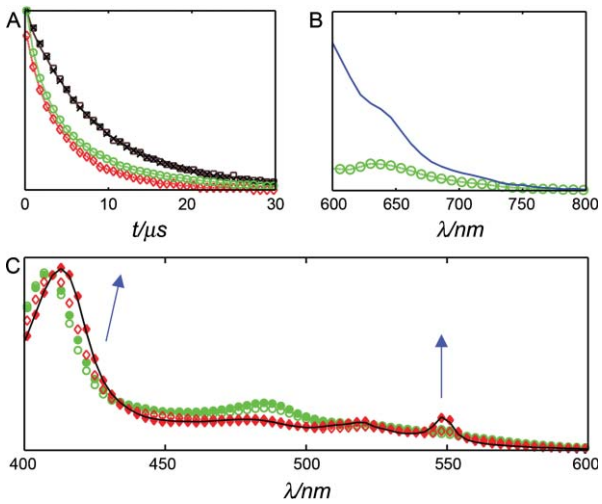


**Fig. 5** (A) Luminescence decay at 77 K ($\lambda_{em}$ = 640 nm) of **1** (□), a non-covalent mixture of **cyt** and **1** (x), **1–SC–cyt** (○) ($\lambda_{ex}$ = 355 nm) and **1** (—) and **1–LC–cyt** (◇) ($\lambda_{ex}$ = 480 nm). (B) Emission spectra of oxidized (○) and reduced (—) **1–SC–cyt**. Room temperature, $\lambda_{ex}$ = 420 nm. (C) UV–Vis spectra of **1–LC–cyt** before (◇) and after (◆) and **1–SC–cyt** before (○) and after (●) exposure to light at 480 nm (77 K in 1 : 1 water–glycerol (v/v)). Chemically reduced (dithiothreitol) **1–LC–cyt** (—) is included for comparison.

are likely to be very small and short-lived absorbance changes at 550 nm.[6] However, indirect evidence for electron transfer has been obtained by comparing the increased emission of **1–SC–cyt** in the reduced ($Fe^{2+}$) form relative to that of the oxidized ($Fe^{3+}$) form (Fig. 5B), indicating that an energy or electron transfer pathway available for emission quenching in the oxidized form is not available in the reduced form.

Further evidence for electron transfer comes from comparing the UV–Vis spectra of bioconjugates **1–LC–cyt** and **1–SC–cyt** before and after prolonged exposure to high intensity laser light at 480 nm (Fig. 5C). Initially, the bioconjugates are present in the oxidized form, but after prolonged excitation at 480 nm, the spectra indicate that the heme group of cytochrome *c* is substantially reduced. Although the mechanism for photoreduction of **1–LC–cyt** and **1–SC–cyt** is unknown, alcohols are frequently used to reduce Ru(III) to Ru(II) (see Scheme 1), and it is therefore believed that the ultimate electron source in our experiments is glycerol.

We have demonstrated methods for the preparation of pure, catalytically and biologically active bioconjugates containing **cyt** and **1**. The combination of emission lifetime, emission from the reduced and oxidized forms, and UV–Vis spectral changes after prolonged exposure to light at 480 nm suggest photoinduced electron transfer between the ruthenium chromophore **1** donor and the **cyt** acceptor. To our surprise, there is little difference between **1–LC–Cyt** and **1–SC–Cyt** with respect to electron transfer rates, suggesting that the former is in a folded conformation resulting in a similar Ru–Fe distance. Work is in progress toward incorporating these light-activated bioconjugates into bioelectronic devices.

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## Notes and references

† Selected data for **4**: White solid; $\delta_H$(300 MHz; $CDCl_3$; $Me_4Si$) 6.90 (s, 2H), 7.36 (ddd, 2H), 7.51–7.58 (m, 2H), 7.89 (dt, 2H), 7.98–8.4 (m, 2H), 8.68 (td, 2H), 8.72–8.75 (m, 2H), 8.76 (s, 2H); *m/z* (HR-ESI) 405.1346 ($M^+$ + H. $C_{25}H_{17}N_4O_2$ requires 405.1352).

‡ Typical procedure for synthesis of bioconjugates **1–LC–cyt** and **1–SC–cyt**: To a solution of 500 mM sodium hydrogen phosphate, 150 mM EDTA (2 mL) in water (16 mL) at 35 °C was added crude Ru(**2**)(tpy) or Ru(**4**)(tpy) in 1 mL of acetonitrile. Purified, reduced **cyt** (2.0 mg, 0.16 μmol) in 0.9 mL water was then added and the resulting solution stirred at 35 °C for 18 h. The reaction mixture was then cooled to room temperature, concentrated, and dialysed into water. The dialysed reaction mixture was purified by IMAC chromatography (HisPrep FF 16/10, GE Healthcare) using a gradient from 0 to 75 mM imidazole in 20 mM $Na_2HPO_4$, 0.5 M NaCl, pH 7.0 in 5 column volumes. The appropriate fractions were pooled, concentrated, and dialysed into water to afford bioconjugate **1–LC–cyt** or **1–SC–cyt** in 40–50% yield.

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