Supporting Information for

Synthesis, characterization and room temperature photo-induced electron transfer in biologically active bis(terpyridine)ruthenium(II)-cytochrome *c* bioconjugates and the effect of solvents on the bioconjugation of cytochrome *c*

Joshua R. Peterson,[a,b] Trevor A. Smith,[c] Pall Thordarson[a]*

[a]School of Chemistry, The University of New South Wales, Sydney, NSW 2052, Australia.
[b]School of Chemistry, The University of Sydney, NSW 2006, Australia.
[c]School of Chemistry, The University of Melbourne, VIC 3010, Australia.

*To whom correspondence should be addressed. School of Chemistry, The University of New South Wales, Sydney, NSW 2052, Australia. Phone: +61-2-9385-4478. Fax: +61-2-9385-6141. E-mail: p.thordarson@unsw.edu.au

Table of Contents:

Oxidation of cytochrome *c* in mixed solvents. The effect of solvent on protein oxidation was assessed for the different solvents by monitoring absorbance at 550 nm for up to 24 hours at 25 and 35 °C (Figure 1 - Panels A and B, respectively). Protein oxidation appeared to be solvent and temperature dependent with 20% tetrahydrofuran and acetonitrile leading to the most rapid oxidation at either 25 °C (within 10 hours) or 35 °C (1 hour). Storage in 20% dimethylformamide led to partial oxidation over 24 hours at 25 °C, while complete oxidation was observed after only 8 hours at 35 °C. Storage for 24 hours in 20% dimethylsulfoxide appeared to be equivalent to storage in water with little or no oxidation regardless of temperature.

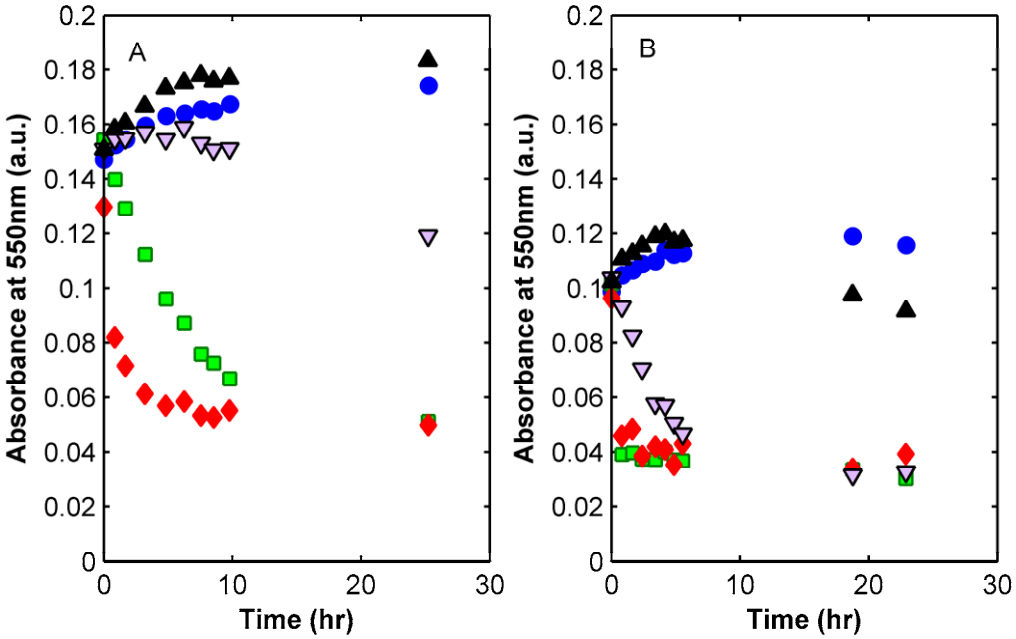

Figure 1. Oxidation of 10 μM iso-1 cytochrome *c* at 25 ºC (Panel A) and 35 ºC (Panel B) over time monitored by absorbance at 550 nm in 20 mM NaH_2PO_4, 20 mM NaCl, 5 mM EDTA, pH 7 (●), and 20 mM NaH_2PO_4, 20 mM NaCl, 5 mM EDTA, pH 7.0 with 20% DMSO (▲), 20% DMF(▼), 20% CH_3CN (■) and 20% THF (♦).

Preparation of reaction mixtures. The reactivity of iso-1 cytochrome *c* CYS102 was measured by preparing reaction mixtures from stock solutions of sodium hydrogen phosphate (1 M, pH 7), sodium chloride (1 M), ethylenediaminetetraacetic acid (EDTA, 200 mM, pH 7), and cytochrome *c* (200 μM) in water. Stock solutions of 4-4'-dithiodipyridine (DTDP) or N-(1-pyrenyl)-maleimide (NPM) were prepared at variable concentrations (2.5, 1 or 0.25 mM) depending on the desired level of organic solvent in the final reaction mixture (5% or 20% (v/v)). Table 1 below shows how the stock solutions were used to prepare typical 1 mL reaction mixtures (or 200 μL reaction mixtures for the case of 100 μM cyt *c*). All reactions were prepared by addition of sodium hydrogen phosphate, sodium chloride, EDTA and cytochrome *c* to water, then initiated by addition of DTDP or NPM.

Table 1. Preparation of cytochrome *c* CYS102 reaction mixtures from stock solutions.

Reaction Mixture	*10 μM cyt c* *50 μM reagent* *5% (v/v) solvent*	*10 μM cyt c* *50 μM reagent* *20% (v/v) solvent*	*100 μM cyt c* *500 μM reagent* *20% (v/v) solvent*
Water (μL)	835	685	47
Cyt *c* (μL)	50	50	100
NaH_2PO_4 (μL)	20	20	4
NaCl (μL)	20	20	4
EDTA (μL)	25	25	5
Reagent (DTDP or NPM)	50 μL of 1 mM stock	200 μL of 0.25 mM stock	40 μL of 2.5 mM stock

Reactivity of cytochrome *c* with N-(1-pyrenyl)-maleimide (NPM). As detailed further below, an initial attempt was made to generate a quantitative standard curve of the luminescent emission of thiol-reacted NPM versus concentration up to 500 μM NPM. However, a standard curve could not be generated because the emission measurements were confounded by solubility, self-quenching, or eximer[1] formation effects. Therefore NPM may not be a suitable detection agent at 10 μM in 5% acetonitrile and reactions with NPM at the desired concentrations of 50 or 500 μM cannot be accurately prepared or quantified if the solvent concentration is to be limited to 5 to 20% (v/v). However, there is benefit to understanding how a compound with limited water solubility will perform in the bioconjugation reaction even with the issues identified. For that reason, reactions were performed using 10 μM cytochrome c and 50 μM NPM in 5 and 20% solvents and the results are shown in Table 2.

Table 2. Results for reactions between N-(1-pyrenyl)-maleimide (NPM) and cytochrome *c*. All reactions were carried out at 10 μM cytochrome *c*, 50 μM NPM in 20 mM NaH_2PO_4, 20 mM NaCl, 5 mM EDTA, 5-20% solvent (v/v), pH 7.0 at 25 °C (deviations as noted in the table). Fluorescence intensities normalized to entry 1. Calculated error for duplicate or triplicate measurements is typically less than ± 10%.

Entry	Solvent	Initial Rate[a] (normalized)	Plateau Intensity[b] (normalized)	Time to 90% of Plateau Intensity hr
	5 % CH_3CN			
1	5 mM EDTA	1.00	1.00	6.3
2	0 mM EDTA	1.02	0.97	4.7
3	20 mM EDTA	0.89	0.80	3.3
4	pH 6.50	0.97	0.98	5.9
5	pH 6.75	0.99	0.91	3.9
6	pH 7.25	0.99	0.89	3.2
7	pH 7.50	0.89	0.78	2.5
8	5 % THF	0.82	1.09	15.7
9	5 % DMF	0.98	1.56	23.5
10	5 % DMSO	0.82	1.00	12.4
11	20 % CH_3CN	2.03	2.14	8.4
12	20 % THF	1.03	1.02	7.5
13	20 % DMF	1.95	2.94	20.8
14	20 % DMSO	1.19	1.79	18.4

[a]Average rate over first 2 hours calculated from the model and normalized to entry 1 (control reaction). [b]Calculated from model and normalized to entry 1 (control reaction).

As shown, EDTA has little effect on the reaction (entries 1 – 3, Table 2) with the possible exception of 20 mM EDTA, which may lead to slightly lower yield (based on plateau fluorescence intensity). Reaction pH, on the other hand, appears to have a significant affect between pH 6.50 and 7.50 (entries 4 – 7 and entry 1 (pH 7.0)), with yield and reaction time tending to decrease with increasing pH reactions. This pH effect is expected based on the literature, which generally indicates a preferred pH range of 7.0 to 7.5 for maleimide-cysteine reactions.[2,3] In the case of these mixed solvent reactions, preparing the phosphate solution between pH 6.5 and 7.0 may result in a more successful reaction as the organic solvents may have a slight basifying effect on the reaction mixture.

The choice of organic solvent appears to have a significant affect as well with dimethylformamide resulting in the highest plateau intensities, but also the longest reaction times (entries 9 and 13). There is apparent benefit to using 20% (v/v) mixtures as opposed to 5% (v/v) mixtures with plateau intensities increasing with solvent concentration in all cases, with the exception of tetrahydrofuran (entries 8 and 12). However, care should be taken in choosing a higher solvent concentration as the stability studies showed that the use of 20% (v/v) solvent mixtures may be undesirable due to protein oxidation and dimerization.

Attempts to make a standard curve of thiol-reacted NPM luminescence. As described above, the luminescent emission of thiol reacted N-(1-pyrenyl)-maleimide (NPM) was measured using up to 500 μM NPM with 5 mM DTT in an effort to generate a standard curve which could be used to determine the extent of reaction between NPM and iso-1 cytochrome *c*. The solutions were prepared with stock solutions of 0.1 mM and 1 mM NPM in acetonitrile resulting in differing levels of acetonitrile in the final reaction mixture. The concentration of acetonitrile and emission intensity of the various samples up to 50 μM NPM is plotted in Figure 2.

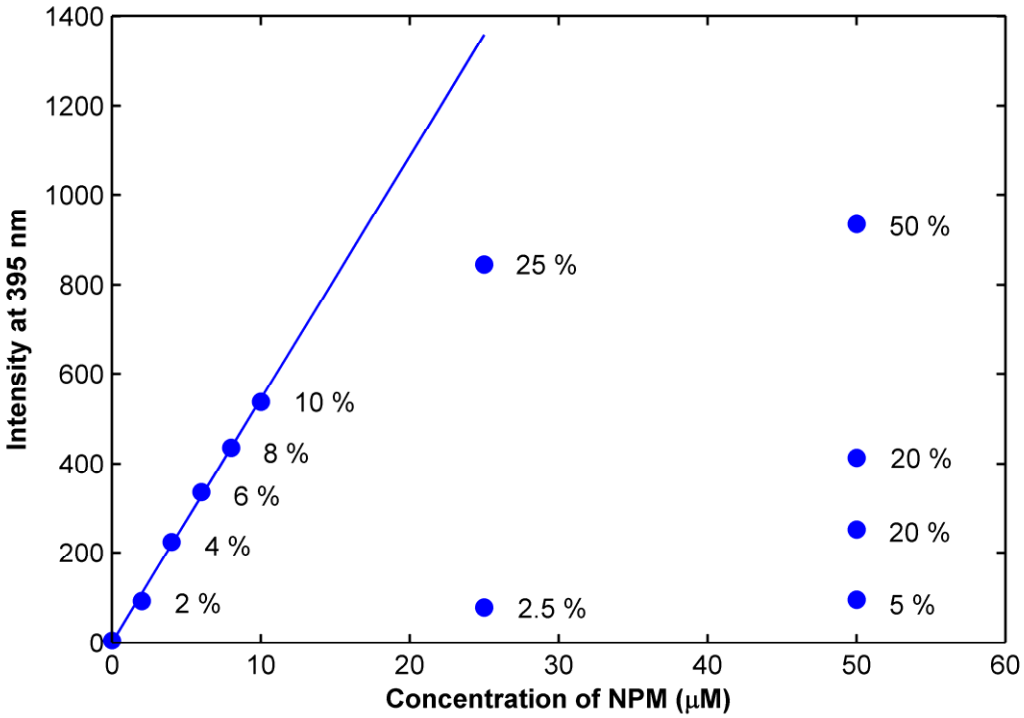

Figure 2. Emission intensity of NPM in CH_3CN/H_2O mixed solvent systems. The level of CH_3CN in each sample is indicated as labeled (%, v/v). λ_{ex} = 342 nm.

As shown in, Figure 2, concentration of acetonitrile has a significant effect on the fluorescence intensity as demonstrated by the results at 25 and 50 μM NPM. At 25 μM NPM, a 10-fold decrease in organic solvent concentration (from 25% to 2.5%) results in an approximately 10-fold decrease in intensity. A similar effect is seen with 50 μM NPM in that the intensity in 50% acetonitrile is 10 times as large as that measured in 5% acetonitrile. Also, there is significant variability between two samples prepared at 50 μM NPM in 20% acetonitrile which indicates that reproducibility will likely be problematic. Furthermore, as shown below in Table 3, there is a steep decline in emission for samples above 50 μM, with samples such as 500 μM NPM in 50% acetonitrile exhibiting immeasurably low intensity compared to 50 μM NPM in 50% acetonitrile.

Table 3. Emission intensity of NPM in CH_3CN/H_2O mixed solvent systems. $\lambda_{ex} = 342$ nm.

Concentration of NPM (μM)	Concentration of Acetonitrile (% v/v)	Emission Intensity
0	-	4
2	2	93
4	4	224
6	6	336
8	8	435
10	10	537
25	25	845
50	5	96
50	20	414
50	50	935
100	10	25
200	20	13
300	30	3
400	40	0
500	50	0

Based on these results, it is clear that a standard curve to determine the extent of NPM reaction with iso-1 cytochrome *c* is not viable, especially if solvent concentrations are to be maintained at 5 – 20% (v/v).

NPM-cytochrome *c* bioconjugation test reaction. To demonstrate successful formation of bioconjugate, a reaction was performed using 10 µM crude reduced cytochrome *c*, 50 µM NPM in 20 mM sodium phosphate, 20 mM sodium chloride, 5 mM EDTA, pH 7.0 with 20% (v/v) dimethylformamide at 25 °C for 24 hours. The crude reaction was concentrated and dialysed into water and analysed by UV-Vis and MALDI-TOF.

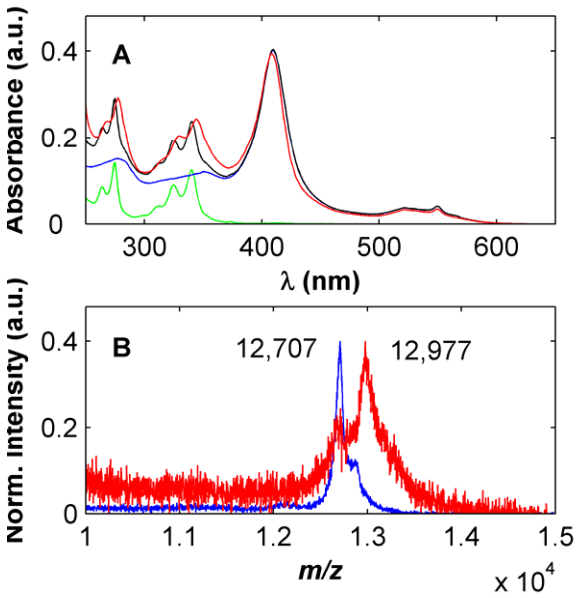

Figure 3. UV-Vis (Panel A) and MALDI-TOF (Panel B) results for a test reaction of 10 µM cytochrome *c*, 50 µM NPM in 20 mM NaH_2PO_4, 20 mM NaCl, 5 mM EDTA, pH 7.0, 20% DMF (v/v), pH 7.0 at 25 °C for 24 hours. Panel A – UV-Vis traces for unreacted NPM (green), unreacted cytochrome *c* (blue) and NPM-cyt *c* bioconjugate (red). The linear sum of NPM and cytochrome *c* spectra (black) is also shown for comparison. Panel B – MALDI-TOF data for unreacted iso-1 cytochrome *c* (blue, expected 12,707) and NPM-cyt *c* bioconjugate (red, expected 13,004).

As the UV-Vis data show (Figure 3, Panel A), the product of the bioconjugation reaction exhibits peaks consistent with cytochrome *c* (between 400 and 550 nm) and NPM (between 250 and 350 nm) and a crude estimate from the spectra indicate that NPM and cytochrome *c* are approximately equimolar. MALDI-TOF data (Figure 3, Panel B) indicate product formation with the main peak shifted to the approximate expected range for NPM-cytochrome *c* bioconjugate. Luminescence data also confirm the formation of product (see supporting information). Yield of the crude product is estimated to be on the order of 30%, although based on previous results this should improve with increased reaction scale and the use of pure iso-1 cytochrome *c* for the starting material.

The luminescent emission of a test bioconjugation reaction between cytochrome *c* and NPM was measured, the results of which are shown in Figure 4. As expected, the NPM-cytochrome *c* bioconjugate product exhibits much higher luminescent emission than the unreacted precursors.

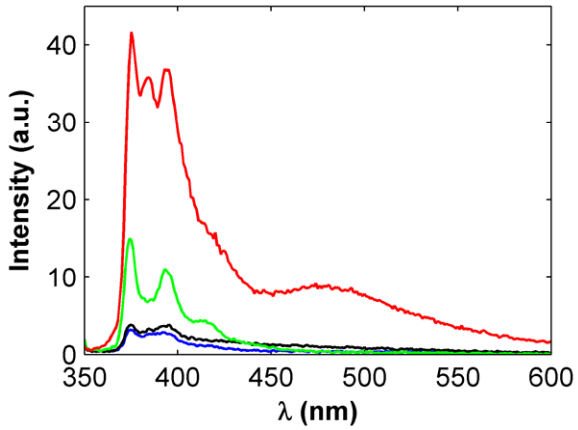

Figure 4. Luminescence emission of water (black), unreacted cytochrome *c* (blue), unreacted NPM (green) and crude NPM-cytochrome *c* bioconjugate (red). λ_{ex} = 342 nm.

Using empirical models to calculate DTDP and NPM reaction data. As indicated in the text, DTDP and NPM reaction data measured by UV-Vis and fluorescence, respectively, was modelled by rational functions in MATLAB to determine quantities such as initial rate, plateau yield (or intensity) and time to 90% of plateau yield (or intensity). Figure 5 shows typical results from an NPM bioconjugation reaction as well as the empirical model used to fit the data.

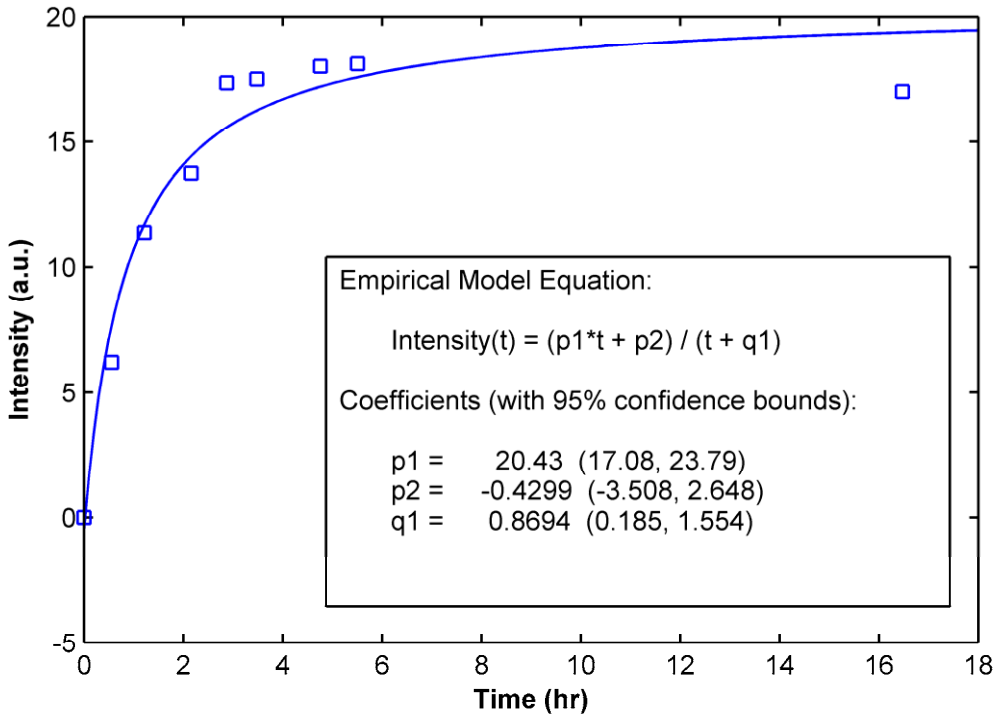

Figure 5. Typical reaction data from an NPM bioconjugation reaction and the empirical model generated with MATLAB.

The initial rate over the first two hours was determined by calculating the intensity at time zero (-0.494) and at two hours (14.1) and calculating the rate as (14.1 – (-0.494))/(2hr) = 7.3 hr^{-1}. Results were averaged for reactions performed in duplicate or triplicate and NPM results were normalized to the control reaction.

The plateau intensity of NPM reactions (or plateau yield for DTDP reactions) was taken to be equal to the calculated intensity at infinite time. From the model reaction shown in Figure 5, this is equal to p1, or 20.4. As above, results were averaged for reactions performed in duplicate or triplicate and NPM results were normalized to the control reaction.

The time to 90% of plateau intensity for NPM reactions (or yield for DTDP reactions) was determined by solving the equation shown in Figure 5 for time, t, with intensity equal to 90%

of the plateau intensity. For the example shown above, Intensity(t) = 18.4 and time = 8.0 hr. Results were averaged for reactions performed in duplicate or triplicate.

References:

1. J. Woodward, J. Tate, P. C. Herrmann and B. R. Evans, J. Biochem. Biophys. Met., 1993, **26**, 121-129.

2. M. Brinkley, *Bioconjugate Chem.*, 1992, **3**, 2-13.

3. G. Gorin, P. A. Martic and G. Doughty, *Arch. Biochem. Biophys.*, 1966, **115**, 593-597; C. C. Lee and E. R. Samuels, *Can. J. Chem.*, 1964, **42**, 168-170; J. D. Gregory, *J. Am. Chem. Soc.*, 1955, **77**, 3922-3923.