Chiang Mai J. Sci. 2009; 36(2) : 236-246
www.science.cmu.ac.th/journal-science/josci.html
Invited Paper

Optimising the Purification of Terpyridine-Cytochrome *c* Bioconjugates

Joshua R. Peterson [a, b] and 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.
*Author for correspondence; e-mail: p.thordarson@unsw.edu.au

ABSTRACT

The synthesis and purification of a terpyridine cytochrome *c* (**tpy-cyt**) bioconjugate is reported. Following previously reported procedures, crude cytochrome *c* was successfully purified by strong-cation exchange chromatography, however, this method was found to be unsuccessful for the purification of **tpy-cyt** bioconjugates. Similar results were obtained with weak-cation exchange chromatography. The results obtained also suggested that metal-coordination to the terpyridine ligand in **tpy-cyt** bioconjugates adversely affects their interaction with cation-exchange column media. Attempts to utilize the affinity of terpyridines for zinc(II) ions to form **tpy-cyt** dimer that could subsequently be purified by size-exclusion chromatography were unsuccessful, however, added further evidence to the role of metal-ion coordination to the chromatographic properties of these bioconjugates. Based on these results, a method using nickel(II) loaded immobilized metal-ion affinity chromatography (IMAC) was then successfully developed and employed to obtain the **tpy-cyt** bioconjugate in good purity. These results will be of significant importance to us and others that are developing redox-protein based bioconjugates that incorporate metal-chelating ligands, such as terpyridine, for application in bioelectronic devices.

Keywords: bioconjugate chemistry, cytochrome *c*, terpyridine, metalloterpyridine, protein purification, chromatography, FPLC, MALDI-TOF MS, Cation-exchange, IMAC, SEC, bioelectronics.

1. INTRODUCTION

Bioconjugate chemistry has recently become an intense area of research at the intersection of biotechnology, chemistry, and nanotechnology.[1-3] For applications in bioelectronics, redox proteins such as cytochrome *c*, are a particularly attractive target. Cytochrome *c* is one of the most heavily studied proteins in science[4,5] and can be obtained easily in large quantities. We have a particular interest in the iso-1 form of yeast cytochrome *c* (from *Saccharomyces cerevisiae*) as it has only one cysteine residue (CYS102)[6,7] making it an especially appealing target for bioconjugation using maleimide-appended ligands.[8] We recently utilised these properties of yeast cytochrome *c* in our synthesis of a Ruthenium(II)-bis(terpyridine) cytochrome *c* bioconjugate that could be activated by light as demonstrated by photophysical studies on this bioconjugate.[9] The key challenge in making this and related cytochrome *c* bioconjugates in our subsequent work towards

the application of these systems in bioelectronics is, however, the purification of these bioconjugates.

Despite the extensive literature on cytochrome *c*, the purification of cytochrome *c* bioconjugates has received very little attention. Chromatographic techniques for the purification of cytochrome *c* itself are mostly based on cation-exchange chromatography, undoubtedly due to the relatively large positive charge that cytochrome *c* possesses at neutral pH. Foucher and co-workers used a phosphate buffered (pH 7.0) sodium chloride gradient on Amberlite IRP 64 cation-exchange resin to separate not only the iso-1 and iso-2 forms of yeast cytochrome *c*, but also other minor species closely related to iso-1 cytochrome *c*.[10] Using this approach as starting point, we explored a number of chromatographic methods for the purification of a model terpyridine-cytochrome *c* bioconjugate **tpy-cyt**, including strong-cation, weak-cation and size-exclusion chromatography before demonstrating here that immobilised metal affinity chromatography seems to be the method of choice for the purification of terpyridine-cytochrome *c* bioconjugates such as **tpy-cyt**.

2. MATERIALS AND METHODS

2.1 Protein Chromatography

Protein and bioconjugate purification was performed using a GE Healthcare Akta Purifier. Cation exchange chromatography (CEX) was performed using either strong cation exchange column (Sigmachrom IEX-S or TSKgel SP-5PW, Supelco) or a weak cation exchange column (HiPrep 16/10 CM FF, GE Healthcare). Immobilized metal affinity chromatography (IMAC) was performed using either a HisTrap HP (1 mL) or a HisPrep FF 16/10 (20 mL, GE Healthcare, charged with Ni^{2+}). Size exclusion chromatography (SEC) was performed using either a Superdex 200 10/300 GL or a Superdex 75 10/300 GL (GE Healthcare). Flow rates were based on manufacturer recommendations for flow and pressure limits and were typically between 0.5 to 2 mL/min. Fractions were collected in 1 mL Eppendorf tubes using either a Frac 901 or Frac 902 (GE Healthcare). Protein solutions were concentrated using 5,000 molecular weight cut-off centrifuge concentrators (Amicon Ultra-15 or Amicon Ultra-4, Millipore) and centrifugation at 4000 rpm in a Sigma 2-6 bench top centrifuge. Samples were dialysed into Milli-Q water using Slide-A-Lyzer Mini Dialysis Units (3,500 molecular weight cut-off, Pierce). Small volume samples (1.5 mL) were centrifuged in a Sigma 1-14 bench top centrifuge.

2.2 Mass Spectrometry

LCMS-ESI was carried out on a Finnigan Mat SpectraSystem HPLC with a Finnigan LCQ-DECA electrospray mass spectrometry unit. MALDI-TOF mass spectra were recorded on either a Micromass Tof Spec 2E or an Applied Biosystems Voyager DE STR MALDI reflectron TOFMS (protein and bioconjugate measurements were made in linear mode). Protein and bioconjugate samples for MALDI were prepared by dilution (1:1) with either a saturated solution of sinapic acid and α-cyano-4-hydroxycinnamic acid (10:1 w/w ratio in acetonitrile/water/trifluoroacetic acid (70:30:0.03, v/v/v)) or a solution of caffeic acid (10 mg/mL in acetonitrile/water/trifluoroacetic acid (80:20:0.1, v/v/v)).

2.3 Cytochrome c Purification

Yeast cytochrome *c* from *Saccharomyces cerevisiae* was purchased from Sigma Aldrich (catalog numbers C-2436). Crude cytochrome *c* (12.0 mg) was diluted in 20 mM sodium dihydrogen phosphate, pH 7.0 (6 mL),

reduced with dithiothreitol (DTT, 192 μL of 200 mM stock solution), and loaded onto a Supelco SigmaChrom IEX-S strong cation exchange column (10 μm resin beads, 7.5 cmh x 0.75 cmd). The protein was eluted using a gradient from 200 mM to 500 mM sodium chloride in 33 mL at pH 7.0 and 2 mL/min. The main peak (eluting from 21.5 to 24.8 mL) was collected and concentrated using a Millipore 10,000 molecular weight cut-off (MWCO) centrifuge concentrator giving pure iso-1-cytochrome *c* in 85% yield based on UV-Vis absorbance of the crude load and the final product (ε_{410} = 9.76 x 10^4 M^{-1} cm^{-1}). MS (ESI) m/z 12,707 ($[M]^+$ requires 12,706). MS (MALDI) m/z 12,710 ($[M]^+$ requires 12,706).

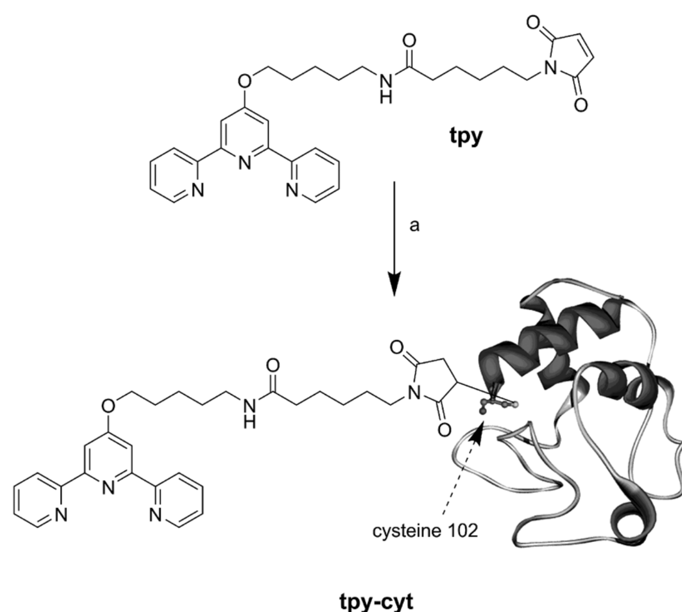
2.4 Bioconjugate Synthesis and Purification

The **tpy-cyt** bioconjugate was synthesised from purified cytochrome c and the previously reported 4'-(maleimide-alkyloxy)-2,2':6',2''-terpyridine **tpy** ligand.[11] A solution of 4'-(maleimide-alkyloxy)-2,2':6',2''-terpyridine (**tpy**) (1.1 mg, 200 x 10^{-5} mmol) in tetrahydrofuran (500 μL) was mixed with purified iso-1 cytochrome *c* (1.08 mg, 8.53 x 10^{-5} mmol) in 50 mM sodium dihydrogen phosphate, 15 mM ethylenediaminetetraacetic acid, 5% tetrahydrofuran, pH 7.0 (10 mL). The mixture was placed at 35 °C for 3 h, concentrated, dialyzed into water, then purified in 20 mM sodium dihydrogen phosphate, 0.5 M sodium chloride, pH 7.0 on immobilized metal affinity chromatography (IMAC, HisTrap HP, 1 mL, GE Healthcare) with an elution gradient from 0 to 250 mM imidazole in 15 mL at 0.5 mL/min. The product was collected between 3.8 and 8.4 mL, concentrated and dialysed into water yielding **tpy-cyt** (0.63 x 10^{-5} mmol, 7%). MS (MALDI) *m/z* 13,224 (with EDTA, $[M]^+$ requires 13,234). MS (MALDI) *m/z* 13,280 (without EDTA, $[M + Fe]^+$ requires 13,290).

3. RESULTS AND DISCUSSION

3.1 Bioconjugate Synthesis

Bioconjugate **tpy-cyt** was synthesised by reaction of the maleimide group of ligand



Scheme 1. Synthesis of **tpy-cyt**. (a) 8.5 μM iso-1 cytochrome *c*, 200 μM **tpy** ligand[11] 50 mM NaH_2PO_4, 15 mM EDTA, 5% THF, pH 7.0, 35 °C.

tpy[11] to the free cysteine (CYS102) of pure, reduced iso-1 cytochrome *c*. A solution of 4'-(maleimide-alkyloxy)-2,2':6',2"-terpyridine **tpy** in tetrahydrofuran was added to a solution of iso-1 cytochrome *c* such that the final reaction conditions were 8.5 μM iso-1 cytochrome *c*, 200 μM ligand **tpy** in 50 mM phosphate buffer, 15 mM ethylenediaminetetraacetic acid (EDTA), 5% tetrahydrofuran, pH 7.0. The mixture was stirred at 35 °C for 3 hours, concentrated, and dialyzed into water. (Scheme 1).

The crude mixture showed evidence of significant product formation by mass spectrometry (MALDI-TOF, data not shown). Following the work of Foucher and co-workers[10] we had successfully purified the crude cytochrome *c* itself by strong cation exchange chromatography (see section 2.3). The purification of the **tpy-cyt** bioconjugate was therefore first attempted by two forms of cation exchange (strong and weak), then by size-exclusion chromatography and finally by immobilized metal affinity chromatography (IMAC) with unexpected results.

3.2 Strong-cation Exchange Chromatography

The first method used for the purification of **tpy-cyt** bioconjugate was strong cation exchange chromatography using a Sigma Chrom IEX-S column. It was expected that the well-established behaviour of iso-1 cytochrome *c* on this form of column chromatography would make interpretation of the chromatograms more straightforward. A sample of crude **tpy-cyt** was run on SigmaChrom IEX-S using a gradient from 200 to 480 mM sodium chloride over 33 mL in 20mM phosphate buffer, pH 7.0 at 2 mL/min (Figure 1).

Small peaks were present in the region of typical cytochrome *c* elution (15 to 25 mL), but unexpectedly, the bulk of the bioconjugate mixture eluted very late in the gradient, even tailing into the 1 M sodium chloride

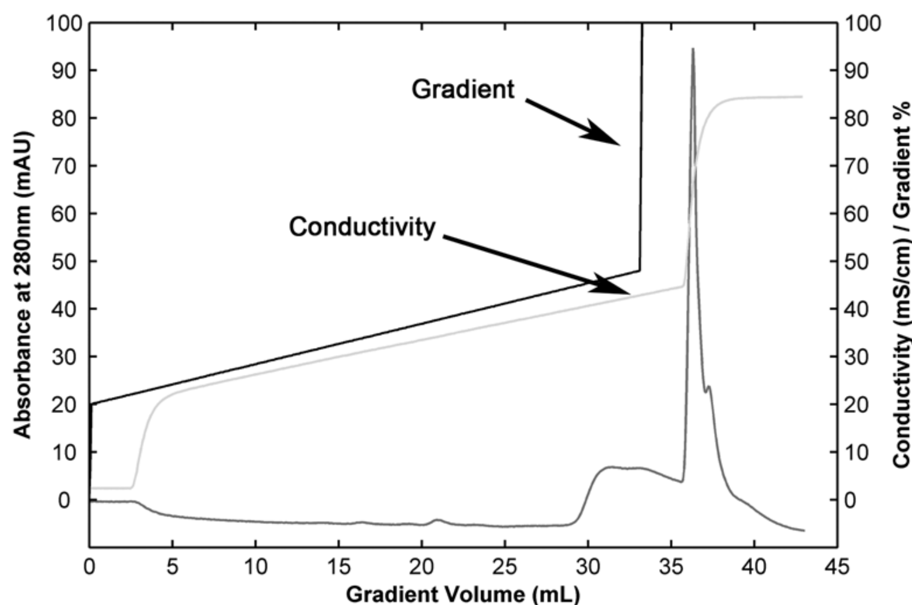


Figure 1. Initial purification of crude **tpy-cyt** on SigmaChrom IEX-S. Protein eluted with a gradient from 200 to 480 mM NaCl over 33 mL in 20 mM NaH_2PO_4, pH 7.0 at 2 mL/min. The gradient and conductivity are also indicated for illustration.

regeneration step. The entire peak from 30 to 43 mL was pooled, concentrated, and buffer exchanged into 20mM phosphate buffer, pH 7.0. A small portion of the sample was analysed by HPLC-MS (ESI) and appeared to be a pure species with a mass of 13,296 which could correspond to bioconjugate **tpy-cyt** with a coordinated copper ion being bound by the terpyridine ligand. Copper-terpyridine complexes have been reported in the literature,[12] although it would seem more reasonable to detect sodium or iron complexes given the abundance of these metals and their likely introduction to the sample during purification and mass spectrometry. Regardless, a bound metal ion would explain why the bioconjugate product required a much higher salt concentration for elution than would be expected for iso-1 cytochrome *c*. The remainder of the product collected above was then re-injected onto the same cation exchange column and eluted with a different gradient – 100 to 1000 mM sodium chloride in 50 mL – in an attempt to improve the purification method (Figure 2).

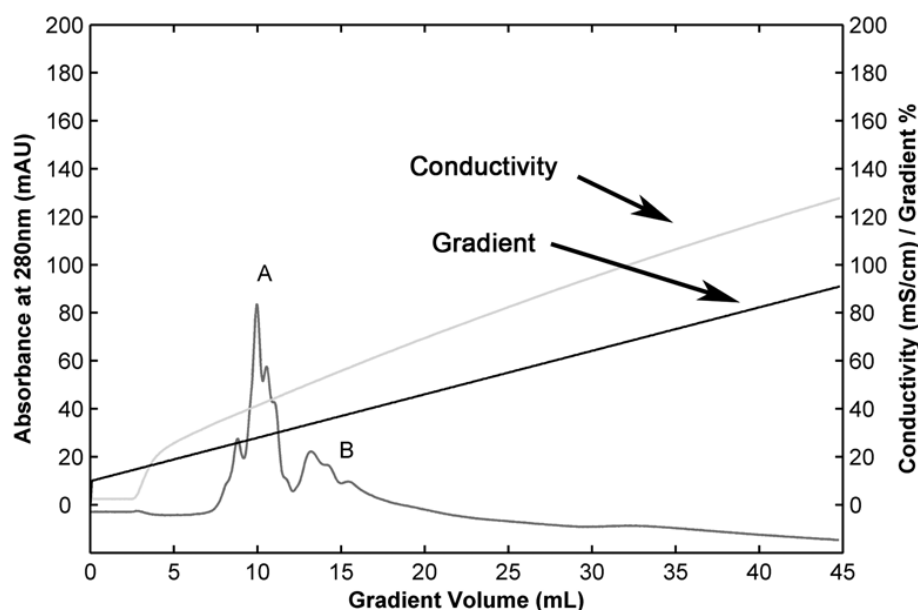


Figure 2. Re-injection of purified **tpy-cyt** on SigmaChrom IEX-S. Protein eluted with a gradient from 100 to 1000 mM NaCl over 50 mL in 20 mM NaH_2PO_4, pH 7.0 at 2 mL/min. The gradient and conductivity are also indicated for illustration.

As seen previously, the bioconjugate sample elutes slowly and extends into a relatively high concentration of sodium chloride in the eluent. In addition, there is little resolution between the peaks detected, and the peaks themselves are unexpected given that the previous mass spectrometry results indicated that the material was fairly pure. Mass spectrometry analysis of this second attempt indicated that Peak A in Figure 2 (the main peak eluting at 10 mL) consisted of a product with mass 12,918 (which could not be identified) and Peak B (the peak eluting at *ca.* 13 mL) contained a product with mass 13,296 (possibly corresponding to bioconjugate **tpy-cyt** with a bound copper ion).

3.3 Weak-cation Exchange Chromatography

Given the unusual behaviour seen on the strong cation exchange column, an attempt was also made to purify bioconjugate **tpy-cyt** with a weak cation exchange column, HiPrep 16/10 CM FF (GE Healthcare). A crude reaction mixture was loaded onto the column and eluted with a gradient from 100 to 500 mM sodium chloride over 160 mL in 20 mM phosphate buffer, pH 6.8 (Figure 3).

While several peaks were eluted, analysis of the main peaks (Peaks A, B, and C in Figure 3) by HPLC-MS (ESI) indicated that a species of 13,295 was present throughout the elution. Peak A included another species at 12,931 that could not be identified. Peak C included the desired product at 13,234 and a product at 13,290, which is likely the desired bioconjugate with an iron ion coordinated to the terpyridine ligand.

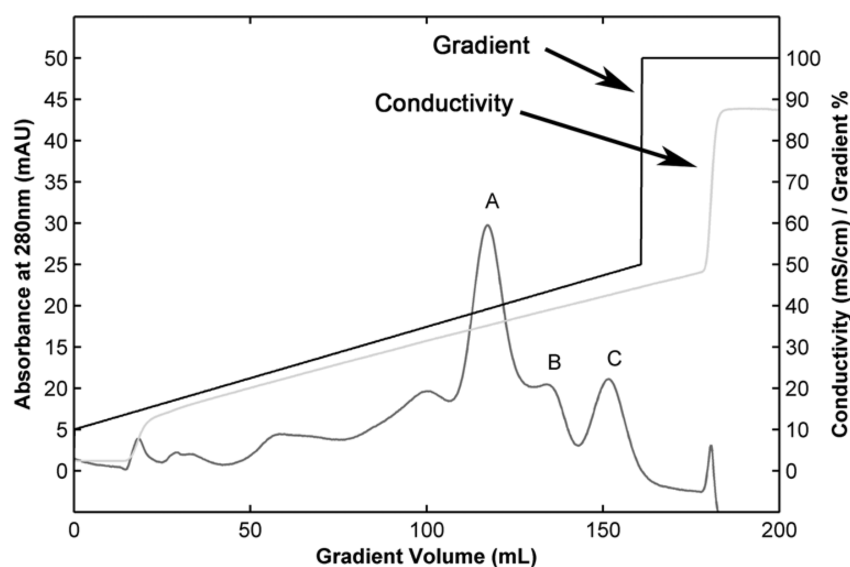


Figure 3. Purification of **tpy-cyt** on weak cation exchange (HiPrep 16/10 CM FF, GE Healthcare). Protein eluted with a gradient from 100 to 500 mM NaCl over 160 mL in 20 mM NaH_2PO_4, pH 6.8. Peaks A, B, and C all indicate presence of bioconjugate by mass spectrometry. The gradient and conductivity are also indicated for illustration.

In general, the results indicated that purification of bioconjugate **tpy-cyt** by cation exchange chromatography would likely be difficult as the elution behaviour appeared to be significantly affected by the binding of metal ions to the free terpyridine ligand. This makes analysis of the chromatograms, pooling of the correct product-containing fractions, and interpretation of the reaction outcomes challenging. For these reasons, cation exchange chromatography was abandoned as a method for the purification of **tpy-cyt** bioconjugates.

3.4 Size-exclusion Chromatography

Given the difficulties using cation exchange chromatography, another mode of chromatography, size exclusion chromato-graphy (SEC), was attempted. Normally, this would not be the method of choice as there is very little difference in the masses of iso-1 cytochrome *c* and the **tpy-cyt** (12,707 as compared to 13,235). Resolution of species

by SEC typically requires a substantial mass difference, even for the most efficient columns. Little and co-workers had reported the separation of cytochrome *c* monomer and dimer (molecular weights of *ca.* 12,707 and 25,414) using SEC in 1967, although the resolution of the two species was not complete.[13] However, modern size exclusion columns can separate cytochrome monomer and dimer, and could theoretically work in this case if the bioconjugates could be coupled together to form a species roughly the same mass as the dimer. It was thought that since terpyridine ligands bind metal ions, then introducing a metal ion may lead to dimerisation of the bioconjugate while unreacted cytochrome *c* remains as monomer. Zinc(II) ions were chosen based on the published reports that terpyridine binds Zn^{2+} ions to form bis-complexes that can be observed by UV-Vis absorbance.[14,15] In addition, zinc(II) ions bind to terpyridine more weakly than other metals such as iron(II) or nickel(II).[16] There was a perceived trade-off in regard to the binding strength – too weak and the dimer complex may not be formed or held together during the size exclusion process, but too strong and it may be difficult to remove the metal from the bioconjugate for analysis and future use. A sample of **tpy-cyt** was diluted in 20 mM phosphate buffer, 1 mM $ZnCl_2$, pH 5.0 and a shift in the UV-Vis spectra was seen, although the increase in absorbance was seen at 316 nm (data not shown). The sample was run on a Superdex 200 10/300 GL (GE Healthcare) size exclusion column in 20 mM phosphate buffer, 1 mM $ZnCl_2$, pH 5.0 (Figure 4).

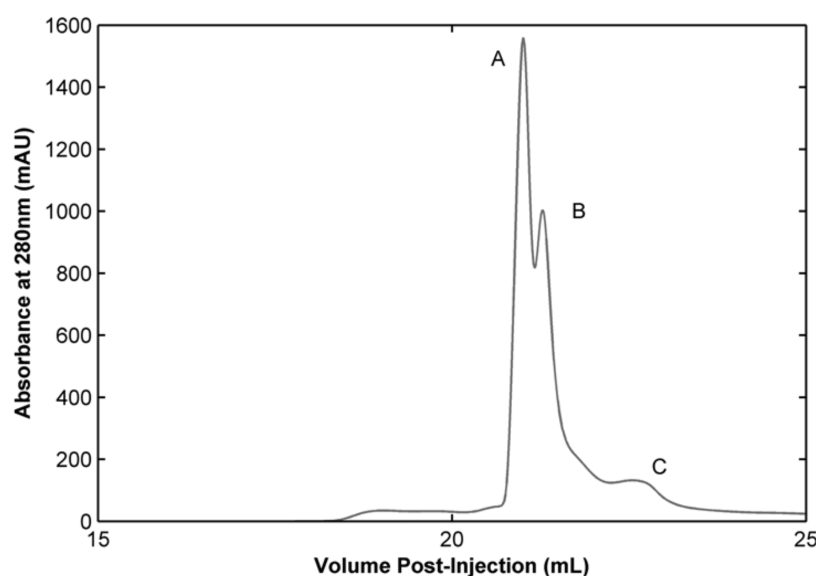


Figure 4. Purification of **tpy-cyt** by size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare) in 20 mM NaH_2PO_4, 1mM $ZnCl_2$, pH 5.0. Analysis of peaks A, B, and C indicate increasing relative amounts of bioconjugate compared to that of unreacted iso-1 cytochrome *c*.

The peaks eluted (A, B, and C in Figure 4) were analysed by MALDI-TOF mass spectrometry and were shown to be mixtures of unreacted iso-1 cytochrome *c* (12,715 mass units), bioconjugate **tpy-cyt** (13,245 mass units), and **tpy-cyt** coordinated with a zinc ion (13,309 mass units, Panel A in Figure 5 below). In addition, the relative abundance of

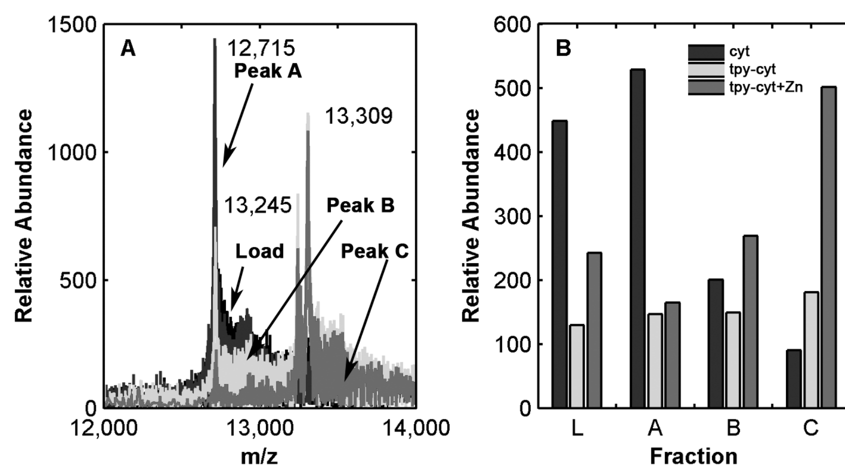


Figure 5. Analysis of SEC purification of **tpy-cyt**. Panel A – MALDI-TOF of load material (black), peak A (drak gray), peak B (light gray), and peak C (gray). Panel B – relative abundance of iso-1 cytochrome c (left bar), **tpy-cyt** (middle bar) and **tpy-cyt** + Zn^{2+} (right bar) in the crude load (L), and peaks A, B and C.

the three species indicated that peak C contained more bioconjugate than peak A (Panel B in Figure 5). This is unexpected as the largest species typically elute earlier on size exclusion chromatography. The result indicates that either the bioconjugate and the bioconjugate coordinated with zinc(II) have a smaller hydrodynamic radius in solution, thus behaving like a smaller protein than native iso-1 cytochrome *c*, or there is a weak non-specific absorption between the bioconjugate and the resin which retards its elution.

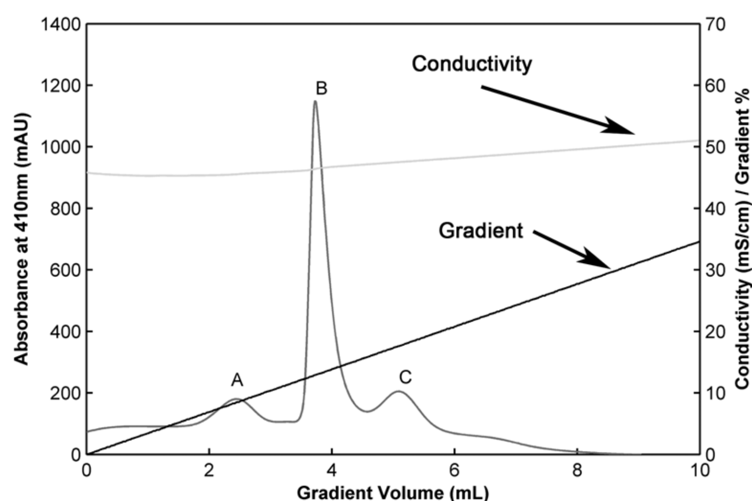


Figure 6. Purification of **tpy-cyt** by IMAC chromatography (HisTrap HP, GE Healthcare) using a gradient from 0 to 250 mM imidazole in 20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.0 in 15 mL at 0.5 mL/min. Peak A – iso-1 cytochrome *c* (2 – 3 mL). Peak B – bioconjugate **tpy-cyt** (3.5 – 4.5 mL). Peak C – unidentified **tpy-cyt** + metal ion. The gradient and conductivity are also indicated for illustration.

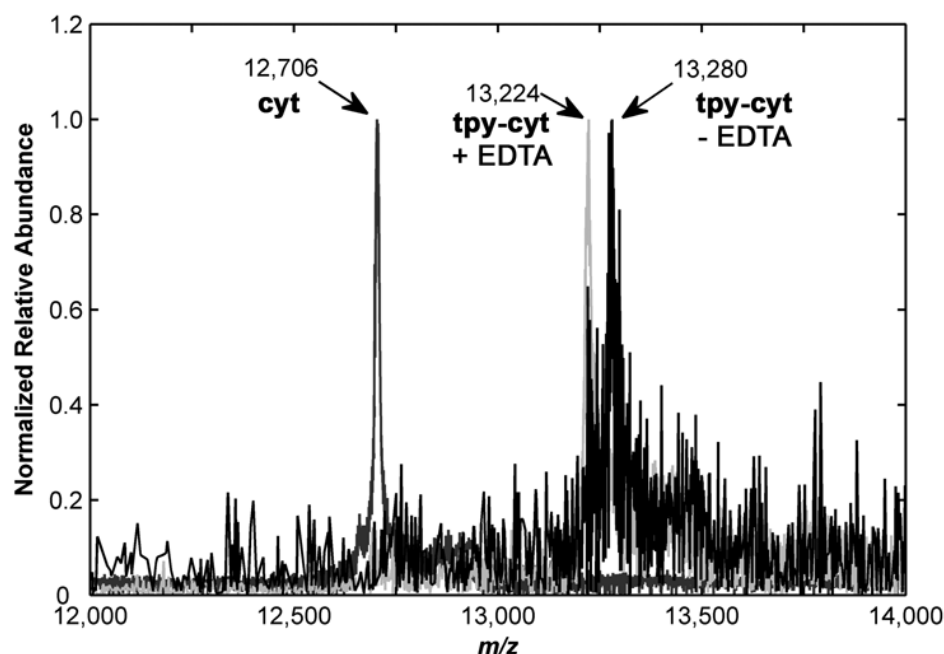


Figure 7. MALDI-TOF of bioconjugate **tpy-cyt** with EDTA (+EDTA, [**tpy-cyt**]$^+$ expected 13,234) and without EDTA (-EDTA, [**tpy-cyt** + Fe]$^+$ expected 13,290). The spectrum of iso-1 cytochrome *c* (**cyt**, expected 12,707) is shown for comparison.

3.5 Immobilized Metal Affinity Chromatography

While the findings were interesting, the resolution was not sufficient to warrant further attempts at using SEC for the purification of the **tpy-cyt** bioconjugate. The unexpected behaviour of the zinc-metal coordinated **tpy-cyt** on a SEC column prompted us to investigate immobilized metal affinity chromatography (IMAC) as a method to purify **tpy-cyt** as it has the ability to separate species based on the ability to chelate to a metal ion (typically Ni^{2+}) . It was believed that the terpyridine moiety in product **tpy-cyt** would bond more strongly to the Ni^{2+} ions than unmodified cytochrome *c*. It is important to note that the reaction mixture must be extensively dialysed into water to remove EDTA prior to purification on IMAC as residual EDTA will interfere with the purification. The dialysed mixture was loaded on to a IMAC column ((HisTrap HP, 1 mL, GE Healthcare) and purified in 20 mM phosphate buffer, 0.5 M sodium chloride, pH 7.0 with an elution gradient from 0 to 250 mM imidazole in 15 mL at 0.5 mL/min (Figure 6).

Analysis by mass spectrometry (MALDI-TOF) indicated that the initial peak (Peak A in Figure 6) was unmodified iso-1 cytochrome *c*, Peak B was product to **tpy-cyt** coordinated to an iron ion, and Peak C could not be conclusively identified, although it was believed to be the desired product **tpy-cyt**, but possibly in a different oxidation state. The eluate was collected between 3.8 and 8.4 mL (Peaks B and C in Figure 6), concentrated and dialysed into water to afford **tpy-cyt** in 7% yield. Mass spectrometry data supported the idea of metal ion coordination by indicating that this product was a species of mass 13,280 in the absence of EDTA (the iron coordinated species is calculated to be 13,289) and 13,2 24 in the presence of EDTA (**tpy-cyt**

alone is calculated to be 13,234). The results shown in Figure 7 indicate that the dialysis with EDTA has reduced the measured mass of **tpy-cyt** by 56 mass units, which corresponds to a bound iron ion.

4. Conclusions

The synthesis and purification of the terpyridine cytochrome *c* bioconjugates **tpy-cyt** has been successfully completed after an extensive study into the optimal method for the purification of this bioconjugate using immobilised metal affinity chromatography (IMAC). Contrary to yeast cytochrome *c* itself, strong-cation exchange chromatography was not capable of separating **tpy-cyt** from the crude protein mixture obtained from the bioconjugation reaction. Weak-cation exchange chromatography and size-exclusion chromatography were also equally unsuccessful for this task; however, the rather unusual chromatographic profiles obtained by the latter technique for zinc-complexed **tpy-cyt** did suggest that metal-ligand interactions were the key for a sucessfula separation technique for these bioconjugates. This was then confirmed by the successful purification of **tpy-cyt** by IMAC. It was also noted that the **tpy-cyt** bioconjugates bind strongly to various metal ions as evident by MALDI studies in the absence and presence of EDTA. The results obtained here will be of significant value for further studies on the chemical modification of redox-active proteins such as cytochrome *c*, especially with metal-chelating ligands including the metallo-terpyridine but metallo-terpyridine cytochrome *c* bioconjugates and related compounds have a number of potential application in the area of light-activated bioelectronic devices.[9]

Acknowledgements

This work was supported by the Australian Research Council (ARC DP0666325) and an ARC Australian Research Fellowship to PT. JRP was funded by the EIPRS and IPA scholarships from The University of Sydney.

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