

Video Article

Synthesis of Protein Bioconjugates via Cysteine-maleimide Chemistry

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Abstract

The chemical linking or bioconjugation of proteins to fluorescent dyes, drugs, polymers and other proteins has a broad range of applications, such as the development of antibody drug conjugates (ADCs) and nanomedicine, fluorescent microscopy and systems chemistry. For many of these applications, specificity of the bioconjugation method used is of prime concern. The Michael addition of maleimides with cysteine(s) on the target proteins is highly selective and proceeds rapidly under mild conditions, making it one of the most popular methods for protein bioconjugation.

We demonstrate here the modification of the only surface-accessible cysteine residue on yeast cytochrome c with a ruthenium(II) bisterpyridine maleimide. The protein bioconjugation is verified by gel electrophoresis and purified by aqueous-based fast protein liquid chromatography in 27% yield of isolated protein material. Structural characterization with MALDI-TOF MS and UV-Vis is then used to verify that the bioconjugation is successful. The protocol shown here is easily applicable to other cysteine - maleimide coupling of proteins to other proteins, dyes, drugs or polymers.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54157/>

Introduction

Bioconjugation involves covalently linking one biomolecule with another or with a synthetic molecule such as a dye, drug or a polymer. Protein bioconjugation methods are now extensively used in many chemistry, biology and nanotechnology research groups with applications ranging from fluorescent dye labelling^{1,2}, making of protein (antibody)-prodrugs³ (antibody drug conjugates — ADCs) synthesis of protein dimers^{4,5}, through to self-assembling protein-polymer hybrids^{6,7} used in nanomedicine⁸ and systems chemistry⁹.

Specificity of the chemistry used for bioconjugation, while not always critical, is of utmost importance for most functional protein bioconjugates, so as to not interfere with the active site of the target protein. The ideal bioconjugation reaction needs to fulfill several criteria, including: i) targeting rare or unique sites on the protein of interest, ii) be selective towards this target, iii) proceed under non-denaturing conditions to avoid protein unfolding and iv) be high-yielding as the target protein is usually only available at sub-millimolar concentration. The maleimide - cysteine Michael addition comes close to fulfilling all these criteria, and has for that reason long claimed a special status in the field of bioconjugate chemistry¹⁰. This is because i) many proteins containing only one cysteine residue on their surface can be genetically engineered there, ii) at the correct pH the reaction is highly selective towards cysteine, iii) it proceeds smoothly in aqueous buffers and iv) it is very fast with the second order rate constant of maleimides to cysteine-containing proteins reported to exceed 5,000 M⁻¹ sec⁻¹ in some cases¹¹. Provided the protein of interest can tolerate a small ($\approx$ 5-10%) amount of organic co-solvent¹², almost any maleimide-functionalized dye, polymer, surface or another protein can be linked to proteins. In addition, maleimides are more specific for cysteines on proteins than iodoacetamides, which are more prone to reacting with other nucleophiles at elevated pH; and more stable than disulfide-based conjugations which need to be kept at acidic pH to prevent disulfide exchange¹³.

Here we report a generic protocol for the conjugation of maleimide-functionalized molecules to a protein containing a single cysteine residue using the reaction between a Ru(II)-based chromophore and the redox protein cytochrome c as an example. This protocol is equally applicable to most other proteins containing an accessible surface cysteine residue and the corresponding maleimide-functionalized target, be it another protein, a fluorescent dye, a chromophore or a synthetic polymer.

Protocol

Note: The following protocol is designed for the synthesis of a protein-dye bioconjugate as shown in **Figure 1**. It is a general protocol for the reaction of a maleimide with free surface cysteine containing proteins, with notes inserted where applicable to assist with membrane protein

bioconjugates, protein-polymer bioconjugates, and synthetic protein dimer (protein-protein) bioconjugates. In this particular case, the protein iso-1 cytochrome *c* has one surface cysteine residue available to react which allows a highly specific labelling to occur. If a protein of interest has multiple cysteine residues, the same protocol applies, albeit with the loss of specificity and product homogeneity. Chemistry targeting surface lysine residues, using *N*-hydroxysuccinimidyl esters or isothiocyanates, may be a simpler approach if specificity is not required.

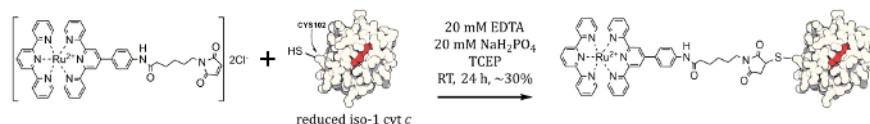


Figure 1. Bioconjugation Reaction Scheme. As an example case, a light harvesting, ruthenium-based antenna molecule will be attached to cytochrome *c* via Michael addition of a pendant maleimide on the ruthenium-based antenna molecule and an exposed cysteine residue (CYS102) on the protein. The red area of the cyt *c* surface indicates the heme group. [Please click here to view a larger version of this figure.](#)

1. Purification of Cytochrome *c*

Note: This step is not applicable to all proteins. However, it is important to know that a protein obtained from a commercial supplier can contain other, undesired protein isoforms which may need to be removed by further purification¹³.

- Dissolve 2.4 g of sodium dihydrogen phosphate ($M_w = 120 \text{ g mol}^{-1}$) in 1 L of ultrapure water to prepare a buffer solution containing 20 mM NaH_2PO_4 . Adjust the pH with 1 M NaOH to pH 7.
Note: The phosphate buffers used in this protocol should be prepared freshly on a daily basis, and filtered through a 0.2 μm cellulose membrane filter prior to use.
- Dissolve 29.22 g of sodium chloride ($M_w = 58.44 \text{ g mol}^{-1}$) in 500 ml of the 20 mM NaH_2PO_4 buffer to make a 20 mM NaH_2PO_4 and 1 M NaCl buffer. This is the elution buffer for the purification in step 1.6).
- Dissolve 12.0 mg of lyophilized cytochrome *c* (cyt *c*) in 6 ml of pH 7 20 mM phosphate buffer.
- Separately dissolve 14.7 mg of dithiothreitol (DTT, $M_w = 154.25 \text{ g/mol}$) in 95.3 μl of ultrapure water to prepare a 1 M stock solution.
Note: The DTT stock solution should be prepared freshly, as this reagent is susceptible to oxidative deactivation in aqueous solution.
- Pipette 60 μl of the 1 M DTT solution into the protein solution to reduce the cyt *c*. The color of the solution will change from dark red to light red upon mixing.
- Filter the reduced protein solution through a 0.22 μm low protein binding PVDF syringe filter before injecting onto any chromatographic media.
- Attach a 3.3 ml strong cation exchange column between the injection loop and UV-Vis detector of a Fast Protein Liquid Chromatography (FPLC) instrument.
- Equilibrate the column with 3 column volumes of ultrapure water, followed by 3 column volumes of 20 mM pH 7 phosphate buffer, using a flow rate of 1 ml/min.
- Load 1 ml of reduced crude cyt *c* into the injection loop, and start a gradient method from 328 - 450 mM NaCl, over 5 column volumes. Monitor the 280 nm and 410 nm channels of the UV-Vis detector, and collect the largest peak.
- Increase the salt concentration to 1 M for 2 column volumes to elute iso-2 cytochrome *c*, and after the column has been flushed, re-equilibrate the column with 2 column volumes of 20 mM phosphate buffer before injecting the next crude aliquot.
- Repeat steps 1.9-1.10 until all the crude protein has been purified.
- Pool the iso-1 containing fractions and concentrate them using a 3.5 kDa Molecular Weight Cutoff (MWCO) spin filter and centrifuging at 3,000 $\times g$.
- Load the concentrated protein into 3.5 kDa MWCO dialysis cassettes and dialyze against ultrapure water overnight, with 2 changes of water.
- Determine the concentration of the pure, concentrated protein solution by taking 10 μl , diluting it to 100 μl with ultrapure water, and taking an absorbance spectrum. Use a low volume, 100 μl quartz cuvette to obtain protein absorbance spectra. Typically, the undiluted protein concentration is on the order of 50 - 100 μM .
 - Use the characteristic 410 nm peak of cyt *c* to quantify the concentration using the Beer-Lambert law with a molar absorptivity value of $97.6 \text{ cm}^{-1} \text{ mM}^{-1}$:
$$A = \epsilon \times c \times l$$
where *A* is the absorbance, ϵ is the molar absorptivity, *c* is the concentration in mM, and *l* is the path length of the cuvette in cm¹³.
- At this point, divide the protein into 1 ml aliquots and keep frozen at -20 °C until they are needed. Cyt *c* can be frozen and thawed without loss of structure or function, but repeat cycles will begin to denature the protein.
Note: Proteins tolerate freezing to different degrees. For example, green fluorescent proteins^{5,9} should not be frozen, instead stored in a refrigerator.

2. Synthesis of Cytochrome *c* Bioconjugates

- Dissolve 0.9 mg of ruthenium(II) bisterpyridine maleimide ($\text{Ru(II)}(\text{tpy})_2\text{-maleimide}$, 0.975 μmol , 6 equivalents) in 600 μl of acetonitrile.
Note: If the maleimide used is soluble in water, prepare a stock solution in water instead of acetonitrile. It is important for the maleimide to be soluble in the reaction buffer. Dimethylsulfoxide, *N,N*-dimethylformamide, and acetonitrile are all commonly used adjuvants to assist in the dissolution of low molecular weight¹², often hydrophobic maleimide reactants.
- Prepare a buffer solution containing 100 mM phosphate buffer and 100 mM ethylenediaminetetraacetic acid (EDTA, $M_w = 292.24 \text{ g/mol}$), which when diluted to 20 mM is at pH 7. Add solid sodium hydroxide until the EDTA is dissolved (several grams typically) and adjust the pH to 7.
- Dissolve 2.87 mg of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP¹⁵, $M_w = 286.65 \text{ g/mol}$) in 1 ml of ultrapure water to prepare a 10 mM stock solution. This step is important to ensure that the cysteine on the protein is fully reduced prior to maleimide coupling¹⁵.

Note: The TCEP stock solution should be prepared freshly for each reaction, as this reagent is susceptible to oxidative deactivation in aqueous solution.

4. Combine 11.4 ml of ultrapure water with 3 ml of 100 mM phosphate/EDTA stock solution in a 50 ml plastic tube. When diluted further with protein and maleimide stock solutions the buffer concentration will be 20 mM.

Note: If the protein being labelled is a transmembrane protein, ensure that a suitable detergent is added to the buffer to solubilize the protein. If a detergent is not used the membrane protein may slowly precipitate, which will negatively impact reaction yields. Use the detergent in all subsequent purification steps.

5. Add 0.15 μmol (3 ml of a 50 μM solution, 1 equivalent) of purified cyt c to the phosphate-EDTA buffer.
6. Add 7.5 μl of the TCEP stock solution (0.075 μmol , 0.5 equivalents) to the protein solution and leave stirring for 5 min to reduce any protein that may have dimerized due to cysteine oxidation.
Note: Do not add TCEP if the protein of interest does not dimerize readily. Check this by running a protein gel under non-reducing conditions.
7. Add 600 μl of the acetonitrile solution of Ru(II) (tpy)₂-maleimide to the reduced, buffered solution of cyt c, and leave the reaction mixture stirring, in the dark, at RT, for 24 hr.
8. Concentrate the reaction mixture using 3.5 kDa MWCO spin filters, centrifuging at 3,000 x g, repeating 2-3 times with fresh 20 mM phosphate buffer until the filtrate runs clear. Remove as much unreacted maleimide from the reaction mixture as possible before the next stage of purification.

Note: At this point, the crude bioconjugation mixture can be stored in the refrigerator, in the dark. It is important to remove unreacted maleimide dye by centrifuge dialysis before storage as the maleimide can slowly and nonspecifically react with lysine residues on the surface of the protein.

3. Purification of Cytochrome c Bioconjugates

1. Dissolve 2.4 g of sodium dihydrogen phosphate and 29.22 g of sodium chloride in 1 L of ultrapure water to prepare a buffer solution containing 20 mM NaH₂PO₄ and 0.5 M NaCl. This is the running buffer for the immobilized metal-affinity chromatography (IMAC) purification.
2. Dissolve 17 g of imidazole ($M_w = 68.077 \text{ g mol}^{-1}$) in 500 ml of the 20 mM NaH₂PO₄ and 0.5 M NaCl buffer. This is the elution buffer for the IMAC purification.
3. Adjust the pH of both buffers to pH 7 using 1 M NaOH or HCl, and filter them through 0.22 μm regenerated cellulose membranes before use in the FPLC.
4. As the IMAC columns purchased are shipped without any metal ions loaded onto the column, prepare the column for a Ni²⁺-based purification by washing the column with 3 ml of ultrapure water, 3 ml of 100 mM nickel acetate solution, and 6 ml of ultrapure water. If the column is not to be used immediately wash through 3 ml of 20% ethanol and store the column in the refrigerator to prevent bacterial growth.
5. Attach a Ni²⁺-loaded 1 ml IMAC column to the FPLC between the injection loop and UV-Vis detector.
6. Equilibrate the column with 3 column volumes of 20 mM phosphate, 0.5 M NaCl buffer using a flow rate of 0.5 ml/min.
7. Load 100 μl of crude reaction mixture (filtered through a 0.22 μm syringe filter) on the Ni²⁺ IMAC column. Wash the column with 3 column volumes of running buffer to elute non-reacted cyt c, followed by an imidazole gradient from 0 to 125 mM over 3 column volumes to elute the ruthenium bisterpyridine-cytochrome c bioconjugate (Ru(II)-cyt c).
8. Wash the column with 250 mM imidazole buffer for 5 column volumes, then re-equilibrate the column with 20 mM phosphate, 0.5 M NaCl and repeat step 3.7 until all the crude bioconjugate has been purified.
9. Pool the bioconjugate fractions and concentrate them using a 3.5 kDa MWCO spin filter, centrifuging at 3,000 x g.
10. Load the concentrated bioconjugate into 3.5 kDa MWCO dialysis cassettes and dialyze against ultrapure water overnight, with 2 changes of water.
11. Determine the concentration of the pure, concentrated bioconjugate solution by UV-Vis, using the same molar absorptivity value as iso-1 cytochrome c (97.6 $\text{mM}^{-1} \text{cm}^{-1}$) at 410 nm. Typically, the bioconjugate concentration is on the order of 50 - 100 μM .
12. Divide the bioconjugate into 25 μl aliquots and keep frozen at -20 °C until they are needed.

4. Characterization of Cytochrome c Bioconjugates

1. Determination of bioconjugate mass by MALDI-TOF MS

1. Dissolve 10 mg of caffeic acid in 1 ml of an acetonitrile/water/trifluoroacetic acid solution (80:20:0.1, v/v/v).
2. Dilute 5 μl of concentrated protein solution with 5 μl of caffeic acid solution.
3. Spot 0.5 μl of caffeic acid solution on the MALDI target plate and allow the solution to dry.
4. Spot 0.5 μl of the sample/matrix solution on top of this caffeic acid spot, allow the spot to dry. Spot another 0.5 μl of sample matrix on top of this to "sandwich" the sample between layers of matrix, and allow to dry.
5. Acquire mass spectra in linear mode using suitable instrument settings for proteins¹⁶.

2. Investigation of bioconjugates by gel electrophoresis

1. Prepare 10 μl of each protein sample to be run by diluting protein samples with premixed lithium dodecyl sulfate (LDS, pH 8.4) buffer (4x) to a final concentration of approximately 20 μg per well. For wells that require reducing conditions, add 1 μl of 500 mM dithiothreitol (DTT) reducing agent (10x).
2. Heat samples at 70 °C for 10 min.
3. Add 50 ml of premixed 1 M MES, 1 M Tris base, 2% SDS, 20 mM EDTA (pH 7.7) running buffer mixture (20x) from a commercial source to 950 ml of ultrapure water to prepare the gel running buffer.
4. Remove the plastic comb from the gel and place the gel in the gel running tank. Fill up the tank with gel running buffer so that the wells are covered with buffer.
5. Load samples carefully onto a precast 12% Bis-Tris, 1 mm, 10-well gel using long pipette tips to assist in loading. Load the prestained 10 protein molecular weight marker (3-188 kDa) into a middle well of the gel to aid analysis.
6. Run the gel at 200 V constant voltage for 35 min.
7. Stain the gel with a commercial Coomassie blue solution for 2 hr, and wash with ultrapure water for 48 hr.

3. Determination of bioconjugate purity by UV-Vis spectroscopy

1. Prepare 120 μ l of 5 μ M solutions of Ru(II) (tpy)₂-maleimide, iso-1 cyt c, and Ru(II)-cyt c.
2. Measure the baseline spectrum of the quartz cuvette containing only ultrapure water, from 250 nm to 650 nm.
3. Measure the spectrum of each component, ensuring the cuvette is rinsed and dried between each measurement.
4. Plot the absorbance of each component as a function of wavelength, and compare the linear sum of the starting materials with the final product to determine if a 1:1 ratio of Ru(II) (tpy)₂-maleimide to cyt c has reacted.

Representative Results

The synthesis of bioconjugates is confirmed by three primary methods: Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), polyacrylamide gel electrophoresis, and Ultraviolet-Visible (UV-Vis) spectroscopy, as shown in **Figures 2, 3 and 4**. A mass increase corresponding to the mass of the appended small molecule, and the lack of an unreacted protein demonstrates the successful covalent linkage of Ru(II) (tpy)₂-maleimide to cyt c and subsequent purification of the bioconjugate. The UV-Vis spectrum of the bioconjugate allows the yield to be calculated with the absorbance at 410 nm, and by comparing the spectrum to a predicted 1:1 addition spectrum of the starting materials the composition of the bioconjugate can be inferred. In the example above, the yield varies somewhat from batch-to-batch but it generally between 15-27% after FPLC purification¹⁵.

In addition, the appearance of a new peak in the chromatogram during purification confirms the synthesis of a new species. This is exemplified in **Figure 5**, where analysis of the different UV-Vis traces can indicate whether or not a species contains the Ru(II) (tpy)₂-maleimide component.

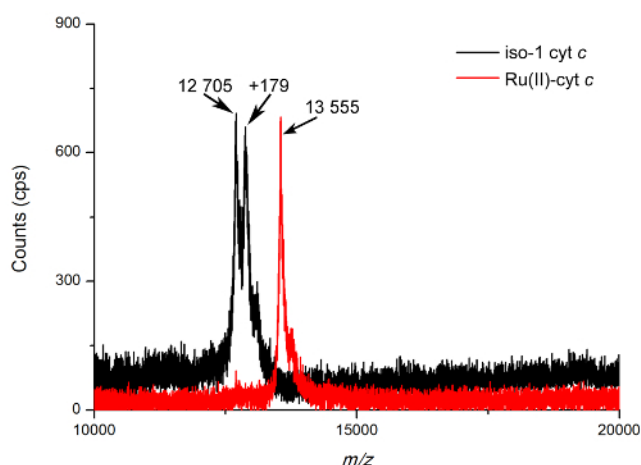


Figure 2. MALDI-TOF Mass Spectra of Proteins. Mass spectra of pure iso-1 cytochrome c (black) and Ru(II)-cyt c (red). Peaks can be observed that correspond to the calculated masses of iso-1 cyt c (12,706 Da) and Ru(II)-cyt c (13,559 Da) with a caffeic acid adduct visible at +179 Da. This adduct is seen in higher amounts in the unreacted cyt c spectra due to a reaction between the α , β -unsaturated carbonyl of caffeic acid and the free thiol of the protein under high-energy MALDI conditions. Matrix adducts are commonly seen in MALDI-MS and can be both covalent and non-covalent in nature. Spectra are baseline corrected, noise filtered, and normalized for comparison. [Please click here to view a larger version of this figure.](#)

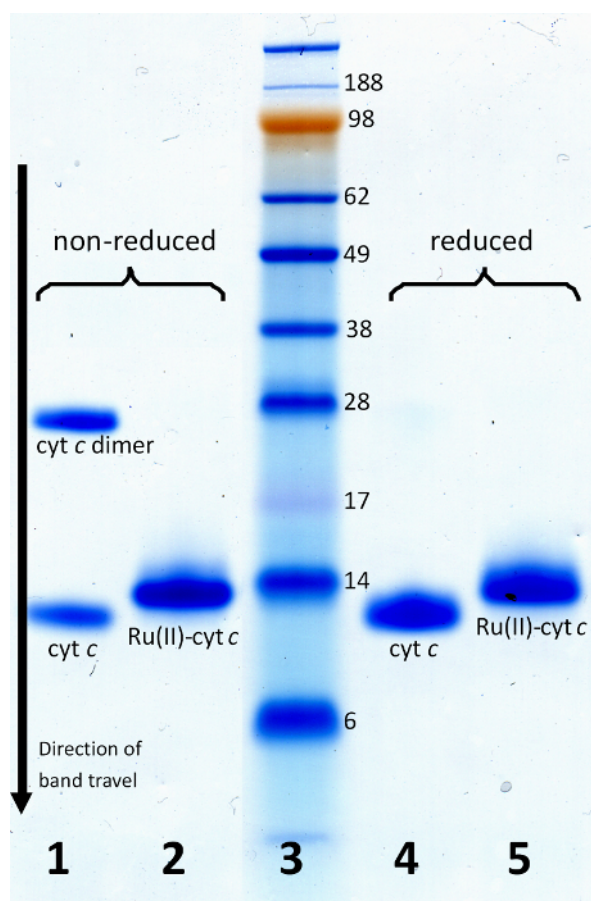


Figure 3. SDS-PAGE of Proteins. 12% Bis-Tris gel of reduced and non-reduced cyt c and Ru(II)-cyt c. Lane 3 contains a pre-stained protein standard, with polypeptide mass annotated to the right of each band (kDa). [Please click here to view a larger version of this figure.](#)

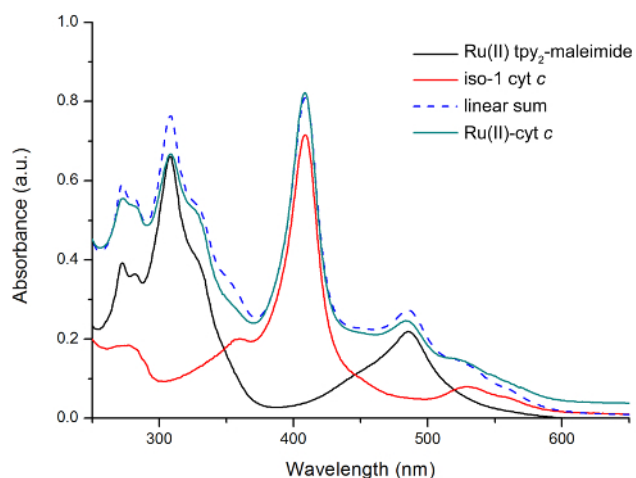


Figure 4. UV-Vis Spectra of Proteins: The absorbance spectrum of Ru(II)-cyt c (green) corresponds closely to the linear addition (dashed blue) of pure, unreacted cyt c (red) and unreacted Ru(II) (tpy)₂-maleimide (black). This demonstrates that the bioconjugate consists of a 1:1 attachment of the maleimide to the protein. [Please click here to view a larger version of this figure.](#)

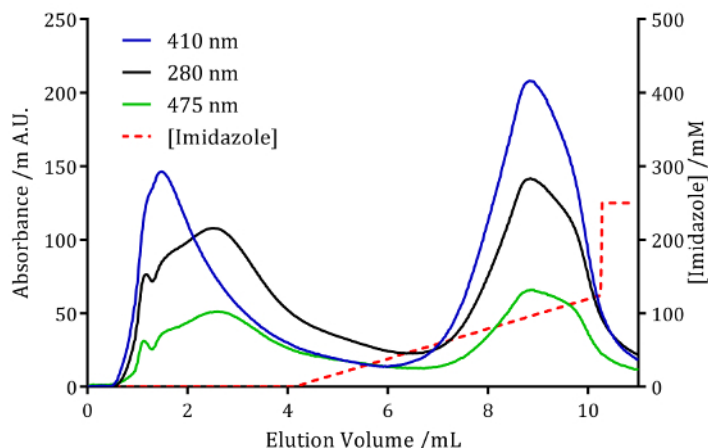


Figure 5. Chromatogram of Ni-IMAC purification of bioconjugates: IMAC trace of Ru(II)-cyt c purification. UV-Vis traces: 410 nm corresponds to the Soret band of cyt c, 280 nm corresponds to both cyt c and Ru(II) (tpy)₂-maleimide, and 475 nm corresponds to the metal-to-ligand charge transfer band of the ruthenium(II) complex. [Please click here to view a larger version of this figure.](#)

Discussion

Purification of the starting materials before a bioconjugation is of utmost importance. Proteins obtained from commercial recombinant sources often contain other isoforms of the protein of interest, which can have different surface chemistry and reactivity. For example, in the described bioconjugation, the commercially available cyt c contains a mixture of both iso-1 and iso-2 cyt c^{12,14,17}. Iso-2 and iso-1 forms of cytochrome c are largely homologous, with the main difference being the presence of a free cysteine residue near the C-terminus of iso-1 cytochrome c. In the example here, purification is achieved with aqueous strong cation-exchange FPLC, however, other forms of FPLC such as anion exchange, affinity, hydrophobic or size-exclusion chromatography may be more applicable. The protein is also reduced in this step to ensure that the cysteine residue on the protein of interest (here cytochrome c) is fully reduced prior to bioconjugation with the maleimide-linked target. In addition, it is useful to ascertain if the maleimide-appended small molecule to be used with a cysteine containing protein is pure and that the maleimide has not undergone a reaction in storage, as maleimides are light and temperature sensitive. This can be quickly and easily checked by ¹H NMR, as the vinylic protons of an intact maleimide will appear as a singlet at 6-6.5 ppm, whereas the protons of an open maleimide will shift upfield; and also mass spectrometry, with an open maleimide ring corresponding to an 18 Da mass increase.

Buffer choice, pH, and the inclusion of adjuvants such as detergents, organic solvents, and reducing agents will have a profound effect on bioconjugation yields^{5,15}. In the case of a Michael addition of a maleimide and a thiol, the pH must be above 6 yet below 8 for a fast, specific reaction to occur. Below pH 6, specificity is high as the maleimide will not be able to react with any amines, but the thiol is protonated and thus is a poor nucleophile for Michael addition leading to a slow reaction. Above pH 8, surface lysine residues can become deprotonated and react with available maleimides, leading to a non-specific covalent attachment of the maleimide component to the protein. Phosphate buffer is used in this bioconjugation because it is a strong buffer in this pH range and does not interact with any of the reagents. Tris(hydroxymethyl) aminomethane (Tris) buffer, for example, would be a poor choice as the amine group in this buffer could potentially react with the maleimide, which would lead to poor yields. Solubility of all reagents is also key to high yields. The addition of small amounts (<10% v/v) of organic solvent can assist in the dissolution of non-polar components, while correct pH and salt concentration are essential for keeping proteins in solution and folded¹². For large membrane proteins with significant hydrophobic surface residues, a surfactant will be necessary to prevent the protein from precipitating¹⁸.

If the protein in the bioconjugation is prone to oxidation and dimerization, the addition of a reducing agent such as TCEP has been shown to improve bioconjugation yields¹⁵. The *in situ* reduction of the protein to its monomeric, free-thiol form allows a greater proportion of active thiol sites to be accessed by the maleimide. However, order of addition and stoichiometry is the key to a successful increase in yield as TCEP will also react with maleimide. By adding the maleimide after TCEP, the TCEP will be consumed by reducing the protein first. Half an equivalent of TCEP to protein is used for the same reason, so that excess TCEP is not available to react unfavorably with the free maleimide. Structural disulfide bridges on the interior of the protein are not likely to be affected by the addition of TCEP or other reducing agents such as DTT, provided that the protein is not held in denaturing conditions such as high temperature or a high concentration of urea. For example, the addition of a large excess of DTT to cyt c prior to FPLC purification does not have a significant effect on the amount of protein recovered after purification. If reduction of structural cysteine bridges by TCEP is suspected, this can be confirmed by running a protein gel under native, non-denaturing conditions.

The Ru(II) bisterpyridine-labelled cytochrome c synthesized in this method is purified *via* Ni²⁺ Immobilized Metal Affinity Chromatography¹⁴. Other purification methods exist, such as size exclusion chromatography, anion/cation exchange chromatography, and specific affinity chromatography such as an antibody stationary phase. In general, the method remains the same as described above, with concentration and dialysis steps following chromatographic purification to return the bioconjugate product to a suitable buffer for storage.

The primary method of determining if a bioconjugation is successful is *via* MALDI-TOF MS. It is the most accurate way to ascertain the small difference in mass between native protein and protein covalently labelled with a small molecule, a difference often less than 1,000 Da. The most difficult part of running a MALDI-TOF MS experiment is matrix choice and spotting technique; however caffeic acid has been found to be a reliable, general purpose matrix for the analysis of the proteins and bioconjugates used in this work. As a potential alternative, sinapinic acid is another commonly used MALDI matrix for the analysis of proteins. Analysis of MALDI-TOF MS data is relatively straightforward. **Figure 2**

shows the typical MALDI-TOF MS spectrum of both pure iso-1 cyt c ($M_w = 12,706$ Da) and pure Ru(II)-cyt c ($M_w = 13,559$ Da), both of which closely correspond to the calculated molecular mass. The purity can also be observed, noting the lack of an iso-2 cytochrome c peak ($M_w = 12,532$ Da) in the unreacted spectrum and the lack of an iso-1 cytochrome c peak in the bioconjugate spectrum. The bioconjugate mass can also be estimated using gel electrophoresis. **Figure 3**, a 12% Bis-Tris protein gel, provides two pieces of evidence for bioconjugation. First, the absence of a dimer band under non-reducing conditions for Ru(II)-cyt c indicates that there is no longer a free cysteine, and second, the slight shift of the bioconjugate band upwards translates to an increase in molecular weight. Protein gels are invaluable and can definitively provide proof of successful bioconjugation not only for small molecule attachments, but also for the synthesis of protein dimers⁵ or protein-polymer bioconjugates⁹.

For proteins containing chromophores such as green fluorescent protein or heme-containing cytochromes, UV-Vis spectroscopy is used to determine the composition and yield of the bioconjugate. The 1:1 linear addition of the starting materials spectra allows the construction of a hypothetical UV-Vis spectrum of the bioconjugate, as shown in **Figure 4**. By comparing the actual bioconjugate spectrum to this 1:1 addition spectrum, the composition can be inferred. For example, if two or more Ru(II) (tpy)₂-maleimides had nonspecifically attached to the protein, the 480 nm band of the bioconjugate would be higher than the predicted spectrum. In addition, the concentration of the bioconjugate can be approximated by using the 410 nm molar absorptivity value of $97.6 \text{ cm}^{-1} \text{ mM}^{-1}$. This is assumed to be the same for the bioconjugate because the Ru(II) (tpy)₂-maleimide has minimal absorbance in this region.

It should be noted that bioconjugation *via* cysteine-maleimide chemistry has several limitations. Firstly, the protein of interest must have a single, accessible cysteine residue, or else protein engineering must be performed to introduce one. Secondly, the cysteine-maleimide bond is susceptible to exchange with other free thiols, such as albumin and glutathione in the context of plasma applications⁹. Such exchange is highly dependent on the solvent accessibility and local charge on the surface of the protein. Maleimide-thiol linkages may still be suitable for plasma applications, but long term stability should be checked using mass spectrometry and gel electrophoresis.

Despite this, the highly specific, high yielding attachment of novel molecules such as fluorescent dyes, redox centers, polymers, and other proteins to proteins *via* cysteine-maleimide chemistry is a powerful technique that allows a range of interesting macromolecular constructs to be accessed. Having large quantities of chemically well-defined protein bioconjugates is key to future studies involving protein binding, self-assembly, enzyme kinetics and protein localization. As demonstrated above, starting material purification, choice of reaction medium, and the addition of supplementary reagents such as reducing agents or surfactants all have a significant impact on bioconjugation yields. Purification is most easily achieved by protein-compatible chromatography, and characterization is accomplished using a combination of MALDI-TOF MS, gel electrophoresis, and UV-Vis spectroscopy.

Disclosures

The authors have nothing to disclose.

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