JOC The Journal of Organic Chemistry

Article

pubs.acs.org/joc

One-Pot Synthesis of High Molecular Weight Synthetic Heteroprotein Dimers Driven by Charge Complementarity Electrostatic Interactions

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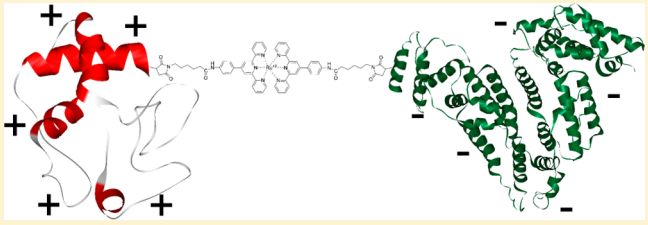
Supporting Information

ABSTRACT: Despite the importance of protein dimers and dimerization in biology, the formation of protein dimers through synthetic covalent chemistry has not found widespread use. In the case of maleimide−cysteine-based dimerization of proteins, we show here that when the proteins have the same charge, dimerization appears to be inherently difficult with yields around 1% or less, regardless of the nature of the spacer used or whether homo- or heteroprotein dimers are targeted. In contrast, if the proteins have opposing (complementary) charges, the formation of heteroprotein dimers proceeds much more readily, and in the case of one high molecular weight (>80 kDa) synthetic dimer between cytochrome *c* and bovine serum albumin, a 30% yield of the purified, isolated dimer was achieved. This represents at least a 30-fold increase in yield for protein dimers formed from proteins with complementary charges, compared to when the proteins have the same charge, under otherwise similar conditions. These results illustrate the role of ionic supramolecular interactions in controlling the reactivity of proteins toward bis-functionalized spacers. The strategy here for effective synthetic dimerization of proteins could be very useful for developing novel approaches to study the important role of protein−protein interactions in chemical biology.

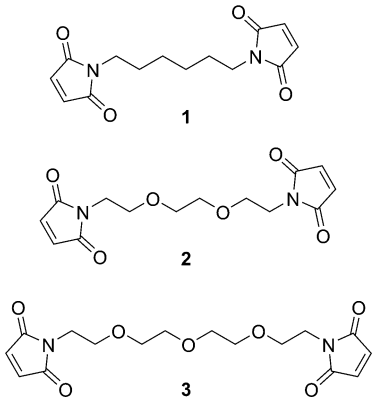


INTRODUCTION

Protein−protein interactions and processes are essential for cell functionality and play an important role in biological systems. These processes include signal transduction, gene expression, and enzymatic regulation.[1,2] Dimerization often allows for biological specificity due to well-defined protein interactions.[3] The ability to control the ubiquitous phenomena of protein dimerization allows scientists to manipulate the regulatory function of organisms and their physical structure.[4] Control of protein dimerization using small molecular ligands allows for applications in gene expression,[5] signal transduction,[6] protein therapeutics[7] and tumor therapy.[8] Additionally, unnatural synthetic protein dimers might provide unique functions in future sensing or nanoscale devices to name but two examples. Although dimerization of proteins has wide applicability, synthetic dimerization by covalent means using small ligands have been largely limited to low molecular weight enzymes/peptides (<10 kDa) which primarily target terminal (N/C) residues in the literature.[9−12]

There have been relatively few examples of isolated high molecular weight protein dimers formed using small synthetic linkers in reasonable yields (>20%). Some notable examples do include a 52 kDa dimer of a monoclonal antibody single-chain fragment (di-scFv),[13] a 128 kDa hemoglobin dimer (di-Hb),[14,15] a 34 kDa human interleukin 1 receptor antagonist (di-IL-1ra),[16] and a 96 kDa lipase−BSA heteroprotein dimer.[17] It is worth noting that Hb has a p*I* of around 7.1−7.5[18] close to neutrality, largely eliminating any unfavorable ionic interactions between the two proteins. For di-scFv and di-IL-1ra, the reaction was facilitated by targeting the corresponding protein terminal residues. The di-IL-1ra was synthesized by native chemical ligation (NCL) of N-terminal modified IL-1ra, while di-scFV was synthesized from scFV modified on the C-terminus by a cysteine—E-TAG sequence.[19] Finally, for the lipase-BSA heteroprotein dimer, the reaction was achieved by an azide−alkyne click reaction. The role and importance of protein charge to probe protein function after amino acid modification has been utilized in the literature, such as the use of protein charge ladders.[20,21] However, the exploitation of global protein charge to facilitate dimer bioconjugate synthesis via supramolecular interactions has been neglected. In the above examples, the linkers or spacers used have not provided any additional function to the resulting synthetic protein dimers.

Received: July 28, 2014
Published: September 18, 2014



Herein, we report the synthesis of protein dimers driven by complementary charge interactions using short-chain commercially available bismaleimide linkers. The linkers used in this study include 1,6-bismaleimidohexane **1**, 1,8-bismaleimidodiethylene glycol **2**, and 1,11-bismaleimidotriethylene glycol **3**, which are inert and neutrally charged spacers, allowing investigation of protein charge complementary effects on synthetic yield. We targeted homo- and heteroprotein dimers incorporating the redox protein iso-1 cytochrome *c* (**Cyt *c***) from *Saccharomyces cerevisiae* (yeast), bovine serum albumin (**BSA**), and a mutant green fluorescent protein derived from *Acropora millepora* (**GFP**)[22] due to their unique property of possessing only a single free cysteine residue. Moreover, extending our previously reported work on light-activated donor–acceptor bioconjugates,[23−25] we also targeted the representative formation of protein-based triad systems, consisting of protein dimers with a charged central synthetic donor **5** or acceptor linker **7**. High molecular weight heteroprotein dimers (>80 kDa) were covalently linked using cysteine–maleimide coupling[26] with site-specific attachment under benign physiological reaction conditions with up to 30% yield due to charge complementarity and favorable electrostatic attraction.

RESULTS AND DISCUSSION

Ionic Properties of Proteins Used for Preparing Dimers. We envisioned that supramolecular ionic interactions might be of considerable importance in the formation of protein dimers. Prior to experimental reaction studies of homo- and heteroprotein dimer bioconjugates, the protein surface charges were analyzed using PyMol (version 1.3) to determine whether the charge surrounding cysteine is localized or a global effect plays the key role based on the total number of charged residues in the protein. The **GFP** and **Cyt c** proteins have a high positive p*I* of 8.3[27] and 10.6,[28] respectively, with 2−8 + charges at physiological pH = 7.4. To complement the positively charged **GFP** and **Cyt c** protein, **BSA** was included in this study, which has a p*I* = 4.7[29] and approximately 13 − charges at pH = 7.4. All three proteins have a solvent-accessible single cysteine (CYS) residue on their surface. The surface CYSs do not appear to be in particularly negatively or positively charged regions on the calculated[30] electrostatic surface of these proteins as shown in Figure 1.

Synthesis of Bis-maleimide Donor and Acceptor Spacers 5 and 7. The required donor and acceptor spacers **5** and **7** were prepared with the electron-donating or -accepting ligands ruthenium(II) bis(terpyridine) and 4,4′-bipyridinium, respectively (Scheme 1). Maleimide-functionalized ruthenium-

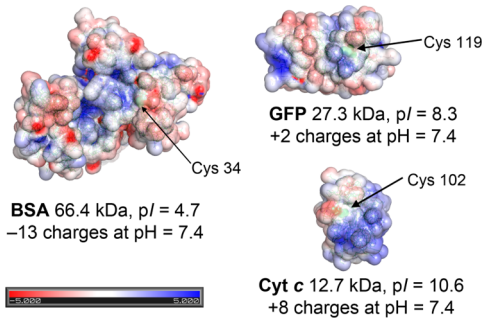


Figure 1. Three proteins used in this study and their key properties. For bovine serum albumin (**BSA**), the sequence of BSA (ExPASy code: P02769) has been aligned onto the X-ray crystallographic structure of human serum albumin (PDB code: 1AO6). Similarly, the modified *Acropora millepora* green fluorescent protein (**GFP**) sequence has been aligned with another *Acropora millepora* GFP structure (PDB code: 2A46). The sequence alignment was performed using ClustalW2 (http:www.ebi.ac.uk/Tools/clustalw2/). The structure for iso-1 cytochrome c (**Cyt *c***) was used without further modification (PDB code: 1YCC). Negative (red), neutral (white), and positive (blue) electrostatic surface features are presented according to the inserted polarized scale. Images were generated with PyMol (Version 1.3, Schrödinger, LLC) using the APBS plug-in to calculate the electrostatic surface potential.[26] The pI values for **BSA**[29] and **Cyt *c***[28] were obtained from the literature, while for **GFP** it has been estimated by theoretical calculations based on its sequence.[27] The number of + or − charges refers to the net whole charges at pH = 7.4 assuming only Lys (+), Arg(+), Glu (−), and Asn (−) are charged at that pH (the N- and C-protein terminus cancel each other out). The target cysteine residues for bioconjugation are colored in green and indicated by an arrow.

(II) bis(terpyridine) **5** was synthesized in 27% yield from the previously reported [Ru(4′-(4-aminophenyl)-2,2′:6′2″-terpyridine)$_2$[(PF$_6$)$_2$ **4**[31] by a HATU-mediated coupling to 6-maleimidocaproic acid. Prior to bioconjugation, **5** was exchanged with chloride salt to increase solubility in aqueous solution and yield. The bis-maleimide functionalized viologen **7** was synthesized from 4,4′-bipyridinium *N*,*N*-di-(propylammonia) hexafluorophosphate **6**[32,33] in modest 2% yield as the main product of this reaction appears to be maleamic acid derivatives of **7** that reduce the yield and make the isolation of **7** difficult.

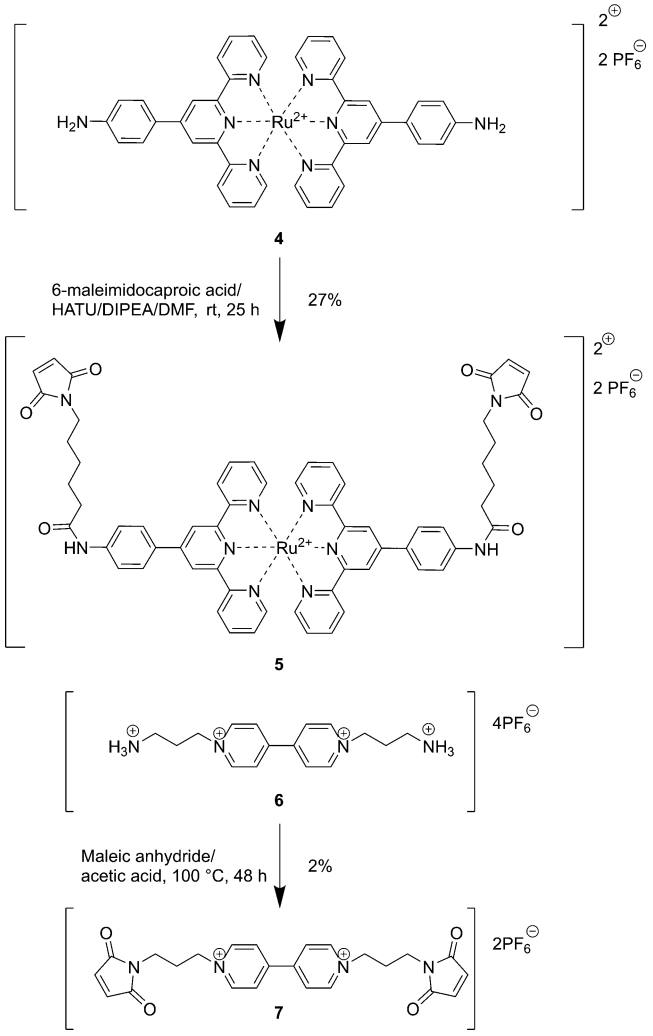
Synthesis of Protein Dimers. Homo- and heteroprotein bioconjugates were synthesized in either a one-or-two-step, one-pot approach using maleimide–thiol chemistry (Scheme 2).[26] Maleimide-functionalized spacers allow chemoselective and site-specific attachment to cysteine residues of target proteins under benign conditions (pH 7) via Michael addition.

It should be noted that the mutant **GFP** (27.3 kDa), derived from *Acropora millepora*, was engineered and expressed to contain only a single free cysteine for functionalization at CYS119[22] allowing for single site-specific modification. The **Cyt *c*** (12.7 kDa) and **BSA** (66.7 kDa) were chosen as protein dimer targets due to the complementarity of their net charge, positively and negatively charged at physiological pH, respectively as shown in Figure 1. Additionally, they offer the unique property that each protein has a single free cysteine residue (CYS102 and CYS34),[34,35] respectively, allowing for chemoselective functionalization with the target bismaleimides **1**, **2**, **3**, **5**, and **7**.

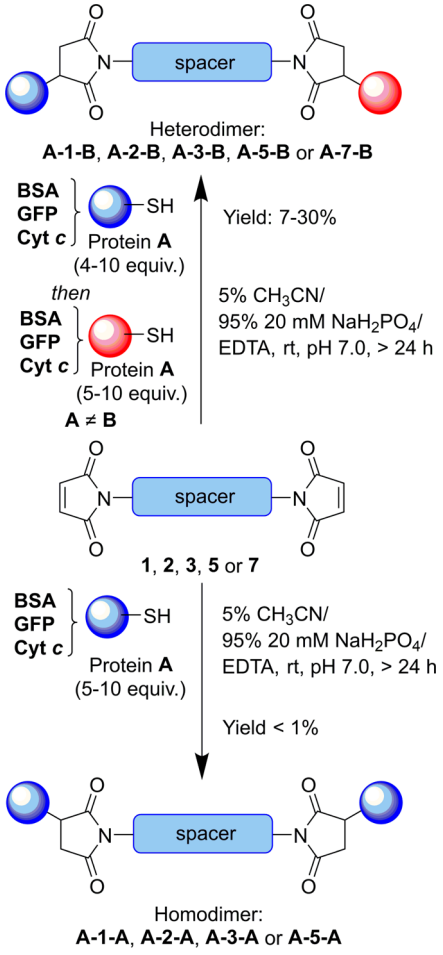
To show how the interactions of **BSA**, **GFP**, and **Cyt *c*** affected dimer yields, the formation of homo- and hetero-

dx.doi.org/10.1021/jo501713t | J. Org. Chem. 2014, 79, 9594−9602

Scheme 1. Synthesis of Bis-maleimide-Functionalized Spacers 5 and 7



Scheme 2. Generic Conditions for Synthesizing Functionalized Protein Dimer Bioconjugates



protein dimers from the structurally simple, commercially available maleimide spacers **1**–**3** was determined by gel electrophoresis with results as shown in Table 1.

Representative gel electrophoresis examples from the synthesis of homo- and heteroprotein dimer bioconjugates using combinations of **Cyt *c***, **GFP**, and **BSA** and 1,11-bismaleimidotriethylene glycol **3** are shown in Figure 2.

As an example, the **Cyt *c*-1-BSA** dimer was synthesized via cysteine–maleimide coupling using spacer **1**. Acetonitrile was used as a cosolvent to solubilize spacer **1**.[24] Spacer **1** in a 20 mM phosphate/EDTA-buffered aqueous solution (pH 7) was reacted with reduced **Cyt *c*** in 5 fold excess for 2 h prior to addition of **BSA** (also in 5-fold excess). The first step ensures formation of monofunctionalized **Cyt *c*** conjugate, partly due to the CYS102 residue being buried in the hydrophobic pocket of the protein, resulting in slower reaction rates (~1 h for completion).[24] Subsequently, **BSA** was added with the more reactive cysteine residue (~2 min for completion)[24] exposed in the hydrophilic region of the protein and stirred overnight. A 5-fold excess of BSA was added to ensure completion, in part because it has been reported that the actual free cysteine available for functionalization is 0.5 mol of the protein.[36]

When the results with spacers **1**–**3** (Table 1) were compared, it was found that only complementary charged

Table 1. Yield of Homo- and Heteroprotein Dimers from BSA, GFP, and Cyt *c* with Spacers 1–3 in 20 mM NaH_2PO_4/EDTA, pH 7.0 in 5% CH_3CN

			yield[b] (%)		
entry	conjugate	charge[a]	**1**	**2**	**3**
1	**Cyt *c*-BSA**	+/−	24	21	21
2	**GFP-BSA**	+/−	13	7	10
3	**Cyt *c*-Cyt *c***	+/+	<1	<1	<1
4	**GFP-Cyt *c***	+/+	<1	<1	<1
5	**GFP-GFP**	+/+	<1	<1	<1
6	**BSA-BSA**	−/−	<1	<1	<1

[a]Overall charges of the at pH 7. See Figure 1 and text for details. [b]Yields were determined by electrophoresis (see the Supporting Information).

heteroprotein dimers could be successfully synthesized in detectable yields (>1%). The heteroprotein dimer **Cyt *c*-1-BSA** resulted in a yield of 24% as shown in Table 1, indicating charge complementarity and electrostatic attraction overcomes steric hindrance of large proteins during dimer formation. Similarly, the complementary charged heteroprotein dimer **GFP-1-BSA** was prepared using a similar procedure and resulted in an estimated yield of 13% (Table 1). Interestingly, the **GFP-1-BSA** dimer resulted in a lower yield compared to **Cyt *c*-1-BSA** due to the reduced net charge of **GFP** relative to **Cyt *c*** with +2 and +8 charges, respectively.[27,28]

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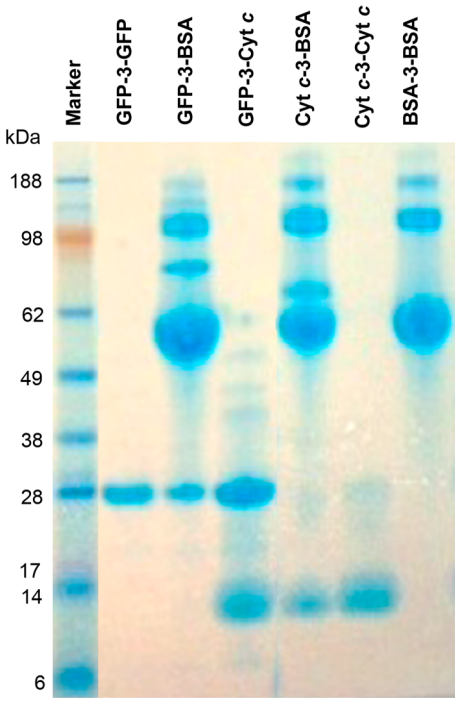

Figure 2. SDS-PAGE gel electrophoresis (reduced) of (attempted) formation of homo- and heteroprotein dimers resulting from the reaction of **BSA**, **Cyt *c***, and **GFP** with 1,11-bismaleimidotriethylene glycol **3** in 20 mM NaH_2PO_4/EDTA, pH 7.0 in 5% CH_3CN. The gels stained with Coomassie blue stain and consolidated into a single image.

In order to demonstrate the necessity of charge complementarity of protein−heteroprotein dimers to obtain higher yields, the synthesis of control like-charged homo- and heteroprotein dimers was attempted. The attempted synthesis of dimers such as **Cyt *c*-1-Cyt *c***, **GFP-1-Cyt *c***, **GFP-3-Cyt *c***, **GFP-3-GFP**, **BSA-1-BSA**, and **BSA-3-BSA** proved to be unsuccessful, which were confirmed by gel electrophoresis as shown in Figure 2, Table 1 and additional gel electrophoresis images for experiments with **1** and **2** (see the Supporting Information).[31]

It should be noted here that when proteins with opposite charges are mixed they might form strong enough electrostatically bound dimers to be detectable by gel electrophoresis. Additionally, all three proteins contain a reactive cysteine surface residue and hence the possibility of homo- and heteroprotein formation via disulfide bridges cannot be discounted. To investigate both issues, admixtures of homo- and heteroprotein pairs were subjected to the exact same conditions as in Scheme 2 but in the absence of bis-maleimides **1**−**3**. The resulting mixtures were then analyzed by gel electrophoresis with and without a reducing agent present. The results clearly show that when our standard gel electrophoresis method under reducing conditions is used no protein disulfide-based dimers can be detected. Furthermore, the strongly denaturing lithium dodecyl sulfate (LDS) surfacant denatures the proteins and electrostatically shields the resulting polypeptide making the survival of any potential dimers formed by pure electrostatic interactions extermely unlikely under the conditions used for gel electrophoresis in our work (see the Supporting Information).

Another slight complication arises in analyzing results from reactions involving **GFP** and **Cyt *c*** as small impurities from **GFP** around the 44 kDa mark could be mistaken for a protein heterodimer formed between **1**, **2**, or **3** and **GFP** with **Cyt *c*** around 40 kDa. A more detailed image analysis using gel analysis software does, however, indicate strongly that these bands are not the result of a **GFP-Cyt *c*** heterodimer at 40 kDa but an unknown **GFP**-related impurity at 44 kDa (see the Supporting Information).

It is noteworthy that the data in Table 1 suggest that PEG-based spacers **2** and **3** gave lower yields for those heteroprotein dimers that were successfully synthesized, compared to the aliphatic spacer **1**. This may be due to the hydrophilic nature of PEG spacers **2** and **3** causing the spacers to lie on the protein surface, hindering access to the maleimide group for reactions with cysteine residues.[37]

To investigate the sensitivity of dimerization to changes in the charge-state of the target protein, the pH dependency of the heteroprotein dimer yield of bioconjugate **GFP-1-BSA** was measured within the main physiological pH range (pH 6.8−7.8) using gel electrophoresis (see the Supporting Information). As shown in the Supporting Information (Figure S11), no significant change in protein dimer yields was observed from pH 6.8−7.4. However, bioconjugate yields increased above pH 7.6, and this is attributed to nonspecific functionalization. This is not unexpected as maleimide−cysteine functionalization is chemoselective at neutral pH; however, side reactions with other amino acid residues start to occur at and above a pH ≈ 8.[26,38] However, the observation that the reaction rate is relatively constant over a pH range of >0.6 suggests that minor changes in the surface charges of the target protein do not play a role; rather, it is the overall charge (mis)match that controls whether dimerization is successful or not.

Gel electrophoresis studies of homo- and heteroprotein dimer yields demonstrate that charge complementarity facilitates and improves synthetic yields in commercially available neutral bismaleimide spacers as shown in Figure 2 and Table 1. To extend this work, electron donor/acceptor bismaleimide spacers **5** and **7** were synthesized to demonstrate applicability to potentially functional bioconjugate dimer systems. Dimer bioconjugates synthesized using functional spacers **5** and **7** were purified and yields were determined after isolation.

To expand the scope of this study, the formation of homo- and heteroprotein dimers from bis-maleimide **5** and **7**, containing typical charged electron donor or acceptor functionalities, was also explored. In cases where these potentially functional protein dimers were obtained in reasonable (>5%) yields, the resulting protein dimers were purified on a preparative scale using fast protein liquid chromatography (FPLC) and characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, gel electrophoresis, and UV−vis spectroscopy. Attempts to synthesize homodimers from **Cyt *c*** and **BSA** with **5** (**Cyt *c*-5-Cyt *c***, and **BSA-5-BSA**) were not very productive; the **BSA-5-BSA** and **Cyt *c*-5-Cyt *c*** were only obtained in 1% yield. On the other hand, a large molecular weight (>80 kDa) heteroprotein dimer **Cyt *c*-5-BSA** was prepared in 30% isolated yield.

The **Cyt *c*-5-BSA** dimer was synthesized via cysteine−maleimide coupling using spacer **5** using a similar methodology as discussed for spacers **1**−**3** (Scheme 2). Prior to bioconjugation, hexafluorophosphate salts of **5** were exchanged with chloride salt to increase water solubility and prevent precipitation of the spacer during the reaction. The use of

dx.doi.org/10.1021/jo501713t | J. Org. Chem. 2014, 79, 9594−9602

acetonitrile as a cosolvent further facilitated the solubility of **5**.[24] The intermediate product **Cyt *c*-5** (not isolated) did not require desalting to remove excess ligand **5** due to low yields from unfavorable homoprotein dimer (**Cyt *c*-5-Cyt *c***) formation as discussed above. Subsequently, **BSA** was added to the intermediate product to form bioconjugate **Cyt *c*-5-BSA**. Confirmation for the formation of a **Cyt *c*-5-BSA** dimer was obtained using MALDI-TOF mass spectrometry with a m/z 80,609 as shown in Figure 3a. In this and other MALDI-TOF spectra obtained here, lower molecular weight species were frequently observed (Figure 3). It needs to be stressed that even for fairly pure protein samples, small impurities or starting materials and even admixtures of these, can give rise to significant peaks in the mass spectra, particularly as lower molecular weight/higher charged species yield a much better MALDI-TOF signal than the target single-charged high-molecular weight species. Hence, gel electrophoresis is a far superior method when it comes to determining purity or relative concentration of proteins in a mixture.

Additionally, the UV−vis spectra in Figure 4 of **Cyt *c*-5-BSA** (black trace) show three primary absorption bands at 280, 410, and 495 nm corresponding to **BSA**, **Cyt *c***, and ligand **5**, respectively.

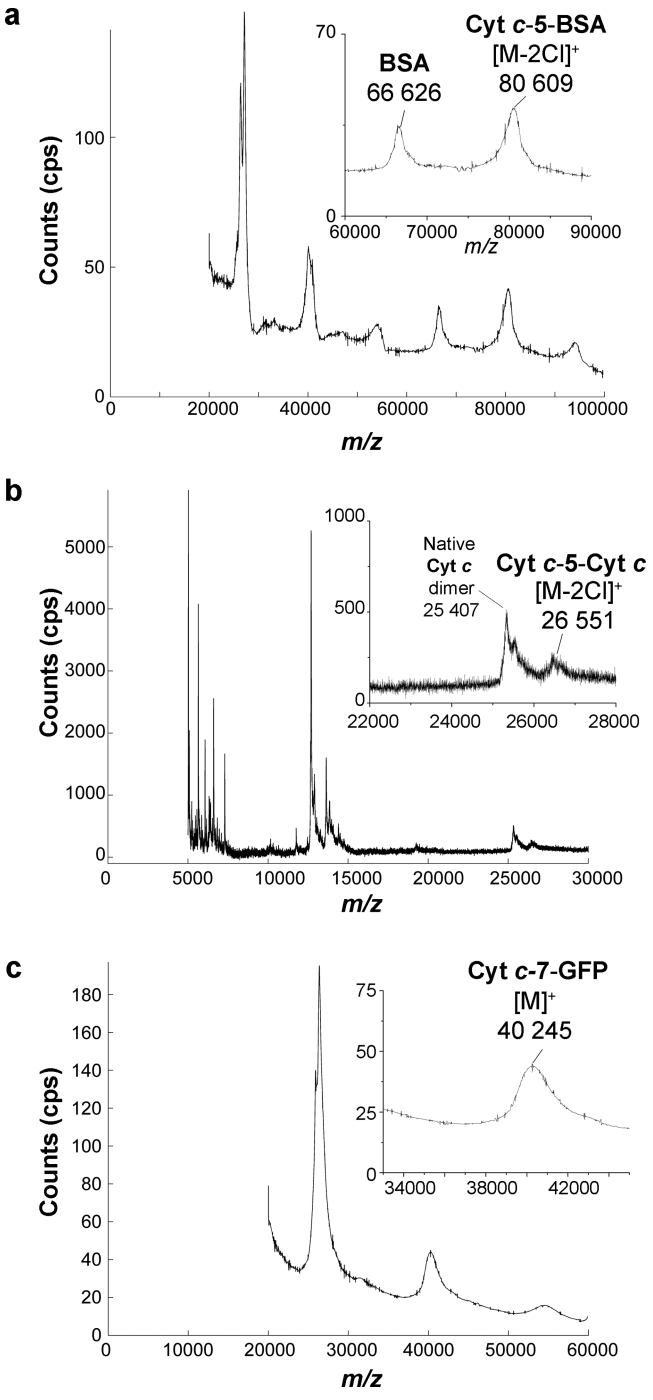

Figure 3. MALDI-TOF mass spectra of (a) **Cyt *c*-5-BSA**; (b) **Cyt *c*-5-Cyt *c***, and (c) **Cyt *c*-7-GFP**. Spectra (a) and (c) were noise reduced. The inserts show the region of interest for the expected products. The spectra are cutoff by the instrument at 5000−20000 Da to lessen the noise level from low molecular or highly charged weight impurities.

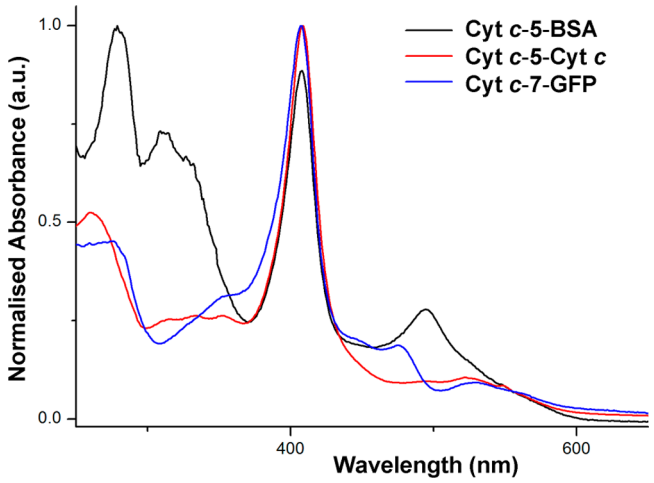

Figure 4. UV−vis spectra of protein dimers: **Cyt *c*-5-BSA** (black); **Cyt *c*-5-Cyt *c*** (red); **Cyt *c*-7-GFP** (blue).

The SDS PAGE gel electrophoresis of **Cyt *c*-5-BSA** in Figure 5 shows a new dimer band, further confirming conjugate formation. The 30% yield for dimer **Cyt *c*-5-BSA** is consistent with previously discussed results indicating charge complementarity facilitates dimer formation.

Attempts to synthesize the homoprotein dimer **Cyt *c*-5-Cyt *c*** resulted in a low yield of 1%. This conjugate was prepared by reacting spacer **5** with an excess of reduced **Cyt *c*** (10-fold) in a 20 mM phosphate/EDTA buffered aqueous solution (pH 7). The dimer formation was confirmed by MALDI-TOF mass spectrometry as shown in Figure 3b with a m/z of 26,551. UV−vis spectroscopy in Figure 4 (red trace) shows characteristic absorption bands at 410 and 495 nm corresponding to **Cyt *c*** and ligand **5** and dimer was further confirmed by reduced gel electrophoresis as shown in Figure 5 showing a new dimer band.

A positively charged heteroprotein dimer (**Cyt *c*** and **GFP**) was also synthesized using an electron-accepting positively charged viologen spacer **7**, and this ligand is also more readily water-soluble than complex **5**. The **Cyt *c*-GFP** pair was chosen here as the former is a good electron donor/acceptor (depending on oxidation state) and the latter an energy donor, making these two proteins interesting as partners with the synthetic electron acceptor **7**. This experiment further demonstrated that reacting large protein dimers of similar charges still results in low yields, independent of the electronic (donor/acceptor) properties or spacer solubility consistent with previously discussed observations. The **Cyt *c*-7-GFP** was

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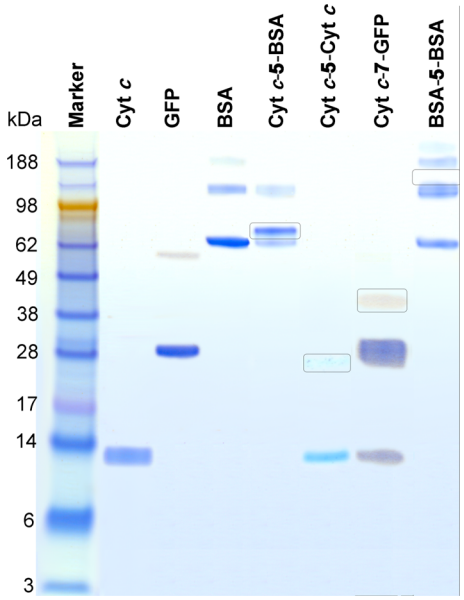

Figure 5. SDS-PAGE gel electrophoresis (reduced) of protein dimer products following purification by chromatography in 20 mM NaH_2PO_4/EDTA, pH 7.0 in 5% CH_3CN. Gels stained with a Coomassie blue stain, consolidated into a single image, and contrast corrected. Dimer bands highlighted in boxes.

prepared by reacting **GFP** (10-fold) with ligand **7** for 3 h, followed by intermediate purification using a G-25 desalting column to remove excess ligand. Finally, reduced **Cyt *c*** (4-fold) was added to the intermediate to yield **Cyt *c*-7-GFP** in isolated 0.1% yield. The dimer was confirmed by MALDI-TOF mass spectrometry as shown in Figure 3c with a m/z of 40,245. UV−vis spectroscopy as shown in Figure 4 (blue trace) shows characteristic absorption bands at 410 and 476 nm corresponding to **Cyt *c*** and **GFP**. The heterodimer was further confirmed by reduced gel electrophoresis, indicated in Figure 5 showing a new dimer band.

From the above, it is clear that the synthesis of similarly charged homo- and heteroprotein dimers results in extremely low yields (<1%) due to steric hindrance and electrostatic repulsion. Further confirmation for this was obtained when the synthesis of a negatively charged homoprotein dimer **BSA-5-BSA** was attempted. Spacer **5** was reacted with **BSA** (40-fold) resulting in a very low isolated yield of 1% and also indicated by the apparent absence of this product in SDS PAGE as shown in Figure 5, noting also that the **BSA-5-BSA** would be fairly close to the natural **BSA-BSA** dimer on SDS PAGE.

CONCLUSIONS

We reported here the preparation of high molecular weight (>80 kDa) heteroprotein dimers in up to 30% yield in the case of **Cyt *c*-5-BSA** with high yields up to 30% utilizing the ionic-charge complementarity of the proteins used in a one-pot synthesis using a functional charged spacer **5**. The formation of this bioconjugate was confirmed by MALDI-TOF (m/z 80609) mass spectrometry, UV−vis spectroscopy and reduced SDS PAGE gel electrophoresis. With simpler noncharged bismaleimides **1**−**3**, heteroprotein dimers yields from 7 to 24% were obtained using various combinations of proteins with complementary (one positive, one negative) charges. In stark contrast, homo- or heteroprotein dimers based on proteins with the same overall charge could either not be synthesized in detectable yields with the investigated bismaleimide spacers or at best, in 0.1−1% yields in some cases using the charged spacers **5** and **7**.

Our studies have therefore shown that favorable supramolecular ionic attraction overcomes steric hindrance even for large bulky proteins such as **BSA** (66.7 kDa). It is noteworthy that the CYS residues targeted in our work do not appear to reside in a particularly positively or negatively electrostatically charged region on these proteins as shown in Figure 1. The dominating factor that controls whether the two proteins form a dimer seems therefore to be the overall supramolecular ionic attraction or repulsion between the two charged proteins. With notable exceptions from the work of Schneider[39,40] and Kraft,[41] ionic interactions have often been ignored in the construction of aqueous supramolecular assemblies and the synthesis of functional protein bioconjugates.[26] It is worth noting in this context the work of Whitesides on "protein ladders",[20,21,42] where chemical modification (e.g., acetylation) of charged surface protein residues was used to control electrostatic interactions between proteins, their stability and activity.

The results obtained in this work also show that it is possible to form synthetic protein dimers in good yield using a functional, relatively rigid spacer with less than desired solubility in water such as the photophysically active spacer **5**. This is important as it will allow us and others to form biohybrid donor−acceptor bioconjugates[23−25] to name but one possible application of this work. Further, as a result of the general approach outlined here, novel protein dimers covalently linked using small organic spacers should be accessible for researchers in chemical biology for potential applications in cancer treatments and other gene-regulation therapeutics.

EXPERIMENTAL SECTION

General Materials and Methods. Compounds **4**[31] and **6**[32,33] were synthesized according to previously reported procedures. All reagents were commercially available, unless otherwise stated. Column chromatography was carried out using silica gel, particle size 40−63 μm. Thin layer chromatography (TLC) was carried out on Al backed precoated silica gel plates with an F-254 fluorescent indicator. Dichloromethane (CH_2Cl_2) and methanol (MeOH) were distilled before use. Dry solvents such as acetonitrile (CH_3CN), dichloromethane, and diethyl ether (Et_2O) were obtained from a dry solvent system utilizing activated columns (typically alumina) to remove water and trace impurities. Dry *N*,*N*-dimethylformamide (DMF) was obtained commercially and used directly from the bottle. For the preparation of salt buffers, ultrapure water ($R > 18 \times 10^6$ Ω) was used and pH values of buffers were adjusted using a calibrated benchtop pH meter and filtered using a 0.45 μm, 47 mm regenerated cellulose membrane, prior to use.

Yeast cytochrome *c* from *Saccharomyces cerevisiae* and bovine serum albumin (BSA) were obtained from commercial sources and the former purified prior to use by strong cation-exchange chromatography to yield pure iso-1 cytochrome *c*.[43] *Acropora millepora* green fluorescent protein was expressed and purified as previously described.[27]

NMR spectra (^1H and ^{13}C) were obtained on 300 and 400 MHz spectrometers, and signals are reported in ppm relative to residual solvent peaks as the internal standard. UV−vis and IR spectra measurements were carried on respective instruments. Low-resolution small molecule mass spectra were obtained on a quadropole mass spectrometer fitted with an electrospray source. High-resolution ESI mass spectrometry was performed on a Quadropole Ion Trap Fourier Transform Ion Cyclotron Resonance mass spectrometer in electrospray mode with a 7 T superconducting magnet. Protein and bioconjugate mass spectra were obtained in linear MALDI-TOF mode while smaller molecules were analyzed in reflectron mode.

dx.doi.org/10.1021/jo501713t | J. Org. Chem. 2014, 79, 9594−9602

Protein and bioconjugate purification was performed using a commercially available low-pressure fast protein liquid chromatography (FPLC) instrument. Cation-exchange chromatography (CEX) was performed using a strong cation exchange column, 7.5 cm × 7.5 mm, 10 μm particle size, and immobilized metal affinity chromatography was performed using a 5 mL, 7 × 25 mm 6% highly cross-linked agarose (particle size 34 μm) affinity column with a polyhistidine chelator charged with Ni^{2+}. Size-exclusion chromatography (SEC) was performed using a 5 mL, 7 × 25 mm cross-linked dextran desalting column with a (1−5) × 10^3 Da fractionation range (Globular proteins).

Gel Electrophoresis (SDS-PAGE). Gel electrophoresis was performed using a microprocessor-controlled programmable power supply and a 50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA (pH 7.3) running buffer mixture (20×) from a commercial source. Samples for gel electrophoresis were prepared by dilution in a premixed lithium dodecyl sulfate (LDS, pH 8.4) buffer (4×). Samples were then reduced (to eliminate disulfide dimers) by adding a 500 mM dithiothreitol (DTT) reducing agent (10 ×). Samples were heated at 70 °C for 10 min to denature the protein. Commercially available 12% bis-Tris, 1 mm, 10-well gels precast polyacrylamide gels with a prestained mixture of 10 proteins (3−188 kDa) as a molecular weight marker were then loaded with 1 μg of protein per well, run at a constant 200 V for 40 min and stained according to the procedure included with a Commassie blue solution.

Purification of Iso-1 Cytochrome *c* (*Saccharomyces cerevisiae*). Crude cytochrome *c* (12.0 mg) was diluted in 20 mM sodium dihydrogen phosphate, pH 7 (6 mL), reduced with dithiothreitol (DTT, 40 μL of 1 M stock solution), and loaded onto a strong cation-exchange column (10 μm resin, 7.5 cm × 7.5 mm). The protein was eluted using a gradient from 328 to 450 mM sodium chloride over 14.3 mL in 20 mM sodium dihydrogen phosphate (pH 7) at a flow rate of 2 mL/min. The main peak (eluting from 15.8 to 18.0 mL) was collected and concentrated using a 3 000 molecular weight cutoff (MWCO) centrifuge concentrator giving pure iso-1 cytochrome *c* in 44% yield based on UV−vis absorbance ($\varepsilon_{410} = 9.76 \times 10^4$)[24,43] of the crude load and the final product (MALDI-TOF: *m*/*z* 12 710 ($[M]^+$ requires 12 706).

[Ru(4′-(4-maleimide-hexylcarboxyamidophenyl)-2,2′:6′2″-terpyridine)$_2$](PF6)$_2$ (5). A solution of 6-maleimidocaproic acid (73.8 mg, 0.349 mmol), *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 136.1 mg, 0.358 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 96.5 mg, 0.746 mmol) in dry dimethylformamide (5 mL) was stirred at room temperature for 1 h. Complex **4**[31] (62.5 mg, 0.0601 mmol) in dry dimethylformamide (5 mL) was added to the reaction mixture and stirred for an additional 25 h in the dark. Dichloromethane (50 mL) was added, the organic phase was washed with 10% w/v citric acid (2 × 10 mL) and water (3 × 10 mL), dried over anhydrous sodium sulfate, and filtered, and dichloromethane was removed in vacuo. The concentrated dimethylformamide phase was diluted and precipitated into dry diethyl ether and solid collected by filtration, washed with diethyl ether, and collected with acetonitrile. The dried solid was purified on silica (acetonitrile/saturated aqueous potassium nitrate/water, 20:1:3 (v/v/v)) followed by concentration of the product containing fractions, collection of the precipitate, washing with water, and collection with acetonitrile to afford the complex **5** as a red solid (22.8 mg, 27%): mp >288 °C dec; IR (CH_3CN:$CHCl_3$ (5:95)) ν_{max}/cm^{-1} 3616, 3534, 3375, 3347, 1709, 1596, 1319, 1136, 1086, 877, 844; ^1H NMR (400 MHz, CD_3CN) δ 8.97 (s, 4H), 8.64−8.61 (m, 6H), 8.16 (ddd, 4H, *J* = 9.0, 2.0, 2.0 Hz), 7.97−7.91 (m, 8H), 7.44−7.41 (m, 4H), 7.17 (ddd, 4H, *J* = 7.6, 5.6, 1.2 Hz), 6.76 (s, 4H), 3.49 (t, 4H, *J* = 7.0 Hz), 2.41 (t, 4H, *J* = 7.4 Hz), 1.77−1.69 (m, 4H), 1.67−1.59 (m, 4H), 1.43−1.34 (m, 4H); ^{13}C NMR (101 MHz, CD_3CN) δ 172.9, 172.2, 159.2, 156.4, 153.4, 148.7, 142.4, 138.9, 135.2, 132.2, 129.3, 128.3, 125.4, 122.0, 120.8, 38.2, 37.6, 28.9, 27.0, 25.6; UV−vis (CH_3CN) λ_{max}/nm (ε/$M^{-1}cm^{-1}$) 494 (4.15 × 10^4), 310 (9.44 × 10^4), 284 (6.61 × 10^4); HRMS (ESI) *m*/*z* calcd for $C_{62}H_{54}N_{10}O_6Ru^{2+}$ ($[M - 2PF_6]^{2+}$) 568.1635, found 568.1638; MS (ESI) *m*/*z* 568.4 ($[M - 2PF_6]^{2+}$).

Ion Exchange of Complex 5. Prior to bioconjugation, complex **5** was exchanged with chloride salt to increase solubility and yield. The hexafluorophosphate salts of complex **5** (10 mg) was dissolved in acetonitrile to which excess tetrabutylammonium chloride was added until precipitation was complete. The mixture was centrifuged to a pellet, and the solution was decanted. The pellet was resuspended in acetonitrile and recrystallized from diethyl ether followed by centrifugation (3×) to give the chloride adducts of complex **5**, which was then subsequently used without further purification or characterization for bioconjugation synthesis.

4,4′-Bipyridinium-*N*,*N*-di(maleimidopropyl) Hexafluorophosphate (7). A solution of maleic anhydride (3.54 g, 36.1 mmol) and 4,4′-bipyridinium-*N*,*N*-di(propylammonia) hexafluorophosphate **6**[32,33] (3.93 g, 6.98 mmol) in glacial acetic acid (100 mL) was stirred at 100 °C for 48 h. The precipitate was collected and dissolved in minimal water and reprecipitated with ammonium hexafluorophosphate. The product was purified on silica (acetonitrile/saturated aqueous potassium nitrate/water, 20:1:3, v/v/v) and precipitated with ammonium hexafluorophosphate to yield viologen **7** as a white solid (96 mg, 2%): mp >164 °C dec; IR (KBr) ν_{max}/cm^{-1} 3451, 3147, 3095, 2946, 1709, 1644, 1446, 1410, 1384, 1169, 837, 694; ^1H NMR (300 MHz, CD_3CN) δ 8.92 (d, 4H, *J* = 6.8 Hz), 8.37 (d, 4H, *J* = 6.8 Hz), 6.81 (s, 4H), 4.59 (t, 4H, *J* = 7.6 Hz), 3.56 (t, 4H, *J* = 6.2 Hz), 2.32−2.24 (m, 4H); ^{13}C NMR (75 MHz, CD_3CN) δ 172.6, 147.2, 135.9, 128.6, 60.9, 35.2, 31.5; HRMS (ESI) *m*/*z* calcd for $C_{24}H_{24}N_4O_4PF_6$ ($[M - PF_6]^+$) 577.1439, found 577.1438; MS (ESI) *m*/*z* 576.63 ($[M - PF_6]^+$).

General Method A for the Formation and Quantitation of Homo- and Heteroprotein Dimers with Bis-maleimide Spacers 1, 2 or 3. All aqueous solutions used were buffered to 20 mM sodium dihydrogen phosphate, 20 mM ethylenediaminetetraacetic acid with the pH adjusted to the target pH (usually 7.0) using a dilute sodium hydroxide or hydrochloric acid solution and a benchtop pH meter. In the case of homodimer formation, a solution of spacer **1**, **2**, or **3** (0.009 μmol) in acetonitrile (100 μL) was added to 1.10−1.75 mL of the buffer solution, followed by additional 0.15−0.80 mL buffer containing the protein of interest in about 4−5 fold excess to final volume of 2.0 mL. The mixture stirred in the dark for 24 h. In the case of heterodimer formation, a solution of spacer **1**, **2**, or **3** (0.009 μmol) in acetonitrile (100 μL) was added to approximately 0.95−1.75 mL of the buffer solution, followed by additional 0.15−0.80 mL buffer containing the first protein of interest in about 4−5-fold excess, and the mixture was then stirred in the dark for 2 h. The second protein in about 0.15 mL of the buffer solution was then added so that the total volume of the reaction mixture was 2.0 mL. This mixture was then stirred in darkness at room temperature for an additional 21−22 h (total reaction time 24 h). The reaction mixtures were then concentrated, dialyzed into water, and analyzed via gel electrophoresis. The relative concentration of monomer and dimer bands was determined using a "gel analysis" function in an image software package.[44]

Before reaction mixtures were analyzed in this manner, the relative intensities (efficiency of staining) was determined by doing gel electrophoresis on an equimolar mixture of two proteins and measurement of the relative intensities of these bands using the gel analysis software to yield a 5:1, 3.7:1, and 1.9:1 ratio of intensities for the **BSA-Cyt *c***, **BSA-GFP**, and **GFP-Cyt *c*** pairs, respectively. The gel analysis software was then used to compare the relative intensities of the sum of the unreacted reactant proteins and the target dimer. The raw relative % of the dimer was multiplied by a factor corresponding to the stoichiometric ratio of the protein to bis-maleimide spacer **1**, **2**, or **3**, which is typically around 10. This number was then divided by the sum of the relative intensity ratios for the pure monomers which was 2 for all the homodimers, 6, 4.7, and 2.9 for the **BSA-Cyt *c***, **BSA-GFP**, and **GFP-Cyt *c*** pairs, respectively, to give the final yield of the dimer. Representative examples of these calculations are given in the Supporting Information.

GFP-1-BSA, GFP-2-BSA, GFP-3-BSA, Cyt *c*-1-GFP, Cyt *c*-2-GFP, and Cyt *c*-3-GFP Dimers. Using general method A, 100 μL of spacers **1**, **2**, or **3** (0.009 μmol) in acetonitrile was added to 950 μL for

dx.doi.org/10.1021/jo501713t | J. Org. Chem. 2014, 79, 9594−9602

BSA and 957 μL for **Cyt *c*** of buffer, followed by the *Acropora millepora* green fluorescent protein **GFP** (0.034 μmol) in 800 μL of buffer solution. After 2 h of stirring, bovine serum albumin **BSA** (0.045 μmol) in buffer (150 μL) or purified, reduced iso-1 cytochrome *c* **Cyt *c*** (0.048 μmol) in buffer (143 μL) was subsequently added to the mixture, which was then stirred for a further 21−22 h (total time = 24 h). The product mixture then analyzed by gel electrophoresis according to general method A.

BSA-1-Cyt *c*, BSA-2-Cyt *c*, and BSA-3-Cyt *c* Dimers. Using general method A, 100 μL of spacers **1**, **2**, or **3** (0.009 μmol) in acetonitrile was added to 1607 μL of buffer, followed by the **Cyt *c*** (0.048 μmol) in buffer (143 μL) was added and stirred for 2 h. Then a bovine serum albumin **BSA** (0.045 μmol) solution in buffer (150 μL) was added and the mixture stirred for a further 21−22 h (total of 24 h). The product mixture then analyzed by gel electrophoresis according to general method A.

BSA-1-BSA, BSA-2-BSA, and BSA-3-BSA Dimers. Using general method A, a 100 μL solution of spacers **1**, **2**, or **3** (0.009 μmol) in acetonitrile was to 1750 μL of the buffer solution, followed by bovine serum albumin **BSA** (0.045 μmol) solution in buffer (150 μL), the mixture was then stirred for 24 h, and the product mixture was then analyzed by gel electrophoresis according to general method A.

Cyt *c* (1), Cyt *c*, Cyt *c* (2) Cyt *c*, and Cyt *c* (3) Cyt *c* Dimers. Using general method A, a 100 μL solution of spacers **1**, **2**, or **3** (0.009 μmol) in acetonitrile was added to 1757 μL of the buffer solution followed by **Cyt *c*** (0.048 μmol) solution in buffer (143 μL), the mixture was stirred for 24 h, and the product mixture was then analyzed by gel electrophoresis according to general method A.

GFP-1-GFP, GFP-2-GFP, and GFP-3-GFP Dimers. Using general method A, a 100 μL solution of spacers **1**, **2**, or **3** (0.009 μmol) in acetonitrile was added to 1100 μL of the buffer solution, followed by **GFP** (0.034 μmol) solution in buffer (800 μL), and stirred for 24 h, and the product mixture was then analyzed by gel electrophoresis according to general method A.

Attempted Formation of Electrostatically and Disulfide-Bonded Protein Heterodimers. On the basis of general method A, heterodimers were prepared by addition of 40.8 μL of **Cyt *c*** (0.009 μmol) in 79.2 μL of buffer solution or 25.7 μL of **GFP** (0.009 μmol) in 94.3 μL of buffer solution to 30 μL of BSA (0.009 μmol). The resulting solutions were then stirred for 24 h. The product mixture was analyzed by gel electrophoresis with and without reducing agent (500 mM dithiothreitol).

Synthesis and Isolation of a Cyt *c*-5-BSA Dimer. A solution of complex **5** (chloride counterion) (0.0410 mg, 0.0340 μmol) in acetonitrile (170 μL) was added to a solution of 94 mM sodium dihydrogen phosphate and 94 mM ethylenediaminetetraacetic acid, pH 7.0 (723 μL) in water (1.87 mL) at room temperature. Purified, reduced iso-1 cytochrome *c* **Cyt *c*** (4.32 mg, 0.340 μmol) in water (636 μL) was then added and the resulting solution stirred in darkness at room temperature for 2 h. Bovine serum albumin **BSA** (45.9 mg, 0.687 μmol) was subsequently added to the mixture and stirred in darkness at room temperature for an additional 21 h. The reaction mixture was then concentrated, dialyzed into water, and purified (FPLC) by immobilized metal affinity chromatography using a gradient from 0 to 250 mM imidazole in 20 mM sodium dihydrogen phosphate, 0.5 M sodium chloride, pH 7.0 in 9 at 0.5 mL min^{-1}. The product fraction (eluting from 5.6 to 10.2 mL) was pooled, concentrated, and dialyzed into water. The pooled fractions were further purified using a strong cation exchange column using a gradient from 320 to 450 mM sodium chloride in 20 mM sodium dihydrogen phosphate, pH 7.0 in 14.4 at 1 mL min^{-1}. The product fraction (eluting from 12.5 to 14 mL) was pooled, concentrated, and dialyzed into water to yield the bioconjugate **Cyt *c*-5-BSA** dimer (0.0340 μmol, 30%). MS (MALDI) *m*/*z* 80 609 ($[M - 2Cl]^+$ requires 80 618).

Synthesis and Isolation of a Cyt *c*-5-Cyt *c* Dimer. A solution of complex **5** (chloride counterion) (0.0375 mg, 0.0311 μmol) in acetonitrile (156 μL) was added to a solution of 94 mM sodium dihydrogen phosphate, 94 mM ethylenediaminetetraacetic acid, pH 7.0 (665 μL), in water (805 μL) at room temperature. Purified, reduced iso-1 cytochrome *c* **Cyt *c*** (3.97 mg, 0.313 μmol) in water (1.5 mL) was then added and the resulting solution stirred in darkness at room temperature for 27 h. The reaction mixture was then concentrated, dialyzed into water, and purified (FPLC) by immobilized metal affinity chromatography using a gradient from 0 to 250 mM imidazole in 20 mM sodium dihydrogen phosphate, 0.5 M sodium chloride, pH 7.0 in 9 at 0.5 mL min^{-1}. The product fraction (eluting from 6 to 10 mL) was pooled, concentrated, and dialyzed into water to yield the bioconjugate **Cyt *c*-5-Cyt *c*** dimer (1.90 nmol, 1%), mixed in with an unreacted **Cyt *c***, with the yield of dimer estimated from gel electrophoresis of this mixture using an approach similar to that approach used in general method A. MS (MALDI) *m*/*z* 26 551 ($[M - 2Cl]^+$ requires 26 550).

Synthesis and Isolation of Cyt *c*-7-GFP Dimer. A solution of viologen **7** (0.78 mg, 1.08 μmol) in acetonitrile (344 μL) was added to a solution of 94 mM sodium dihydrogen phosphate, 94 mM ethylenediaminetetraacetic acid, pH 7.0 (1.46 mL) in water (4.55 mL). *Acropora millepora* green fluorescent protein **GFP** (1.79 mg, 0.0688 μmol) was added at room temperature in darkness and stirred for 3 h. The reaction mixture was then concentrated to ca. 1 mL and desalted (FPLC) by size-exclusion chromatography using 20 mM sodium dihydrogen phosphate, pH 7.0 at 0.5 mL min^{-1}. The product fraction (eluting from 1.5 to 3.1 mL) was pooled and concentrated (527 μL). Purified, reduced iso-1 cytochrome *c* **Cyt *c*** (3.68 mg, 0.290 μmol) in water (264 μL) and a solution of 94 mM sodium dihydrogen phosphate and 94 mM ethylenediaminetetraacetic acid, pH 7.0 (213 μL) was then added to the desalted product fraction and the resulting solution stirred in darkness at room temperature for 17 h. The reaction mixture was then concentrated, dialyzed into water, and purified (FPLC) by immobilized metal affinity chromatography using a gradient from 0 to 250 mM imidazole in 20 mM sodium dihydrogen phosphate, 0.5 M sodium chloride, pH 7.0 in 6 at 0.5 mL min^{-1}. The product fraction (eluting from 5.5 to 8.2 mL) was pooled, concentrated, and dialyzed into water to yield the bioconjugate **Cyt *c*-7-GFP** dimer (0.49 nmol, 0.1%). MS (MALDI) *m*/*z* 40 245 ($[M]^+$ requires 40 236).

Synthesis and Isolation of a BSA-5-BSA Dimer. A solution of complex **5** (chloride counterion) (0.0410 mg, 0.0340 μmol) in acetonitrile (170 μL) was added to a solution of 94 mM sodium dihydrogen phosphate, 94 mM ethylenediaminetetraacetic acid, pH 7.0 (723 μL) in water (2.50 mL) at room temperature. Bovine serum albumin **BSA** (90.9 mg, 1.36 μmol) was then added and the resulting solution stirred in darkness at room temperature for 23 h. The reaction mixture was then concentrated, dialyzed into water, and purified (FPLC) by immobilized metal affinity chromatography using a gradient from 0 to 125 mM imidazole in 20 mM sodium dihydrogen phosphate, 0.5 M sodium chloride, pH 7.0 in 6 at 0.5 mL min^{-1}. The product fraction (eluting from 5.7 to 8.1 mL) was pooled, concentrated, and dialyzed into water to yield bioconjugate **BSA-5-BSA** (0.43 nmol, 1%), mixed with unreacted **BSA**, with the yield of dimer estimated from gel electrophoresis of this mixture using an approach similar to that approach used in general method A.

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR for compounds **5** and **7**, SDS-PAGE gel electrophoresis (reduced) of protein dimers using spacers **1** and **2** from attempted formation of electrostatically and disulfide-bonded dimers and from a pH dependency study on heteroprotein dimer yield of **GFP-1-BSA**, FPLC traces of heteroprotein dimer **Cyt *c*-5-BSA**, gel analysis trace showing impurities accompanying **GFP**, and representative examples from gel analysis software showing of how protein dimer yields were calculated. This material is available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/jo501713t | J. Org. Chem. 2014, 79, 9594−9602

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the facilities of the Mark Wainwright Analytical Centre of the University of New South Wales. We acknowledge the Australian Research Council (ARC) for a Discovery Project Grant DP120101604, ARC Centre of Excellence CE140100036 and a Future Fellowship (FT120100101) to P.T. and support from the Faculty of Science UNSW to C.P.M. and P.T. We thank the Australian Government for a Ph.D. Scholarship to D.H. and the ARC and UNSW for a Ph.D. Scholarship to E.V.N.

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