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ortho-Methoxyphenols as Convenient Oxidative Bioconjugation Reagents with Application to Site-Selective Heterobifunctional Crosslinkers

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ABSTRACT: The synthesis of complex protein-based bioconjugates has been facilitated greatly by recent developments in chemoselective methods for biomolecular modification. The oxidative coupling of *o*-aminophenols or catechols with aniline functional groups is chemoselective, mild, and rapid; however, the oxidatively sensitive nature of the electron-rich aromatics and the paucity of commercial sources pose some obstacles to the general use of these reactive strategies. Herein, we identify *o*-methoxyphenols as air-stable, commercially available derivatives that undergo efficient oxidative couplings with anilines in the presence of periodate as oxidant. Mechanistic considerations informed the development of a pre-oxidation protocol that can greatly reduce the amount of periodate necessary for effective coupling. The stability and versatility of these reagents was demonstrated through the synthesis of complex protein-protein bioconjugates using a site-selective heterobifunctional crosslinker comprising both *o*-methoxyphenol and 2-pyridinecarboxaldehyde moieties. This compound was used to link epidermal growth factor to genome-free MS2 viral capsids, affording nanoscale delivery vectors that can target a variety of cancer cell types.

INTRODUCTION

Site-specific and chemoselective bioconjugation remains a challenging task for many proteins. The reactions used for this purpose must be compatible with near-neutral aqueous solutions and be kinetically competent at low micromolar concentrations. They also push the limits of functional group tolerance to proceed in the presence of amines, carboxylic acids, thiols, and electron rich aromatic rings that are not intended for modification. Despite these challenges, an increasing number of techniques have been developed to achieve desired high levels of selectivity through the modification of both native and artificially-introduced amino acids[1-4] or the use of tag strategies.[5-10] In our own lab, these reactions have enabled the synthesis of a variety of complex protein-based materials, including drug delivery systems,[11-16] water remediation materials,[17] and artificial light harvesting systems.[18-21]

To build these materials, our group has devoted considerable attention to the advancement of reliable and efficient oxidative coupling reactions (Figure 1a).[22-25] These strategies rely on the addition of either anilines or protein N-termini to *o*-iminoquinones and *o*-quinones[26] that are generated *in situ* using potassium ferricyanide or sodium periodate. These reactions exhibit high chemoselectivities and can proceed in aqueous media at high dilution, but there are a few limitations of the *o*-aminophenol and catechol substrates that have potentially limited the widespread adoption of these methods. As both types of substrates are oxidized in the presence of mild oxidants (such as ferricyanide), these electron rich aromatic rings show considerable instability to storage under ambient conditions. This requires the preparation of the *o*-aminophenol through chemoselective dithionite reduction of a precursor *o*-nitrophenol moiety immediately prior to use. As such, the requisite NHS esters and/or amines are not commercially available and must be synthesized from the parent phenol through nitration.[22] Furthermore, the necessity to reduce the nitrophenol group can limit the types of molecules that can be used with this conjugation procedure since some reductively sensitive moieties (e.g. azo dyes, alkoxyamines, and oximes) will be incompatible with this protocol. Similar issues are associated with the catechol substrates.

Herein, we describe the use of *o*-methoxyphenol substrates as air-stable, commercially available reagents that can be used in oxidative coupling reactions with anilines in the presence of sodium periodate with short reaction times (c.a. 10 min) and excellent chemoselectivity. We further demonstrate the versatility of this reaction by applying these reagents to the development of a site-selective heterobifunctional linker that also involves an N-terminal selective condensation reaction of 2-pyridinecarboxaldehydes (2PCA) recently reported by our laboratory.[27] The synthetic tractability and storability of these *o*-methoxyphenol reagents addresses many of the challenges associated with previous oxidative coupling reactions. Thus, these reagents substantially improve the practicality of this useful class of bioconjugation reactions.

RESULTS AND DISCUSSION

One useful variant of these oxidative coupling strategies is the application of *o*-azidophenols as iminoquinone precursors that can be activated through photolysis.[28] As this reaction was being developed, an informative control experiment was performed wherein an *o*-azidophenol was treated *in the absence of light* with periodate and toluidine, and the reaction was monitored by RP-HPLC. Surprisingly, ~40% conversion to the coupled product was observed, wherein the azido group in the starting material had been converted to the carbonyl group (Figure 1b). We hypothesized that this occurred through the nucleophilic addition of water to the periodate-activated aromatic ring, leading to an azidohydrin intermediate that expels hydrazoic acid to generate the presumed *o*-quinone for coupling. This experiment suggested that the placement of other leaving groups *ortho* to the phenol could potentially allow for air-stable oxidative coupling partners that would be easier to access. Crucial to the success of such an approach, however, would be the identification of a substituent that was both electron rich enough to promote oxidation and also capable of serving as a good leaving group.

Previous studies from the Snyder[29] and Quideau[30] groups indicated that such oxidative dearomatizations of *o*-methoxyphenols are efficient in the presence of hypervalent iodine species. To apply this in an oxidative coupling context, 2-methoxy-*p*-cresol was treated with sodium periodate and *p*-toluidine in 10 mM pH 7.0 phosphate buffer. After 30 min, HPLC analysis indicated that the desired coupling had occurred, but the conversion was quite low (~25%, data not shown). Careful examination of the reaction revealed that the *o*-methoxyphenol was not fully soluble in the reaction conditions, suggesting that the use of a lower ionic strength buffer, such as BisTris, would promote a more efficient coupling. This hypothesis was validated, as the buffer switch resulted in a near quantitative coupling after 30 min at room temperature (Figure 1c). Other *ortho*-leaving groups were examined, such as bromo, chloro, and fluoro; however, no reaction was observed in any of those cases. Furthermore, since *ortho*-methoxyphenols are ubiquitous, air-stable, commercially available, and inexpensive (see sample substrates in Figure 1d), the optimization of this new version of the coupling reaction was pursued.

Reaction Optimization in Macromolecular Couplings

While the above couplings on small molecule substrates were promising, the combination of poor solubility of the simple cresol derivative in phosphate buffer and the ultimate application of the reaction to proteins led us to pursue optimization of the reaction within the macromolecular context. For these studies, an *o*-methoxyphenol-PEG-5k-OMe (MP-PEG) was readily synthesized (see Supporting Information) and used in gel shift assays for coupling to bacteriophage MS2 viral capsids bearing aniline moieties[31] introduced using amber stop codon suppression techniques (Figure 2a). The 180 protein monomers of each 27 nm capsid contained one instance of this residue on the exterior surface. Previous work in our group has shown MS2 to be an attractive scaffold for creating nanoscale biomaterials for drug delivery and cellular imaging, due to its ability to carry and protect cargo within its protein shell.

First, a time screen (Figure 2b) with 200 μM PEG, 20 μM protein, and 1 mM periodate revealed that the reaction reached completion in 10 min, and thus 10 min coupling times were used in all subsequent screens and experiments unless noted otherwise. The reaction was quite tolerant of a variety of buffers, Figure 2c. Tris buffer was a notable exception, likely because its 1,2-aminoalcohols participate in competing oxidative cleavage reactions with sodium periodate. Similarly, the corresponding *o*-aminophenol oxidative coupling reaction also proceeded with reduced yield with periodate in this buffer (see Supplementary Figure S1e). As such, phosphate buffer was used in all remaining experiments. An equivalent screen indicated that high levels of conversion could be reached with as little as 2.5 equiv of PEG reagent; however, a notable increase in yield was observed when 5 equiv were used instead (Figure 2d). The use of 10 equiv provided little improvement in yield. Similar to our previously reported oxidative couplings, the efficiency of this reaction was found to be pH independent over the range from 6-8 (Figure 2e).

In terms of storage, the MP-PEG reagent was left under ambient conditions (i.e. room temperature with no precautions taken to exclude oxygen) for over a year and showed identical reactivity over that time period, in sharp contrast to the aminophenol reagents, which showed noticeable color changes in a matter of hours under identical conditions. This tradeoff in air stability, however, is also reflected in the greater difficulty in oxidation: potassium ferricyanide is completely incapable of oxidizing the *o*-methoxyphenol congener even when present in great excess, while the *o*-aminophenols are oxidized essentially instantaneously. Although many proteins are quite compatible with sodium periodate (especially over the short reaction times and low equivalents generally used in these couplings), a screen was carried out to identify other oxidants capable of initiating the transformation. Despite an extensive screen of oxidants (see Supporting Information), we were unable to identify another water-soluble, commercially available oxidant to effect the desired oxidation controllably.

Methods to Limit Periodate Exposure

The large difference in reaction time required to reach completion for the *o*-aminophenol (<2 min) and *o*-methoxyphenol couplings (10 min) and the necessity for a strong oxidant suggested that *o*-quinone generation from the *o*-methoxyphenol couplings was rate determining. This mechanistic insight presented a method to limit protein exposure to periodate for cases in which proteins of interest are highly susceptible to oxidation. In particular, if *o*-quinone generation were rate limiting, then

use of a pre-oxidation protocol to generate the *o*-quinone with subsequent introduction of the aniline-containing protein could provide a considerable decrease in both the reaction time and the amount of periodate used.

To probe this hypothesis, an oxidative coupling reaction was performed under a stricter set of conditions (20 μM *p*aF MS2, 100 μM PEG, and 500 μM sodium periodate final concentrations, compared to the 1 mM periodate used previously). As shown in Figure 3b, pre-oxidation for as little as 2 min prior to introduction of the MS2 allowed for a dramatic increase in yield of the coupled products relative to a reaction that was set up under identical conditions but without pre-oxidation. Thus, use of a pre-oxidation protocol enabled use of half the periodate (i.e. a total of 2.5 equiv relative to the *o*-methoxyphenol since the overall reaction involves two 2-electron oxidations) and reaction times could also be reduced by at least half.

Such an approach was also examined for *o*-aminophenol-based couplings with limited success (see Supplementary Figure S2b)— a fact that can be rationalized if oxidation is also rate-limiting in those couplings. Slow oxidation would produce *o*-iminoquinones in the presence of remaining *o*-aminophenols that could serve as nucleophile in the coupling reaction. Evidence for this pathway was found in small molecule couplings where masses corresponding to dimers and trimers were observed in the absence of toluidine as a nucleophile.

The success in implementing a pre-oxidation strategy indicated that a method to further reduce periodate exposure could be possible. In particular, if a suitable reductant could be identified to quench excess periodate without reducing the generated *o*-quinone, then a pre-oxidation followed by mild reductive quench would dramatically lower the amount of periodate present in solution. Our previous buffer screen indicated that Tris was incompatible with this oxidative coupling reaction (*vide supra*) and suggested that vicinal diols could serve as an appropriate reductant. Indeed, mannose was found to be effective at rapidly quenching periodate (Figure 3c) while also allowing for useful levels of conversion to be achieved in a pre-oxidation/mannose quenching protocol (Figure 3d). This procedure is likely to find utility with proteins that show sensitivity to oxidative conditions.

Application to Heterobifunctional Linkers

To exploit the latent reactivity of the *o*-methoxyphenol, the functional group was incorporated into a heterobifunctional crosslinker. This class of reagents is commonly used to join oligonucleotides, proteins, molecular complexes, and surfaces to protein substrates using two differentially reactive ends (Figure 4a).[32-36] Furthermore, the ability to tune the length and properties of the spacer arm and to target specific functional groups on a protein surface by altering the reactive ends allows for these reagents to be used in a diverse array of applications.

Recently, our group has identified 2-pyridinecarboxaldehyde (2PCA) reagents to be highly specific for the modification of protein N-termini under mild conditions.[27] Since *o*-methoxyphenol and 2PCA reactions demonstrate exceptional site-selectivity and require different reaction conditions, incorporating these two functional groups into a single heterobifunctional linker has the potential to be a powerful tool for making well-defined protein conjugates (Figure 4b). First, the 2PCA group can be reacted with the N-terminus of a protein of interest to install a single oxidative coupling partner. After removal of the excess linker, the N-terminally modified protein can be coupled to anilines on a second biomolecule. The *o*-aminophenol and *o*-catechol versions of these reagents would be expected to condense with the free aldehyde groups, thus highlighting the advantages of the methoxy derivatives.

Two heterobifunctional crosslinkers with hydrophilic (**5**) and hydrophobic (**6**) spacers were synthesized (Figure 4c). To evaluate their reactivity, proof-of-concept studies were first conducted with RNase A and *p*aF MS2 viral capsids. RNase A has previously demonstrated high levels of N-terminal modification by 2PCA reagents.[27]

Reactions with 25 μM RNase A and 10 mM crosslinker in pH 7.5 phosphate buffer at 37 °C for 16 h showed good conversions to *o*-methoxyphenol linker-protein conjugates (MP-RNase A), as analyzed by ESI-TOF mass spectrometry (Figures 5a-c). After purification to remove excess crosslinker, SDS-PAGE analysis showed that the MP-RNase A conjugate was successfully conjugated to *p*aF MS2 by oxidative coupling in the presence of $NaIO_4$ (Figure 5d). A screen of varying concentrations of $NaIO_4$ for the oxidative coupling reaction showed a strong dependence on oxidant concentration with the highest conversion occurring at 2 mM. Most importantly, the selectivity of this strategy was confirmed by the lack of RNase A-MS2 conjugates when reactions were conducted with no $NaIO_4$, with wild-type MS2 (lacking *p*aF residues), or with unmodified RNase A (see Supplementary Figure S3).

Strategy Validation: A Receptor-Targeted Imaging Agent

With the ability to attach protein substrates to the exterior of MS2 capsids using the heterobifunctional crosslinkers, this strategy was applied to the construction of a targeted imaging agent by attaching the human epidermal growth factor (EGF). EGF has become a popular targeting biomolecule for cancer therapy because it is known to bind to the epidermal growth factor receptor (EGFR), which is overexpressed in human carcinomas, at low nanomolar affinity.[37-38] Commercially available EGF was readily modified with *o*-methoxyphenol crosslinkers **5** or **6**, as determined by ESI-TOF mass spectrometry (Figures 6a-c). In addition to the anticipated modification, a second addition of the crosslinkers without the loss of water was observed. This is most likely the result of a competing aldol-type reaction, also at the N-terminal position. Previous investigations into this type of product have been consistent with this outcome.[27]

Surprisingly, the optimal oxidative coupling conditions (4:1 protein to MS2 monomer ratio, 2 mM $NaIO_4$, pH 6.5, 5 min)

found for the conjugation of the linker-RNAse A conjugates to *p*aF MS2 afforded low yields when MP-EGF linker conjugates were used. Optimization studies with the MP-linker conjugates found that yields could be improved by increasing the concentration of $NaIO_4$ to 5 mM or 8 mM and extending the reaction time to 10 min (Supplementary Figure S6). Similar to studies with RNAse A, performing the reaction at pH 5.5–6.5 afforded the highest yields. Of note, the addition of 150 mM NaCl increased the yield of the reaction. NaCl may serve to minimize any repulsive electrostatic interactions between EGF and the MS2 viral capsid, as both have a negative surface charge. With these optimal conditions, only two equivalents of the MP-EGF conjugate were required to achieve sufficient yields. Selectivity was confirmed by the lack of coupling in reactions performed with no $NaIO_4$, with wild-type MS2, or with unmodified EGF (Supplementary Figure S6).

The levels of modification were quantified by optical densitometry based on fluorescence of an Oregon Green 488 dye covalently attached on the interior surface (vide infra). Modification levels were found to range between 19% and 31% (55-56 copies per capsid), depending on the amount of the MP-EGF that was used (Figure 6d). Importantly, excess MP-EGF conjugate could be readily removed by using 100 kDa MWCO ultracentrifugation spin concentrators, as demonstrated by the lack of an EGF protein band in the Coomassie-stained gel.

Transmission electron microscopy of the EGF-MS2 conjugates confirmed that the capsids remained intact and were not distorted by the reaction conditions (Supplementary Figure S8). Additionally, size exclusion chromatography (SEC) and dynamic light scattering (DLS) studies indicated an increase in capsid diameter that was consistent with the addition of EGF to the exterior surfaces (Supplementary Figure S8).

The stability of the EGF-MS2 conjugates was assessed over time (Supplementary Figure S9). Over 48 h, storage of these conjugates at 4 °C resulted in minimal decomposition. Surprisingly, storage at 23 °C resulted in the loss of close to half of the EGF attached to MS2 over the same time period. These results are in contrast to previous stability studies performed with the product of 2PCA and RNase A, which was stable at 23 °C. The increased steric bulk or repulsive electrostatic interaction of EGF with MS2 may account for the reduced stability of the 2PCA-EGF linkage.

Evaluation of EGF-MS2 Conjugates

The cell-targeting ability of the EGF-MS2 conjugates was evaluated with flow cytometry studies. A fluorescently labeled EGF-MS2 conjugate was synthesized by first alkylating the mutated cysteine residue inside of *p*aF N87C MS2 capsids with Oregon Green 488 maleimide dye (EGF-MS2-OG). Then, EGF was attached to the exterior of the MS2 capsid via heterobifunctional crosslinker **5**. Cell binding studies were conducted with an EGFR-positive human epidermoid carcinoma cell line, A431, and an EGFR-negative breast cancer cell line, MCF7. These cell lines were incubated with varying concentrations of MS2-OG conjugates for 1 h at 4 °C (Figures 7a-b). Dose-dependent binding of EGF-MS2-OG to A431 cells was observed, while A431 cells incubated with MS2-OG (lacking EGF) under the same conditions showed no binding. The specificity of the EGF-MS2-OG agent was also verified by lack of binding to EGFR-negative MCF7 cells even at the highest concentration of MS2 conjugates. Finally, incubation of the A431 cells with EGF-MS2-OG and increasing concentrations of free soluble EGF decreased the binding of EGF-MS2-OG (see Supplementary Figure S10), confirming the specific EGF/EGFR interaction.

Confocal microscopy images of live cells further verified that EGF-MS2-OG conjugates bind A431 cells (Figure 7c). Increased binding was observed over time at 37 °C. After 90 min of incubation, images showed accumulation of green fluorescence inside the cells, suggesting that EGF-MS2-OG may be internalized through receptor-mediated endocytosis. Similar to the flow cytometry findings, MS2-OG revealed no binding to the cells even after 2 h at 37 °C.

CONCLUSIONS

The oxidative coupling of *o*-methoxyphenols and anilines detailed above provides a convenient platform for the effecient preparation of complex bioconjugates. The excellent air stability of these derivatives and their convenient commercial availability make them an attractive option for bioconjugation. The short reaction times (10 min) and high effciencies mirror the previously-reported oxidative couplings of *o*-aminophenols while also enabling pre-oxidation protocols that are ineffective with the latter substrates. The improved storability and unique reactivity facilitated the development of a heterobifunctional linker strategy that has the potential to enable the synthesis of complex bioconjugates. The ability to construct well-defined protein-protein conjugates rapidly with predictable attachment sites is difficult to achieve and relies heavily on protein engineering techniques. This crosslinker strategy lessens the need for protein engineering and offers the ability to incorporate endogenous or commercially available proteins into these conjugates. Further optimization and application of both reaction types continue in our laboratories.

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION AVAILABLE

Supporting information, including full experimental details, characterization of small molecules, and supplementary figures, is provided. This information is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Stephanopoulos, N.; Francis, M. B. *Nat. Chem. Biol.* **2011**, *7*, 876−884.
2. Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. *ACS Chem. Biol.* **2014**, *9*, 592−605.
3. Spicer, C. D.; Davis, B. G. *Nat. Commun.* **2014**, *5*, No. 4740.
4. Boutureira, O.; Bernardes, G. J. L. *Chem. Rev.* **2015**, *115*, 2174−2195.
5. Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269–272.
6. Rush, J. S.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 12240–12241.
7. Gautier, A.; Juillerat, A.; Heinis, C.; Correa Jr., I. R.; Kindermann, M.; Beaufils, F.; Johnsson, K. *Chem. Biol.* **2008**, *15*, 128–136.
8. Halo, T. L., Appelbaum, J.; Hobert, E. M.; Balkin, D. M.; Schepartz, A. *J. Am. Chem. Soc.* **2009**, *131*, 438–439.
9. Zhang, C.; Spokoyny, A. M.; Zou, Y.; Simon, M. D.; Pentelute, B. L. *Angew. Chem. Int. Ed.* **2013**, *52*, 14001–14005.
10. Zhang, C.; Welborn, M.; Zhu, T.; Yang, N. J.; Santos, M. S.; Van Voorhis, T.; Pentelute, B. L. *Nat. Chem.* **2016**, *8*, 120–128.
11. Kovacs, E. W.; Hooker, J. M.; Romanini, D. W.; Holder, P. G.; Berry, K. E.; Francis, M. B. *Bioconjugate Chem.* 2007, *18*, 1140-1147
12. Wu, W; Hsiao, S. C.; Carrico, Z. M.; Francis, M. B. *Angew. Chem. Int. Ed.* 2009, *48*, 9493-9497.
13. Tong, G. J.; Hsiao, S. C.; Carrico, Z. M.; Francis, M. B. *J. Am. Chem. Soc.* 2009, *131*, 11174-11178.
14. Stephanopoulos, N.; Tong, G. J.; Hsiao, S. C.; Francis, M. B.; *ACS Nano.* 2010, *4*, 6014-6020.
15. Netirojjanakul, C.; Witus, L. S.; Behrens, C. R.; Weng, C. H.; Iavarone, A. T.; Francis, M. B. *Chem. Sci.* 2013, *4*, 266-272.
16. Finbloom, J. A.; Han, K.; Aanei, I. L.; Hartman, E. C.; Finley, D. T.; Dedeo, M. T.; Fishman, M.; Downing, K. H.; and Francis, M. B. *Bioconjugate Chem.* 2016, *27*, 2480-2485.
17. Esser-Kahn, A. P.; Francis, M. B. *Angew. Chem. Int. Ed.* 2008, *47*, 3751-3754.
18. Miller, R. A.; Presley, A. D.; Francis, M. B. *J. Am. Chem. Soc.* 2007, *129*, 3104-3109.
19. Ma, Y.; Miller, R. A.; Fleming, G. R.; Francis, M. B. *J. Phys. Chem. B.* 2008, *112*, 6887-6892.
20. Stephanopoulos, N.; Carrico, Z. M.; Francis, M. B. *Angew. Chem. Int. Ed.* 2009, *48*, 9498-9502.
21. Miller, R. A.; Stephanopoulos, N.; McFarland, J. M.; Rosko, A. S.; Geissler, P. L.; Francis, M. B. *J. Am. Chem. Soc.* 2010, *132*, 6068-6074.
22. Behrens, C. R.; Hooker J.M.; Obermeyer, A.C.; Romanini, D.W.; Katz, E. M.; Francis MB. *J Am Chem Soc.* 2011, *133*, 16398−16401.
23. Obermeyer, A. C.; Jarman, J. B.; Netirojjanakul, C.; El Muslemany, K.; Francis, M. B. *Angew. Chem. Int. Ed.* 2014, *53*, 1057−1061.
24. Obermeyer, A. C.; Jarman, J. B.; Francis, M. B. *J. Am. Chem. Soc.* 2014, *136*, 9572−9579.
25. ElSohly, A. M.; Francis, M. B. *Acc. Chem. Res.* 2015, *48*, 1971-1978.
26. Liu, B.; Burdine, L.; Kodadek, T. *J. Am. Chem. Soc.* **2006**, *128*, 15228–15235.
27. MacDonald, J. I.; Munch, H. K.; Moore, T.; and Francis, M. B. *Nature Chem. Bio.* 2015, *11*, 326-331.
28. El Muslemany, K. M.; Twite, A. A.; ElSohly, A. M.; Obermeyer, A. C.; Mathies, R. A.; Francis, M. B. *J. Am.*

Chem. Soc. 2014, *136*, 12600−12606.
29. Snyder, S. A.; Kontes, F. *J. Am. Chem. Soc.* 2009, *131*, 1745-1752.
30. Deffieux, D.; Gossart, P.; Quideau, S. *Tetrahedron Lett.* 2014, *55*, 2455-2458.
31. Carrico, Z. M.; Romanini, D. W.; Mehl, R. A.; Francis, M. B. *Chem. Commun.* 2008, 1205-1207.
32. Hermanson, G. T. Heterobifunctional Crosslinkers. *Bioconjugate Techniques*, 3rd Edition; Academic Press: San Diego, CA, 2013, pp 299-339.
33. Friedhoff, P. *Anal. Bioanal. Chem.* 2004, *381*, 78–80
34. Rappsilber, J.; Siniossoglou, S.; Hurt, E. C.; Mann, M. *Anal. Chem.* 2000, *72*, 267–275.
35. Shriver-Lake, L. C.; Donner, B.; Edelstein, R.; Breslin, K.; Bhatia, S. K.; Ligler, F. S. *Biosens. Bioelectron.* 1997, *12*, 1101–1106.
36. Ye, Y.; Sun, Y.; Zhao, H.; Lan, M.; Gao, F.; Song, C.; Lou, K.; Li, H.; Wang, W. *Int. J. Pharm.* 2013, *458*, 110–117.
37. Normanno, N.; De Luca, A.; Bianco, C.; Strizzi, L.; Mancino, M.; Maiello, M. R.; Carotenuto, A.; De Feo, G.; Caponigro, F.; Salomon, D. S. *Gene* 2006, *366*, 2–16.
38. Yewale, C.; Baradia, D.; Vhora, I.; Patil, S.; Misra, A. *Biomaterials* 2013, *34*, 8690–8707.

Figure Captions:

Figure 1. Previously reported oxidative couplings and initial experiments with a methoxyphenol variant. (**a**) Oxidative couplings of aminophenols in the presence of sodium periodate and potassium ferricyanide are summarized. (**b**) Control experiments from a related light-activated azidophenol coupling exhibited notable conversion to coupled product in the presence of a strong oxidant but in the absence of light, prompting consideration of other derivatives for oxidative couplings. (**c**) A 2-methoxy-*p*-cresol substrate undergoes highly efficient oxidative coupling with toluidine in the presence of sodium periodate as the oxidant (see HPLC trace of the crude reaction mixture to the right). (**d**) *Ortho*-methoxyphenols are ubiquitious, air-stable, and commercially available, making them convenient reagents for oxidative coupling reactions.

Figure 2. Reaction optimization in the context of macromolecular couplings. All reactions were optimized for the conversion of the reaction described in scheme (**a**) by gel shift assays and quantified by optical densitometry. (**b**) Time screens reveal that the reaction reaches completion in 10 min. (**c**) Most buffers are compatible with this reaction, with Tris serving as a notable exception (due to the presence of a vicinal aminoalcohol). (**d**) Five equiv of *o*-methoxyphenol are sufficient to achieve high levels of conversion. (**e**) The reaction is essentially pH independent over the range of 6–8.

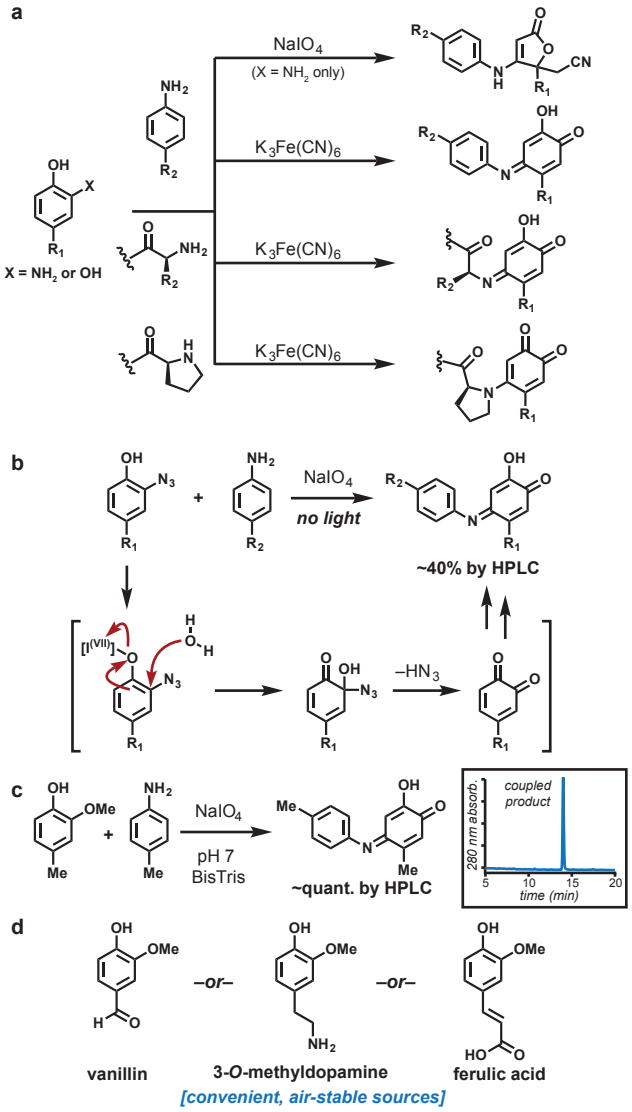
Figure 3. Development of methods to reduce periodate equivalents/exposure to proteins. All reactions were optimized for the conversion of the reaction described in scheme (**a**) by gel shift assays and quantified by optical densitometry. (**b**) Pre-oxidation of the *o*-methoxyphenol component for as little as 2 min prior to addition of the aniline-containing protein reduces the concentration of periodate required for the reaction (500 μM vs 1 mM) while maintaining high reaction conversions. (**c**) *Ortho*-aminophenol PEG (AP-PEG) was used as a control to assess the effectiveness of mannose in quenching periodate. Incubation of periodate with mannose prior to addition of the PEG reagent completely inhibits the reaction. (**d**) Combining a pre-oxidation and mannose quench provides useful levels of conversion after addition of *p*aF-MS2.

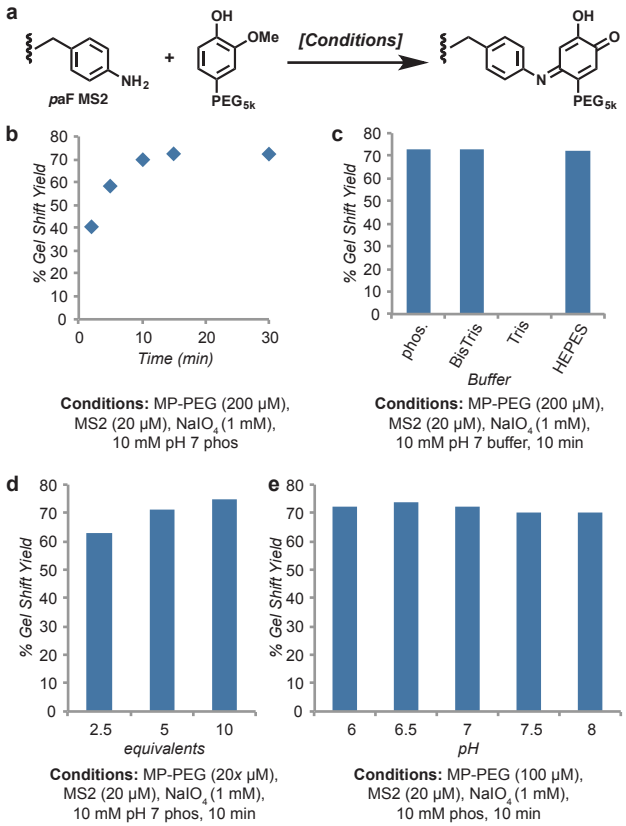
Figure 4. Heterobifunctional crosslinkers. (**a**) Representative heterobifunctional crosslinkers that bear orthogonally reactive groups at the end of a spacer region. Common reactive moieties employed in this class of reagents are depicted. (**b**) Selective N-terminal reactivity of 2-pyridinecarboxaldehyde (2PCA) and design of a new site-selective heterobifunctional crosslinker comprised of 2PCA and *o*-methoxyphenol moieties. (**c**) Synthesis of *o*-methoxyphenol/2PCA heterobifunctional crosslinkers **5** and **6**. Two different crosslinkers were synthesized with variable hydrophilic (**5**) or hydrophobic (**6**) spacers.

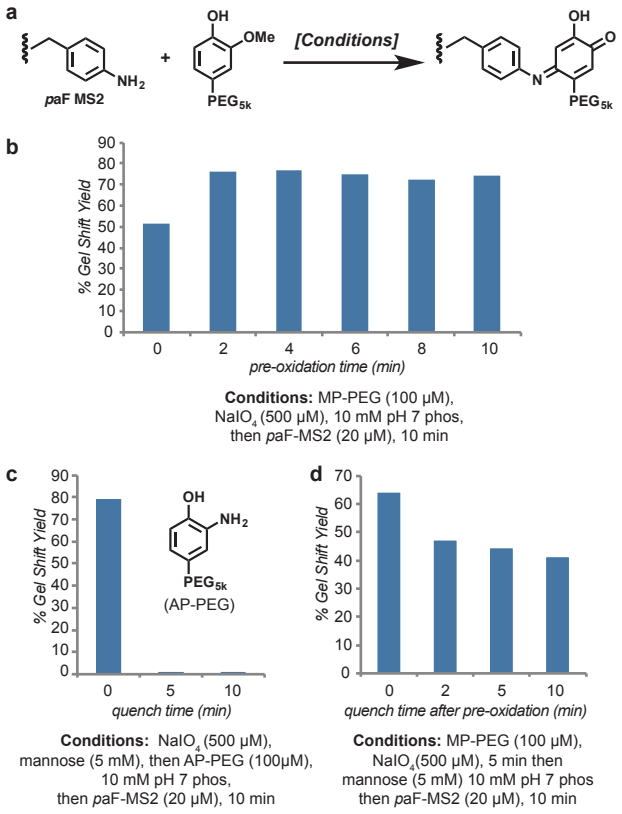
Figure 5. Synthesis of a protein-protein conjugate using *o*-methoxyphenol/2PCA heterobifunctional crosslinkers. (**a**) RNase A is modified to good conversions with crosslinker **5** or **6**, as demonstrated by reconstructed ESI-TOF mass spectra (**b**) and (**c**), respectively. (**d**) Conjugation of RNase A to MS2 in the presence of $NaIO_4$ is confirmed by SDS-PAGE analysis.

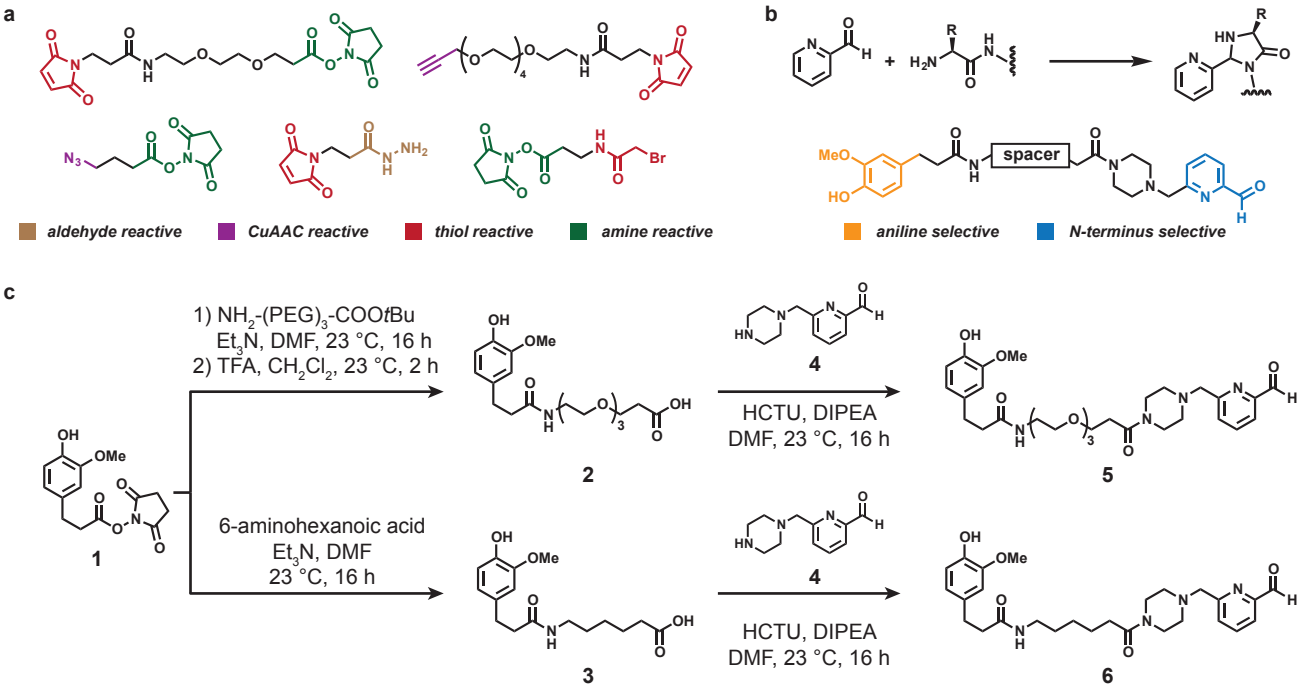
Figure 6. Synthesis of EGF-MS2 conjugates using *o*-methoxyphenol/2PCA heterobifunctional crosslinkers. (**a**) EGF is modified to good conversions with crosslinker **5** or **6**, as demonstrated by reconstructed ESI-TOF mass spectra (**b**) and (**c**), respectively. (**d**) Conjugation of MP-EGF with a PEG spacer to MS2 in the presence of $NaIO_4$ is confirmed by SDS-PAGE analysis after spin concentration to remove unbound EGF.

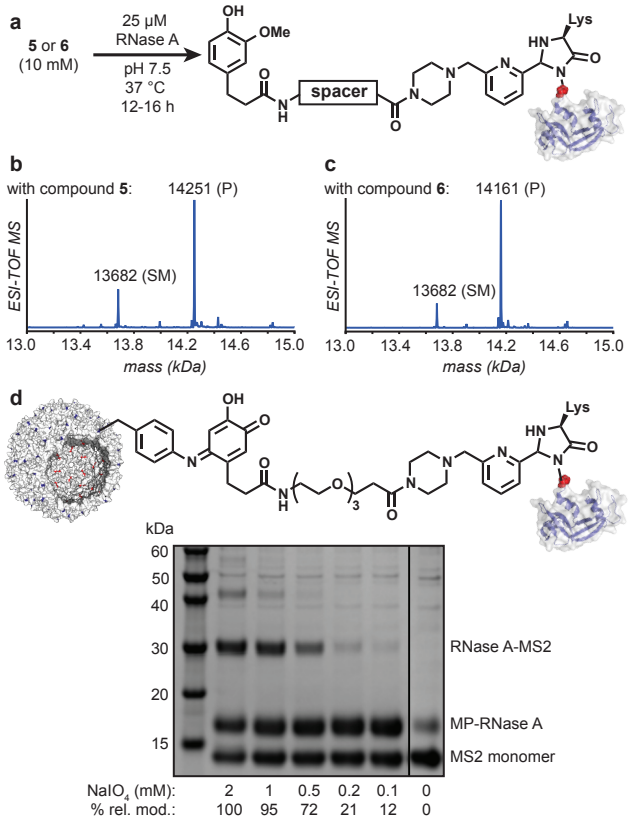
Figure 7. Binding studies of EGF-MS2 conjugates. Flow cytometry data of EGF-MS2-Oregon Green 488 indicate that conjugates bind to EGFR-positive cells (**a**) and do not bind to EGFR-negative cells (**b**). (**c**) Live cell confocal microscopy indicates that these conjugates bind EGFR-positive cells and are rapidly internalized. Cells treated with no agent or capsids lacking EGF do not exhibit significant fluorescence signal in the green channel.

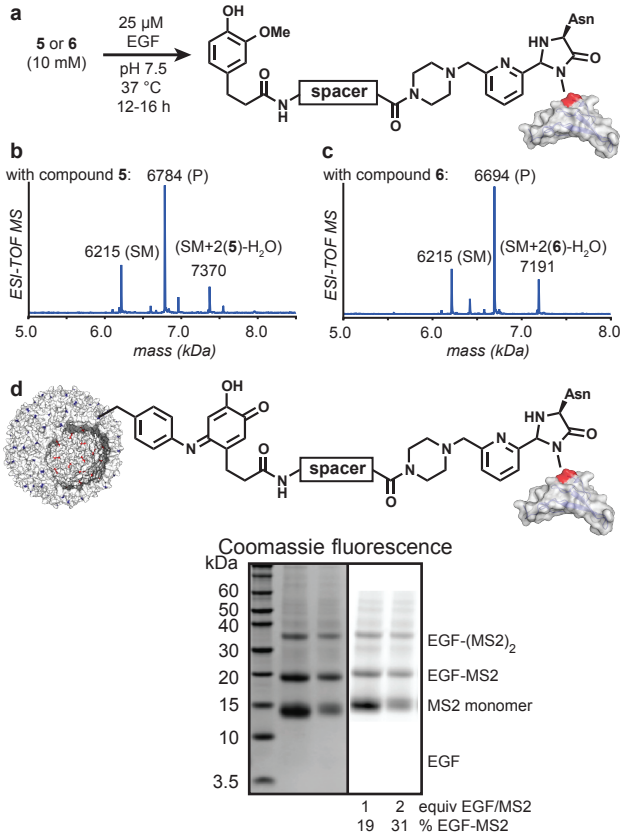

a
NaIO4
(X = NH2 only)
K3Fe(CN)6
K3Fe(CN)6
K3Fe(CN)6
X = NH2 or OH
b
NaIO4
no light
~40% by HPLC
–HN3
c
NaIO4
pH 7
BisTris
~quant. by HPLC
coupled product
280 nm absorb.
time (min)
d
–or–
–or–
vanillin
3-O-methyldopamine
ferulic acid
[convenient, air-stable sources]

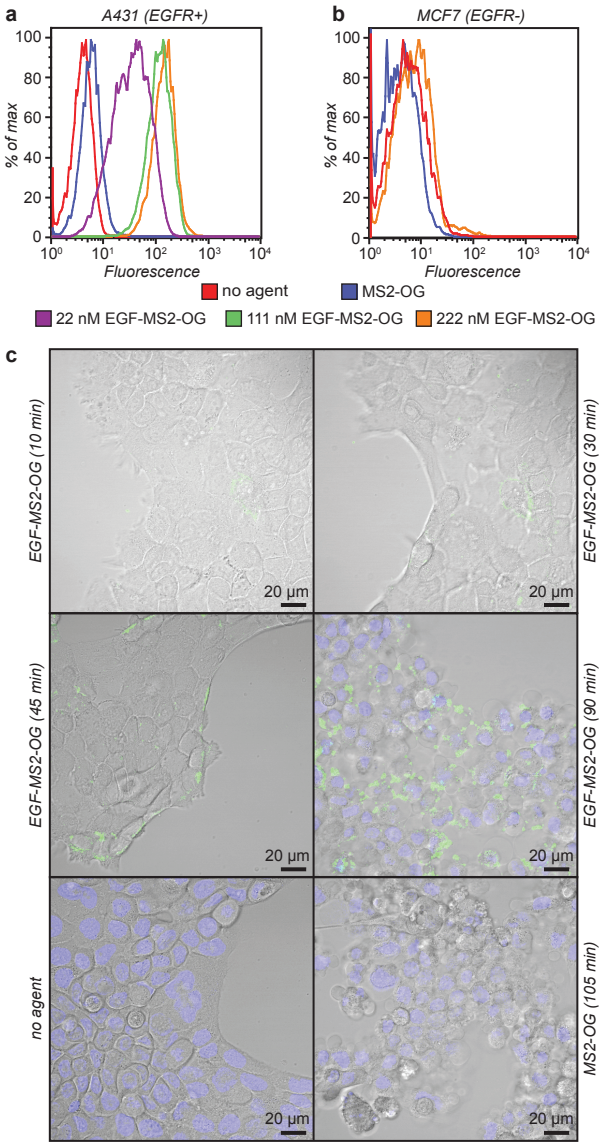

a
OH
OMe
[Conditions]
OH
O
NH2
N
paF MS2
PEG5k
PEG5k
b
% Gel Shift Yield
Time (min)
c
% Gel Shift Yield
phos.
BisTris
Tris
HEPES
Buffer
Conditions: MP-PEG (200 μM), MS2 (20 μM), NaIO4 (1 mM), 10 mM pH 7 phos
Conditions: MP-PEG (200 μM), MS2 (20 μM), NaIO4 (1 mM), 10 mM pH 7 buffer, 10 min
d
% Gel Shift Yield
equivalents
e
% Gel Shift Yield
pH
Conditions: MP-PEG (20x μM), MS2 (20 μM), NaIO4 (1 mM), 10 mM pH 7 phos, 10 min
Conditions: MP-PEG (100 μM), MS2 (20 μM), NaIO4 (1 mM), 10 mM phos, 10 min

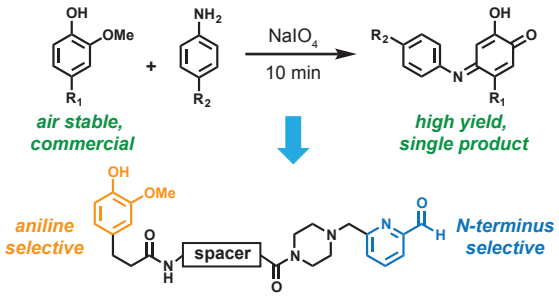

a
OH
OMe
[Conditions]
OH
O
NH2
paF MS2
PEG5k
N
PEG5k
b
% Gel Shift Yield
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
pre-oxidation time (min)
Conditions: MP-PEG (100 μM),
NaIO4 (500 μM), 10 mM pH 7 phos,
then paF-MS2 (20 μM), 10 min
c
% Gel Shift Yield
90
80
70
60
50
40
30
20
10
0
OH
NH2
PEG5k
(AP-PEG)
0
5
10
quench time (min)
Conditions: NaIO4 (500 μM),
mannose (5 mM), then AP-PEG (100μM),
10 mM pH 7 phos,
then paF-MS2 (20 μM), 10 min
d
% Gel Shift Yield
70
60
50
40
30
20
10
0
0
2
5
10
quench time after pre-oxidation (min)
Conditions: MP-PEG (100 μM),
NaIO4(500 μM), 5 min then
mannose (5 mM) 10 mM pH 7 phos
then paF-MS2 (20 μM), 10 min


a
b
aldehyde reactive
CuAAC reactive
thiol reactive
amine reactive
aniline selective
N-terminus selective
spacer
c
1) NH2-(PEG)3-COOtBu
Et3N, DMF, 23 °C, 16 h
2) TFA, CH2Cl2, 23 °C, 2 h
6-aminohexanoic acid
Et3N, DMF
23 °C, 16 h
HCTU, DIPEA
DMF, 23 °C, 16 h
1
2
3
4
5
6


a
5 or 6
(10 mM)
25 µM
RNase A
pH 7.5
37 °C
12-16 h
OH
OMe
spacer
Lys
HN
b
with compound 5:
14251 (P)
13682 (SM)
ESI-TOF MS
13.0 13.4 13.8 14.2 14.6 15.0
mass (kDa)
c
with compound 6:
14161 (P)
13682 (SM)
ESI-TOF MS
13.0 13.4 13.8 14.2 14.6 15.0
mass (kDa)
d
kDa
60
50
40
30
20
15
RNase A-MS2
MP-RNase A
MS2 monomer
NaIO4 (mM): 2 1 0.5 0.2 0.1 0
% rel. mod.: 100 95 72 21 12 0


a
5 or 6
(10 mM)
25 μM
EGF
pH 7.5
37 °C
12-16 h
OH
OMe
spacer
Asn
HN
b
with compound 5:
6784 (P)
6215 (SM)
(SM+2(5)-H2O)
7370
ESI-TOF MS
5.0
6.0
7.0
8.0
mass (kDa)
c
with compound 6:
6694 (P)
6215 (SM)
(SM+2(6)-H2O)
7191
d
Coomassie fluorescence
kDa
60
50
40
30
20
15
10
3.5
EGF-(MS2)2
EGF-MS2
MS2 monomer
EGF
1 2 equiv EGF/MS2
19 31 % EGF-MS2


a
A431 (EGFR+)
b
MCF7 (EGFR-)
% of max
Fluorescence
no agent
MS2-OG
22 nM EGF-MS2-OG
111 nM EGF-MS2-OG
222 nM EGF-MS2-OG
c
EGF-MS2-OG (10 min)
EGF-MS2-OG (30 min)
EGF-MS2-OG (45 min)
EGF-MS2-OG (90 min)
no agent
MS2-OG (105 min)
20 µm


OH
OMe
R1
+
NH2
R2
NaIO4
10 min
R2
OH
O
N
R1
air stable,
commercial
high yield,
single product
OH
OMe
aniline
selective
O
N
H
spacer
N
N
O
N
O
H
N-terminus
selective
site-selective heterobifunctional crosslinker