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Development of Oxidative Coupling Strategies for Site-Selective Protein Modification

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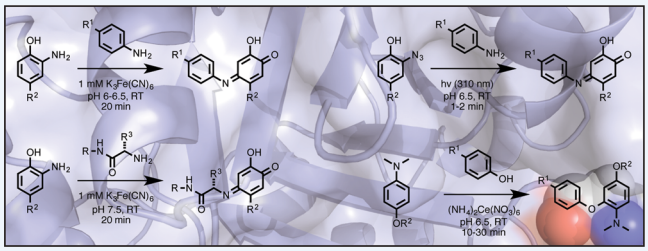
CONSPECTUS: As the need to prepare ever more complex but well-defined materials has increased, a similar need for reliable synthetic strategies to access them has arisen. Accordingly, recent years have seen a steep increase in the development of reactions that can proceed under mild conditions, in aqueous environments, and with low concentrations of reactants. To enable the preparation of well-defined biomolecular materials with novel functional properties, our laboratory has a continuing interest in developing new bioconjugation reactions. A particular area of focus has been the development of oxidative reactions to perform rapid site- and chemoselective couplings of electron rich aromatic species with both unnatural and canonical amino acid residues.



This Account details the evolution of oxidative coupling reactions in our laboratory, from initial concepts to highly efficient reactions, focusing on the practical aspects of performing and developing reactions of this type. We begin by discussing our rationale for choosing an oxidative coupling approach to bioconjugation, highlighting many of the benefits that such strategies provide. In addition, we discuss the general workflow we have adopted to discover protein modification reactions directly in aqueous media with biologically relevant substrates.

We then review our early explorations of periodate-mediated oxidative couplings between primary anilines and *p*-phenylenediamine substrates, highlighting the most important lessons that were garnered from these studies. Key mechanistic insights allowed us to develop second-generation reactions between anilines and anisidine derivatives. In addition, we summarize the methods we have used for the introduction of aniline groups onto protein substrates for modification.

The development of an efficient and chemoselective coupling of anisidine derivatives with tyrosine residues in the presence of ceric ammonium nitrate is next described. Here, our logic and workflow are used to highlight the challenges and opportunities associated with the optimization of site-selective chemistries that target native amino acids.

We close by discussing the most recent reports from our laboratory that have capitalized on the unique reactivity of *o*-iminoquinone derivatives. We discuss the various oxidants and conditions that can be used to generate these reactive intermediates from appropriate precursors, as well as the product distributions that result. We also describe our work to determine the nature of iminoquinone reactivity with proteins and peptides bearing free N-terminal amino groups.

Through this discussion, we hope to facilitate the use of oxidative approaches to protein bioconjugation, as well as inspire the discovery of new reactions for the site-selective modification of biomolecular targets.

INTRODUCTION

The development of new methods for covalent bond formation that takes place rapidly, under mild conditions, and with high levels of chemoselectivity has transformed the ways in which chemists approach the synthesis of new materials with advanced functional properties. One very promising area capitalizes on reactions that proceed in water in the presence of polar functional groups, enabling the generation of multicomponent structures that comprise both biological and abiotic components. Many of these hybrid materials have provided new avenues for drug delivery, molecular sensing, catalysis, and controlled material growth, representing a compelling new frontier for the design and generation of molecular complexity.

To maintain the folded structure of most biomolecules, modification strategies must take place in buffered aqueous solution at near-neutral pH and temperatures below 37 °C. Very few reactions in the canon of organic chemistry meet these requirements, and fewer still possess sufficiently high rate constants to proceed readily at the micromolar concentrations of most biomolecular substrates. To fill this void, recent years have seen much research activity toward developing chemical strategies that complement and extend the tried-and-true methods of the field.[1−4] Many of these reactions fall under the moniker of "click chemistry", originally defined by Sharpless

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and co-workers as reactions that are modular, high yielding, stereospecific, and free of byproducts.[5] In addition to these small molecule-based strategies, a growing number of powerful enzymatic labeling methods have been developed to achieve protein bioconjugation with high degrees of sequence specificity.[6] Together, these new synthetic tools have had an enabling impact on the field of Chemical Biology, and they are rapidly effacing the divisions between organic synthesis and biosynthesis.

Over the past decade, our laboratory has invested considerable effort developing new oxidative coupling strategies for protein bioconjugation. In their most general form, these reactions involve the addition of electron-rich aromatic species to electrophiles generated *in situ*. Both carbon−heteroatom and carbon−carbon bonds can be formed in this fashion, and the products are stable toward pH extremes, heating, nucleophiles, oxidants, and reductants. Through reaction screening, highly specific coupling pairs have been developed, and most of these reactions proceed rapidly at high dilution. This feature allows whole biomolecules to be joined efficiently despite substantial steric bulk and the presence of hundreds of polar spectator groups. In a previous Account, we detailed the use of these strategies for the synthesis of targeted delivery materials for *in vivo* applications.[7] Here, we instead focus on the ways in which these strategies were developed, with the goal of providing both insight into this new reaction class and a blueprint for how protein bioconjugation reactions can be developed.

From the onset, we recognized a number of advantages to an oxidative manifold. First, the majority of biomolecules can tolerate a reasonable measure of oxidative conditions, as we have confirmed many times during our studies. The functional groups of most concern include free cysteines, methionines, tryptophans, and vicinyl diols on carbohydrate groups. Indeed, during the course of our studies, we have observed unwanted oxidations under some conditions that we screened. Fortunately, there exists a wealth of chemical reagents with variable redox potentials and preferences for mechanistically distinct pathways (e.g., two electron vs one electron), suggesting that many of these undesired side reactions could be avoided through judicious oxidant and substrate choices. Second, the large number of water-soluble oxidants in the form of salts facilitates reaction screening. In addition, because most oxidants have pH-variable redox potentials, buffer pH can be used to tune reactivity. Third, using buffered solutions facilitates the stabilization of charged or highly polarized intermediates that result from oxidative conditions. Fourth, in most cases, substrates prior to oxidation are nucleophilic while their oxidized counterparts are electrophilic. This, in effect, allows the unoxidized starting material to behave as a masked or "protected" functional group that can be unveiled by oxidation. This trait is particularly useful because the majority of intrinsically reactive species on proteins and nucleic acids behave as nucleophiles that can be used to intercept these oxidized species. Finally, most oxidants are easy to handle, requiring little if any precaution in excluding air or water.

Throughout the course of these studies, we have focused on developing new reactions directly in buffered aqueous media at low concentrations on biologically relevant substrates. Although such an approach can present its own set of challenges, such as structural elucidation of the resultant products, chemoselectivity issues can be explored upfront and the reoptimization of reactions for aqueous conditions is unnecessary. Furthermore, as will be described below, the unique properties of water as a solvent[5] allow for distinct modes of reactivity that either are not replicable in organic solvents or are highly inefficient. While knowledge of the product structures is crucially important, structural confirmation studies are often among the last steps in our workflow. Instead, our early screening studies rely heavily on either matrix-assisted laser desorption ionization time of flight (MALDI-TOF) or liquid chromatography/mass spectrometry (LC/MS) studies, most often in the context of small peptides bearing the functional group of interest using natural or unnatural amino acids. Adducts are then subjected to tandem mass spectrometry to determine the residue(s) involved in the observed reactivity. In many cases, these studies, when combined with mechanistic considerations, give hints to possible product structures. When high levels of chemoselectivity have been achieved or when structural information may facilitate improving reaction efficiencies, small molecule model systems are prepared for full structural elucidation studies, typically by combinations of 1D and 2D NMR characterization techniques.

DEVELOPMENT OF A NEW OXIDATIVE BIOCONJUGATION STRATEGY

The central design lead for these strategies was provided by a new coupling reaction reported by our laboratory in 2006.[8] We observed that in the presence of sodium periodate, *N*-acyl phenylenediamines (such as **1**, Figure 1a) underwent a clean trimerization reaction to afford red dye derivatives (**3**) described by Bandrowski in the late 1890s.[9] This reaction proceeded in minutes in aqueous solution buffered at pH 6.5 and was unaffected by the presence of complex biomolecules and polar functional groups. From this starting point, we first

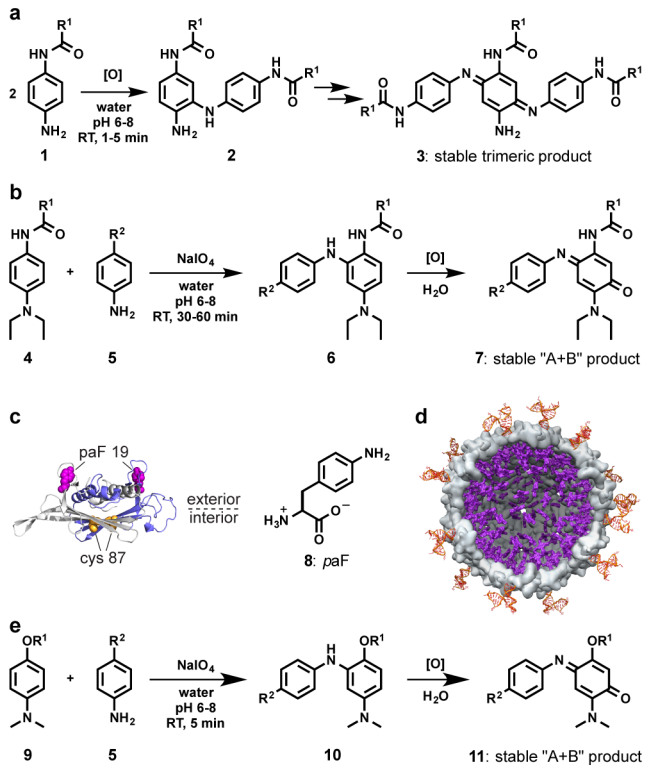

Figure 1. Early examples of oxidative coupling reactions involving (a,b) *N*,*N*-dialkyl phenylenediamines and (e) anisidines. Anilines on MS2 protein dimers (*p*aF residues) are indicated in part c. In part d, DNA aptamers appear in orange and porphyrins appear in purple.

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sought to stop the trimerization reaction through the addition of alkyl groups to the aniline nitrogen, Figure 1b. The resulting *N*,*N*-dialkyl phenylenediamine **4** was unable to form the trimer, as expected; however, this species retained its ability to react with primary anilines, leading to the efficient formation of a stable "A + B" product. Steric hindrance from the *N*,*N*-dialkyl substitution presumably prevented addition of a second aniline at the *ortho* position. Similar to the trimeric dye derivatives, these compounds exhibited high levels of stability, with no degradation observed over 12 h from pH 2−11.

Initial screening studies quickly determined that derivatives of general structure **4** couple rapidly to aniline groups on proteins (**8**) in the presence of periodate with little-to-no participation by native functional groups, Figure 1c,d. The most impressive feature of this strategy is its compatibility with a broad range of molecules, including proteins, nucleic acids, polymers, and small molecule chromophores. The optimized conditions use pH 6−6.5 phosphate buffer with 1 mM periodate. Coupling times range from 10 min for small molecules to 1 h for macromolecules. Following the reaction, periodate is quenched with tris(2-carboxyethyl)phosphine (TCEP) or removed by gel filtration through a Sephadex column.

To generate protein substrates for these reactions, we have shown that aniline groups can be introduced directly using amber codon suppression[10] or through native chemical ligation using an aniline-containing cysteine derivative.[8] Alternatively, ketones introduced at the N-termini of proteins through transamination[11,12] can be coupled to aniline-containing alkoxyamines through oxime formation.[13] Finally, labeling lysine residues with isatoic anhydride provides ready access to aniline derivatives.[8] However, these substrates show diminished reactivity since they are vinylogous amides and suffer the usual lack of site selectivity of lysine modification reactions.

Subsequent studies showed that the functional group tolerance and high efficiency of this reaction under dilute conditions were useful to prepare complex bioconjugates for targeted delivery applications.[10,14,15] This coupling chemistry was used to attach DNA aptamers[16] to the external surfaces of MS2 capsids, providing a means to target tyrosine kinase 7 proteins overexpressed on leukemia cells. Elaboration with porphyrin groups inside of the capsids allowed prodigious amounts of singlet oxygen to be produced upon photoillumination, resulting in efficient killing of only the targeted cells.[15] In the broader sense, these initial investigations demonstrated the potential of oxidative coupling reactions to prepare biomolecular materials of high complexity.

ANISIDINES AS ANILINE COUPLING AGENTS

While reactions involving *p*-phenylenediamine substrates provided a reliable method to label aniline-containing proteins, extended reaction times (up to 2 h) in the presence of periodate resulted in competitive background oxidation of native sulfur-containing residues. Mechanistic considerations of two aspects of this reaction pointed to a rate-determining step associated with oxidation of the phenylenediamine substrate. First, periodate treatment of **4** alone (i.e., in the absence of aniline) led to little observable oxidation of the substrate within the first 5 min. Second, the clean trimerization of **1** suggested that oxidation must be rate determining; otherwise, a low concentration of the nucleophilic species would be expected in solution, and the trimerization would be greatly mitigated. We anticipated that replacement of the *N*-acyl substituent with a more electron-donating moiety would facilitate the oxidation event and increase the overall rate of the reaction. In practice, we found that *N*,*N*-dialkyl-*p*-anisidine derivatives (**9**) undergo rapid oxidative coupling (2−5 min) with aniline derivatives when treated with periodate, Figure 1e.[17] This reaction was used to integrate fluorescein donors and porphyrin acceptors on the interior and exterior surfaces of a viral capsid to achieve photocatalysis at broad wavelengths.[17] To date, this reaction remains among the fastest techniques developed within our laboratory, and the stability of the anisidine coupling partner renders this reaction an excellent choice for many applications.

OXIDATIVE COUPLINGS WITH ELECTRON-RICH NATURAL AMINO ACIDS

The use of *p*-phenylenediamine and anisidine substrates in oxidative couplings with aniline derivatives served as a starting point for another distinct set of bioconjugation reactions. In particular, we envisioned single electron oxidation of these substrates (general structure **4** or **9**) to generate a highly electrophilic radical cation that would be subject to reaction with either a tyrosine or a tryptophan residue. In the former case, beautiful work from the Kodadek laboratory had demonstrated that the use of $Ru(bpy)_3^{2+}$ under visible light illumination effectively generates tyrosyl radicals capable of cross-linking proteins through an oxidative dimerization reaction.[18] Here, we recognized that the key to the success of our proposed strategy would be identification of an appropriate oxidant and substrate to differentiate between the tryptophan and tyrosine residues while minimizing undesired reactions with other side chains.

We began by exploring oxidative conditions to effect the coupling of **4** or **9** with peptide substrates under aqueous, unbuffered conditions.[19] Angiotensin II (a tyrosine-containing peptide) and melittin (a tryptophan-containing peptide) were screened in parallel to differentiate the reactivity of the two most electron-rich aromatic amino acids. Initial screens focused on the identification of an oxidant capable of delivering a single, stable adduct of **4** with the peptides. Common two-electron oxidants proved incapable of controllably delivering coupled products, with either no reaction or overaddition of the substrate observed. A screen of one-electron oxidants, however, identified ceric ammonium nitrate (CAN) as a promising oxidant for the desired transformation, having controllably coupled **4** to both angiotensin and melittin. Subsequent MS/MS experiments clearly demonstrated modification only at the electron-rich residues. Further buffer and pH screens revealed that 50 mM, pH 6.0 bis(TRIS) proved optimal to maintain reactivity while effectively buffering the solution (10 mM buffer solutions do not effectively buffer 1.5 mM solutions of CAN). Although phosphate buffers cause precipitation of CAN from aqueous solutions, this feature provides a convenient method to remove cerium salts upon completion of the reaction.

N,*N*-Dialkylated anilines bearing an additional electron donating group in the *para* position were the most reactive, with **4** and **9** proving optimal. Less electron-rich substrates were unreactive, and primary anilines exhibited overmodification. Importantly, studies with compound **9** demonstrated efficient reactivity with angiotensin but no reactivity with melittin (Figure 2b−d), and MS/MS analysis of this coupling confirmed modification only at the tyrosine residue. Together, these data pointed to a chemoselective coupling of anisidine derivatives with tyrosines. At this point, small molecule model studies were

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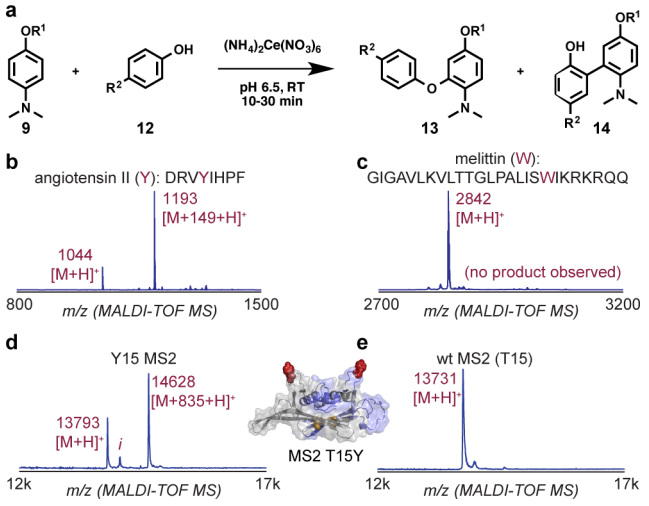

Figure 2. Addition of anisidines to tyrosine residues in the presence of CAN. (a) Two isomeric products are obtained. Reaction selectivity was confirmed on (b,c) peptides and (d,e) MS2 capsids bearing or lacking a tyrosine in position 15 (red).

undertaken to determine the structure of the conjugates, as summarized in Figure 2a.

The above reactions constitute an effective means of performing oxidative couplings of electron-rich canonical amino acids with aniline derivatives.[19] A few features of these reactions are worthy of mention. First, the use of CAN as oxidant allows one of the key limitations of oxidative couplings utilizing periodate to be circumvented: the oxidative degradation of 1,2-diols present in sugars on glycoproteins. While CAN is a powerful oxidant, subsequent studies on many proteins have indicated that it is quite compatible with a variety of substrates if extended periods of incubation with high concentrations are avoided (3:1 with respect to the ansidine coupling partner greatly mitigates any unwanted side oxidations). The use of bis(TRIS) buffer appears to be unique in maintaining reactivity while buffering the solutions. The basis of this phenomenon remains unclear; however, a notable color change takes place upon addition of solutions of CAN to bis(TRIS), potentially signifying the formation of a complex responsible for the subsequent chemistry.

OXIDATIVE COUPLINGS OF AMINOPHENOLS AND CATECHOLS

In our earliest work, we explored the coupling of phenylenediamines (**1**) with *o*-aminophenols (**15**, Figure 3a) generated on viral capsids through tyrosine modification.[20] Determination of the product structure proved challenging; the clean reactivity observed on proteins could never be translated to small molecule analogues. Returning to aminophenol substrates several years later, we recognized that a pitfall of the earlier studies was that both coupling partners can be subject to oxidation. Thus, we hoped that utilizing toluidine-derived coupling partners would greatly simplify the reaction with putative *ortho*-iminoquinone intermediates. Here, we believed this reaction would benefit from the higher degree of polarization in the alkene relative to a *para*-iminoquinone, promoting a faster conjugate addition than any reactions we had previously explored.

At the outset of these studies, however, we were cognizant that in our proposed coupling, a rate limiting oxidation could

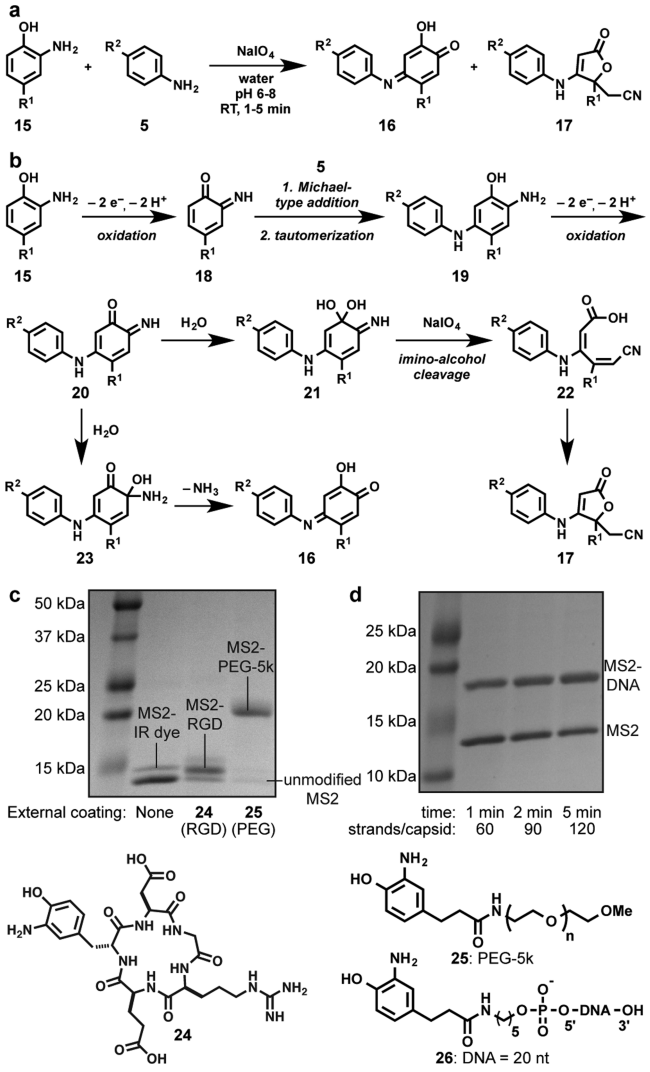

Figure 3. Oxidative couplings with aminophenols. The overall reaction is shown in part a, and proposed mechanisms are provided in part b. The modification of *p*aF MS2 capsids internally labeled with an IR dye appears in part c, and the attachment of DNA to *p*aF MS2 capsids is shown in part d.

complicate this bioconjugation reaction since unoxidized aminophenol would be present in solution and could potentially participate in unwanted reactions. Studies describing the oxidation of *o*-aminophenol substrates with various substitution patterns demonstrated that facile dimerization of such substrates can take place. Surprisingly, relatively subtle structural alterations resulted in dramatically different outcomes during the reactions.[21]

Despite these reservations, we elected to examine this reaction in buffered aqueous conditions under periodate-mediated oxidation. A pH 6.5 phosphate-buffered solution of 2-amino-*p*-cresol and toluidine (1:1) was treated with periodate for 5 min. HPLC analysis of the crude reaction mixture showed an uneven mixture of two products and no starting materials. Purification provided sufficient material for structural characterization of the major product through X-ray crystallographic analysis, which revealed that an unprecedented oxidative ring contraction of the aminophenol-containing ring had occurred, giving rise to compound **17** (Figure 3a).[22] A reasonable

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mechanistic hypothesis for its formation is provided in Figure 3b.

The minor product could not be obtained in sufficient quantity for structural determination. The high-resolution mass spectrum of this compound suggested that only one nitrogen atom was present in the structure, and thus, we anticipated that use of the corresponding catechol in the coupling reaction would provide an alternative route to the minor product through similar reactivity.[23,24] Indeed, a single product that coeluted by HPLC with the minor product above resulted from oxidative coupling with toluidine. This aniline/catechol coupling is highly efficient and provided material for structural characterization. NMR analysis revealed the product to be that expected from direct conjugate addition/oxidation of the *o*-quinone species, **16**.

A time screen in the context of protein-based substrates revealed that the reaction often reached completion within 30 s. This exceptionally high rate of reaction suggested that although periodate was used as an oxidant, side reactions with other oxidation-sensitive functional groups could likely be mitigated. As a demonstration of the compatibility of this reaction with sugars, in couplings of aminophenol-labeled DNA to aniline-labeled antibody Fc domains,[13] oxidation of the glycans could be completely suppressed by the addition of 10 mM mannose (10× with respect to periodate). As a testament to the versatility of this protocol, a number of hybrid biomolecular materials have been prepared using this reaction.[13,25−27] Additional examples of MS2 capsid modification appear in Figure 3c,d.

Here, a practical note concerning the introduction of aminophenols onto coupling partners of interest is worth mention. The catechol and aminophenol substrates are not stable to prolonged storage in solution. Instead, we prefer to use an *o*-nitrophenol moiety that can be readily reduced with sodium dithionite ($Na_2S_2O_4$) prior to the desired coupling. The nitrophenol congener is very stable and can be stored indefinitely in solution. In addition, we prefer the aminophenol to the catechol due to the ability to store it as the nitrophenol congener, despite the fact that the catechol derivative gives a single product.

BEYOND PERIODATE: FERRICYANIDE-MEDIATED OXIDATIVE COUPLINGS

Although the periodate-mediated coupling of aminophenols with aniline derivatives is an extremely reliable reaction, two products are produced, and some unwanted oxidation was observed in a few scenarios (albeit surmountable by judicious choice of equivalents or through the use of appropriate additives). This prompted us to screen other oxidants for this coupling, which revealed a few other reagents that were capable of initiating the desired coupling to varying degrees. These were CAN, Ag(I) salts, Cu(II) salts, and potassium ferricyanide [$K_3Fe(CN)_6$]. Of these, we pursued ferricyanide since it is a mild oxidant and reactions employing this reagent gave excellent levels of conversion.[28] An additional attractive feature was that, unlike CAN and Ag(I) salts, buffer incompatibilities were not of concern. Importantly, we found this oxidant maintains the highest levels of compatibility with oxidation-sensitive functional groups; proteins containing free thiols, sugars, methionine residues, and tryptophan residues are not oxidized by $K_3Fe(CN)_6$, even in vast excesses of reagent over long periods of time.

Further exploration of this coupling with toluidine revealed that a single product (**16**, Figure 4a) is formed, likely because

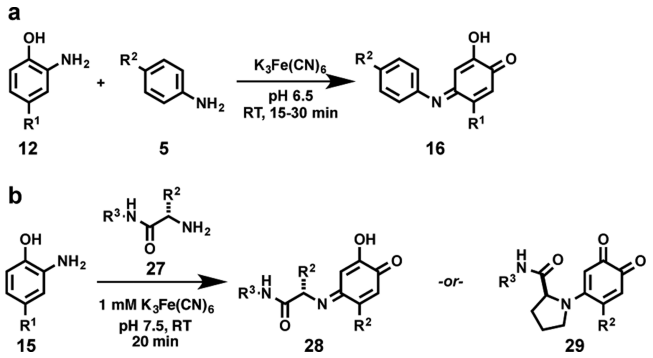

Figure 4. Modification of (a) anilines and (b) N-terminal residues using aminophenols and ferricyanide.

ferricyanide cannot perform a concerted two-electron oxidation necessary to generate the ring-contracted product. These products are exceedingly stable over a broad rage of pH values (2−10) and temperatures (50 °C for 18 h showed no loss in product). Additionally, large excesses (500 equiv) of many nucleophilic and reducing species are generally compatible with the product at room temperature over a period of 18 h, with the only major exception being the use of anisidine wherein a ∼75% reduction in product was observed.[28]

While this reaction is slower than the periodate-mediated coupling (typically 15−30 min versus 2−5 min), comparable levels of conversion are generally achieved. A pH screen using MS2 viral capsids bearing aniline side chains showed 6.0−6.5 to be optimal. Interestingly, at higher pH (7.0−7.5), we observed small amounts of secondary modifications (higher pH values had not been assessed with the periodate-mediated coupling). At this time, the precise site of these extra modifications was unknown (see the section below), though we were able to suppress such unwanted reactivity effectively through the addition of 1−10 mM imidazole.[24,28]

N-TERMINAL CHEMISTRY AND APPLICATIONS

As mentioned above, coupling of *o*-quinone/iminoquinone intermediates with aniline derivatives was found to be highly selective under slightly acidic conditions; however, at higher pH, further adducts were observed. Literature precedents from Messersmith,[23] Kodadek,[24] and others[29−32] describing the reaction of native amino acids and proteins with putative *o*-quinone precursors are well documented in the literature. Here, we hoped to both identify the source of this reactivity and develop conditions to achieve an efficient and selective reaction.

To determine the nature of the observed reactivity and optimize for selectivity,[24] we again chose to use angiotensin and melittin as model peptides that contain many of the common reactive functional groups found on proteins, including free N-terminal amino groups, lysine, arginine, histidine, tryptophan, and tyrosine residues. These peptides were treated with 2-amino-*p*-cresol and ferricyanide for 20 min under varying pH (5.5−8.5), and the reactions were analyzed by MALDI-TOF.[33] As previously observed, higher levels of reactivity were observed on both peptides with increasing pH, and nearly quantitative conversion was achieved on angiotensin at or above pH 7.5. Importantly, in each case, only a single modification was observed. Tandem MS/MS experiments on

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the crude coupling with angiotensin clearly demonstrated that the N-terminal amino acid was involved in the reaction. Given the pH dependence of the reaction, we deduced that the N-terminal amino group was responsible for the reaction, giving rise to a product of analogous structure to that from coupling with toluidine derivatives (Figure 4b). This hypothesis was bolstered by the emergence of the characteristic purple/red color of the product during the course of the reaction. Furthermore, an N-terminal pyroglutamate peptide was completely unreactive, confirming the necessity of a free N-terminal amino group. Next, we probed the effect of the N-terminal residue on the reaction efficiency by screening a series of X-ADSWAG peptides (X = canonical amino acids except cysteine, due to known cross reactivity). While all N-termini were reactive (average yield of 65% by MALDI-TOF analysis), the N-terminal proline peptide consistently showed the highest levels of reactivity in terms of both rate and conversion. In transitioning to protein-based substrates, the necessity of an N-terminal proline was more stringent in order to achieve high levels of conversion. However, useful levels have been achieved with other amino acids.[33]

Interestingly, we found the coupling efficiency of these reactions could not be improved through the use of more equivalents of the aminophenol coupling partner. In fact, the use of excess aminophenol during the coupling resulted in notably worse conversion. We attribute this result to the fact that at higher equivalents of **15**, the rate-limiting oxidation allows the unoxidized aniline-containing starting material to undergo competitive homocoupling, which has been observed in reactions using either high numbers of equivalents of the aminophenol or no coupling partner at all. Mechanistic studies of this reaction were simplified by performing couplings with the catechol derivative where dimerization was not observed. Kinetic studies of the N-terminal proline coupling with 4-methylcatechol by monitoring the appearance of the distinct UV−vis absorption at 520 nm allowed a second order rate constant of 44 ± 4 M^{-1} s^{-1} to be determined when using a vast excess of oxidant, which allowed us to overcome the rate determining oxidation. The corresponding reaction with aniline derivatives was too fast to allow an accurate determination of the second order rate constant with this method.[33]

PHOTOCHEMICAL COUPLINGS AND THEIR APPLICATIONS

When aminophenols are used, both the periodate and the ferricyanide coupling reactions presumably involve the formation of a highly reactive iminoquinone intermediate. We have recently discovered an interesting way to access this intermediate photochemically, thus avoiding the use of any additional oxidants.[34] This method involves the irradiation of *o*-azidophenols, which lose nitrogen to afford a nitrene species that immediately undergoes redox tautomerization with the neighboring phenol (Figure 5a).[35−37] These intermediates couple rapidly to aniline groups as described above, yielding the same products after additional aerobic oxidation. This reaction can be quite efficient for the modification of anilines on proteins, requiring as little as 2 min of irradiation at 310 nm to achieve nearly complete conversion for small molecules and >60% yield after 5 min of irradiation for PEG molecules. The *o*-azidophenols can be stored for extended periods at 0 °C in the absence of light.

Because irradiation is required to initiate coupling, this method offers particular promise for generating patterned

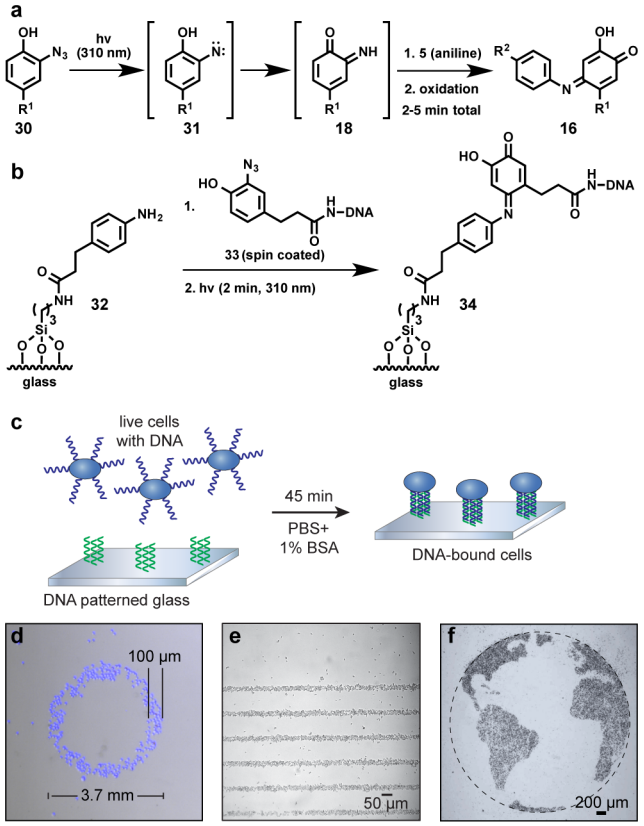

Figure 5. Photochemical coupling reactions based on azidophenols. The basic reaction is shown in part a and its application to DNA surface patterning is shown in part b. (c) Live cells bearing complementary DNA strands can be captured by the surface patterns, as shown for (d) vascular smooth muscle cells and (e,f) yeast.

arrays of biomolecules on surfaces. The use of traditional photolithography for this purpose is hampered by the fact that most photoresists react chemically with biomolecules, rendering them incompatible. Via the azidophenol coupling reaction, a "resist-free" version of photopatterning has been achieved on aniline-coated glass substrates prepared using silane chemistry.[34] A solution of DNA strands bearing azidophenol groups at their 5′-termini is then applied via spin coating. Irradiation through an appropriate photomask results in the conjugation of the DNA molecules to the surface in the exposed regions (Figure 5b). The unbound DNA can be removed from the surface through simple rinsing to leave the desired pattern behind.

One application of this patterning technique is the capture of living cells through DNA hybridization, Figure 5c. We have previously published methods for the attachment of synthetic DNA strands to both mammalian[38,39] and nonmammalian[40] cells, giving them the ability to adhere to surfaces bearing the sequence complements. This methodology is ideal for placing cells in controlled environments for use in diagnostic assays and synthetic biology applications. The results of this process using photopatterned DNA are shown in Figure 5d−f.[34] We are currently exploring applications of this method for the generation of well-defined arrays containing multiple cell types, with the goal of studying the ways in which neighboring cell interactions govern cancer cell morphology, migration, and drug response. In addition, we are using this method to

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combine different types of microorganisms to study metabolite sharing and other symbiotic behaviors.

CONCLUSIONS

Through these studies, a new set of bioconjugation reactions has been developed based on oxidative coupling pathways.

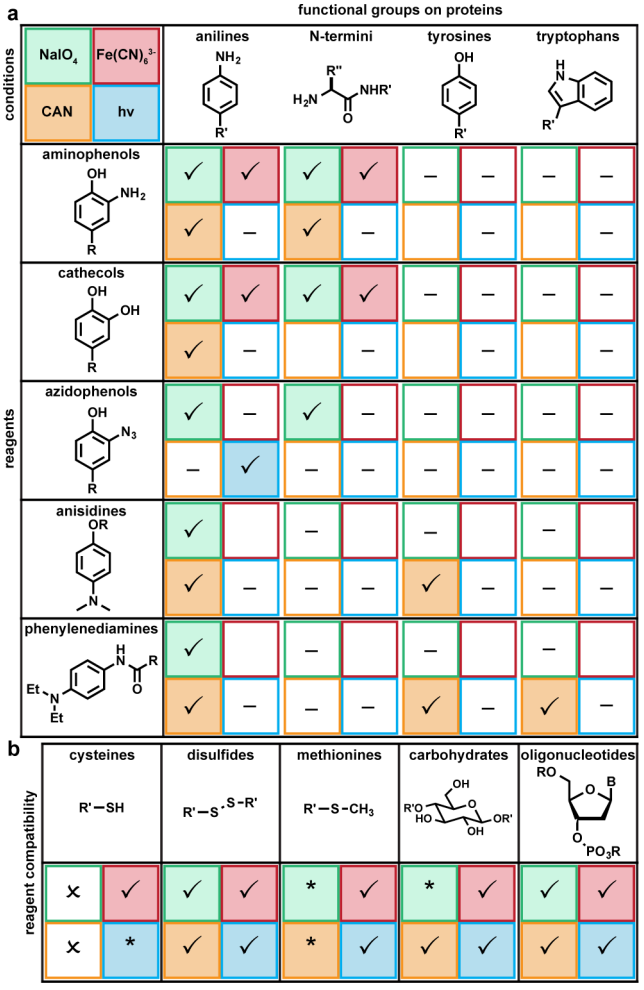

Figure 6. Chemoselectivity and functional group compatibility of the new oxidative protein modification reactions. (a) Successful combinations of reagents, protein functional groups, and oxidants are indicated by the check marks. The dashes indicate combinations known not to react, and empty boxes indicate that the combination has not yet been evaluated. (b) The compatibilities of the four basic sets of reaction conditions are indicated for oxidation-sensitive protein functional groups. An ∗ indicates that side reactions can be suppressed through the use of additives.

Many of these reactions are the first of their kind, providing both useful methods for synthetic group attachment and design leads for future strategies. Taken as a whole, these methods offer a particularly attractive set of features. First, they exhibit very high coupling rates, with typical reactions occurring in 2−20 min even at micromolar concentrations. This capability avoids prolonged exposure to the reaction conditions, minimizes opportunities for biomolecule denaturation, and is highly advantageous for time-critical applications, such as radiolabeling. In addition, the rapid rates of these reactions are only minimally influenced by steric factors, allowing the efficient coupling of full-sized biomolecules in similar amounts of time. Our studies have indicated that the products are highly stable with respect to hydrolysis, reducing agents, oxidants, and strong nucleophiles. The oxidative strategies described herein also rely on easily prepared, inherently water-soluble functional groups and use oxidants that can be easily removed from the product mixtures. Most significantly, by subtly changing the reaction conditions, the reactant structures, or both, these methods can be used to target a variety of natural and unnatural functional groups with high selectivity, offering a greater range of substrates for which they can be used, as summarized in Figure 6. Considering the broad range of electron-rich aromatic and heterocyclic molecules that has yet to be explored in this chemistry, it is likely that many additional transformations can be discovered through the application of the reaction development workflow that has been established. The development of new oxidative coupling strategies and the synthesis of complex biomolecular materials through their use are proceeding apace in our laboratory.

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Notes

The authors declare no competing financial interest.

Biographies

Adel M. ElSohly grew up in Mississippi. In 2007, he received B.S. degrees in Mathematics, Chemistry, and Pharmaceutical Sciences and an M.S. degree in computational chemistry from the University of Mississippi where he worked with Prof. Gregory Tschumper studying weak, noncovalent interactions. He then joined the laboratory of Prof. Scott A. Snyder at Columbia University where he worked on the total synthesis of myrmicarin and securinega alkaloids. In 2012, he moved to UC Berkeley as a postdoctoral fellow in the laboratory of Prof. Matthew Francis where his research has focused on the development of new bioconjugation reactions and materials for biomedical applications.

Matthew B. Francis received his undergraduate degree in Chemistry from Miami University in Oxford, OH, in 1994. From 1994 to 1999, he attended graduate school at Harvard University, working with Prof. Eric Jacobsen. He then moved to UC Berkeley, where he was a Postdoctoral Fellow working with Prof. Jean Fréchet. Matt started his independent career in the UC Berkeley Chemistry Department in 2001, and he is also a faculty member at the Lawrence Berkeley National Laboratory.

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