Heterotelechelic Organometallic PEG Reagents Enable Modular Access to Complex Bioconjugates

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ABSTRACT: Organometallic oxidative addition complexes (OACs) have recently emerged as a powerful class of reagents for the rapid and chemoselective modification of biomolecules. Notably, the steric and electronic properties of the ligand and aryl group can be modified to tune the kinetic profile of the reaction and permit regioselective *S*-arylation. Using the recently developed dicyclohexylphosphine-based bidentate *P*,*N*-ligated Au(III) OACs, we computationally and experimentally examined the effects of sterically bulky and electron deficient aryl substrates to achieve selective *S*-arylation. With this mechanistic insight, aryl substrates based on 4-iodoanisole and 3,5-dimethyl-4-iodoanisole were incorporated as end groups to generate a heterotelechelic bis-Au(III) poly(ethylene glycol) (PEG). This reagent performed rapid and regioselective *S*-arylation with a model biomolecule, designed ankyrin repeat protein (DARPin), to form a protein-polymer OAC *in situ*. This OAC mediated a second *S*-arylation with biologically relevant thiolated small molecules (metal chelator, saccharide, and fluorophore) and macromolecules (polymer and therapeutic peptide). It is envisioned that this approach could be utilized for the rapid construction of biomacromolecular heteroconjugates with *S*-aryl linkages.

Selective chemical labeling of proteins remains among the most popular set of tools used by researchers to probe and manipulate protein behavior across several fields such as molecular biology, proteomics, and medicine.[1–3] As the specificity and tunability of these labeling techniques improve, complex macromolecular heterostructures become more accessible, thereby allowing researchers to study deeper intricacies of the protein space.[4] Cysteine residues are common targets for protein modification, due to their nucleophilicity and low proteomic abundance, which allows for more specific labeling.[1,5–7] Conventional cysteine modification techniques, such as Michael addition or pyridyl disulfide exchange are useful, but the bond lability stemming from these traditional bioconjugates can lead to reversibility of the linkage.[8–10] Alkylation of cysteine residues is generally irreversible, however, the kinetics of the currently available reactions are relatively slow.[11] Alternatively, *S*-arylation chemistry generates a *S*-*C*(sp^2) linkage to the biomolecule that is hydrolytically resistant, and the reaction can be fast depending on the reagent used.[12–14]

Notably, palladium OACs have been used to forge protein homo- and heterodimers with stable *S*-aryl linkages *via* an elegant oxidative addition relay process.[15,16] This two-step reaction relies on the reinsertion of the zerovalent Pd-based species into a second, adjacent aryl halide/pseudohalide site after regeneration from the first bioconjugation process. To form the protein heterodimers, the OAC can then complete a second *S*-arylation upon the addition of a separate cysteine-containing protein. Alternatively, Pd(II) reagents containing an *N*-hydroxysuccinimide (NHS) ester can undergo amine acylation followed by *S*-arylation at a cysteine residue of a second protein to generate protein heterodimers.[17] In a

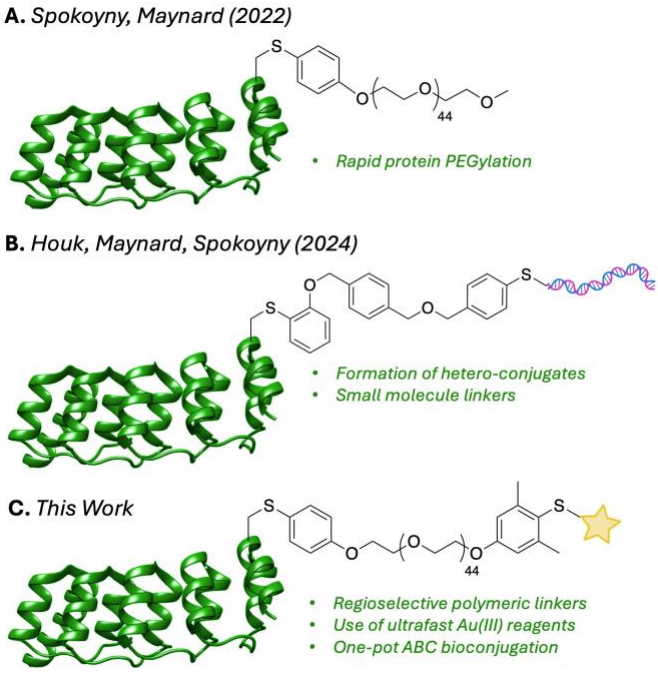
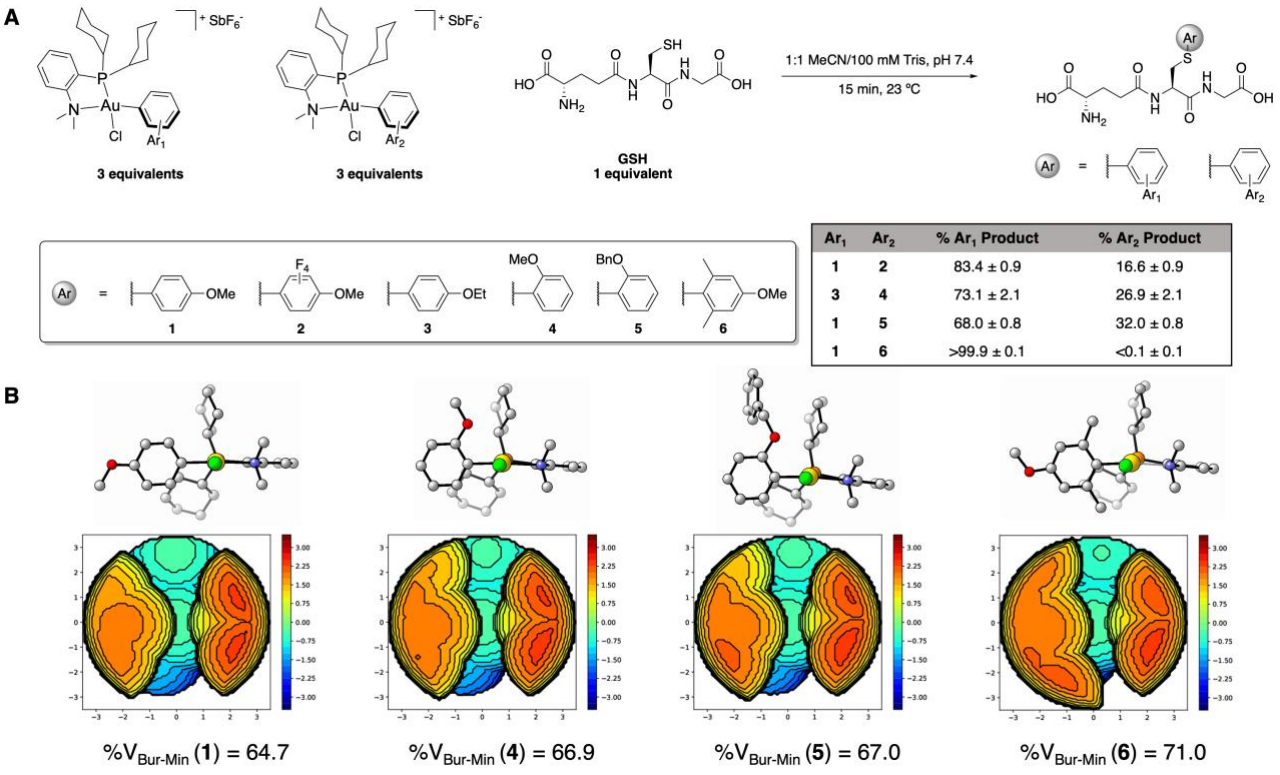


Figure 1. A. Rapid PEGylation of DARPin using organometallic Au(III) PEG reagents. **B**. Construction of heteroconjugates using bis-Au(III) reagents. **C**. Formation of heterodimeric block copolymer protein conjugates using polymeric bis-Au(III) reagents. Star indicates payloads of interest including a polymer, a therapeutic peptide, and biologically relevant small molecules.

similar linker strategy that utilizes multiple orthogonal nucleophiles, *ortho*-pyridinium sulfones have been used to rapidly undergo cysteine *S*-arylation while a pendant fluoride performed S_NAr with a phenol on a dye payload.[18] These approaches employ different reagents for sequential functionalization, however, modification of the local cysteine environment can also be leveraged for this purpose. For example, engineering a π-clamp sequence near a *C*-terminal cysteine (Phe-Cys-Pro-Phe) permits chemoselective S_NAr of the π-clamped cysteine to form protein heterodimers.[19] In each of the examples noted above, heteroconjugate linkers were limited to oligomers and small molecules. Pd-based reagents have been used to mediate catalyst transfer polymerization (CTP) for the formation of protein-polymer conjugates, wherein polymerization could be terminated by addition of thiol-containing species, including a second protein.[20] This innovative graft-from strategy represents one of the first reports of a macromolecular linker that forges hydrolytically stable *S*-aryl bonds between two distinct proteins. However, CTP is typically limited to aromatic monomer groups, which limits the degree of polymerization accessible in water.

We have previously reported Au(III) OACs for cysteine PEGylation, providing nearly quantitative conversion to PEGylated product in one minute (**Figure 1A**).[21] Recently, we investigated the kinetics of various Au(III) OACs, finding that OACs containing the dicyclohexylphosphine-based (PCy_2) bidentate *P*,*N*-ligand performed *S*-arylation more rapidly than the previously used di-1-adamantylphosphine- (PAd_2) and di-*tert*-butylphosphine-based *P*,*N*-ligated Au(III) OACs.[22–24] This acceleration was due to the ultrafast coordination of the thiol to the cationic Au(III) center with the smaller diphosphine substituent. In that same report, it was also demonstrated how one can intentionally slow down coordination of the thiol to the Au(III) center by increasing the steric bulk around the metal through the addition of *ortho*-substituents on the aryl group. Consequently, tethering two sterically distinct Au(III) sites with a linker enables one to prepare protein-oligonucleotide conjugates in one pot (**Figure 1B**).[22] However, the linkers were limited to small molecules and OACs were comprised of the PAd_2-containing *P*,*N*-ligand that imparted slower bioconjugation kinetics. Therefore, we sought to demonstrate that this selectivity could be achieved across macromolecular linker scaffolds, and while using the PCy_2-based *P*,*N*-ligand complex, which demonstrates faster bioconjugation. Computational modeling of both the electronic and steric properties of this system guided the substrate design to maximize selectivity, and a heterotelechelic polymer was prepared such that the terminal PCy_2-based *P*,*N*-ligated Au(III) OACs possessed differentiated rates of *S*-arylation (**Figure 1C**). Ultimately, this polymeric OAC mediates the regioselective one-pot formation of ABC block macromolecules including a protein-block copolymer conjugate, a protein heterodimer, and protein small molecule conjugates.

We have used both electronically and sterically disparate PAd_2-based *P*,*N*-ligated Au(III) OACs to afford selective *S*-arylation.[22] We sought to investigate both types of substrate modifications to access selectivity with the ultrafast PCy_2-based *P*,*N*-ligated Au(III) OACs. Accordingly, Au(III) OACs with tetrahydro- (**1**) and tetrafluoroaryl (**2**) groups with SbF_6^- counterions were then synthesized to examine the effects of electronics on the reaction (**Figure 2A**). A methoxy substituent on the aryl group was incorporated *para* to the Au center to simulate the electronics of a functionalized linker. Competition experiments were performed with **1** and **2** in the presence of glutathione (**GSH**) as a model thiol-containing peptide, which resulted in a modest ~5:1 selectivity for the non-fluorinated *S*-arylated product (**Figure 2A**, Figure S29). Density functional theory (DFT) calculations using methanethiol as a model thiol (See SI for details) indicate that the reductive elimination from the electron poor tetrafluoroaryl-containing OAC (**2**) is 1.1 kcal/mol higher than the tetrahydroaryl-containing OAC



Ar_1	Ar_2	% Ar_1 Product	% Ar_2 Product
1	2	83.4 ± 0.9	16.6 ± 0.9
3	4	73.1 ± 2.1	26.9 ± 2.1
1	5	68.0 ± 0.8	32.0 ± 0.8
1	6	>99.9 ± 0.1	<0.1 ± 0.1

Figure 2. **A**. General competition experiment scheme along with competition experiment results for sterically different Au(III) OACs. See pages S47-S51 for additional details. **B**. Au(III) OAC geometries for substrates **1**, **4**, **5**, and **6** calculated at the ωB97X-D/6-311+G(d,p), SDD, CPCM(Water)//B3LYP-D3/6-31G(d), LANL2DZ, CPCM(Water) level of theory along with their corresponding buried volume plots.

(**1**) (SI Figures S46-48), which is in agreement with the experimental results.

center and slow coordination to the Au atom, we prepared an OAC based on 2-iodo-*m*-xylene, again with *para*-methoxy

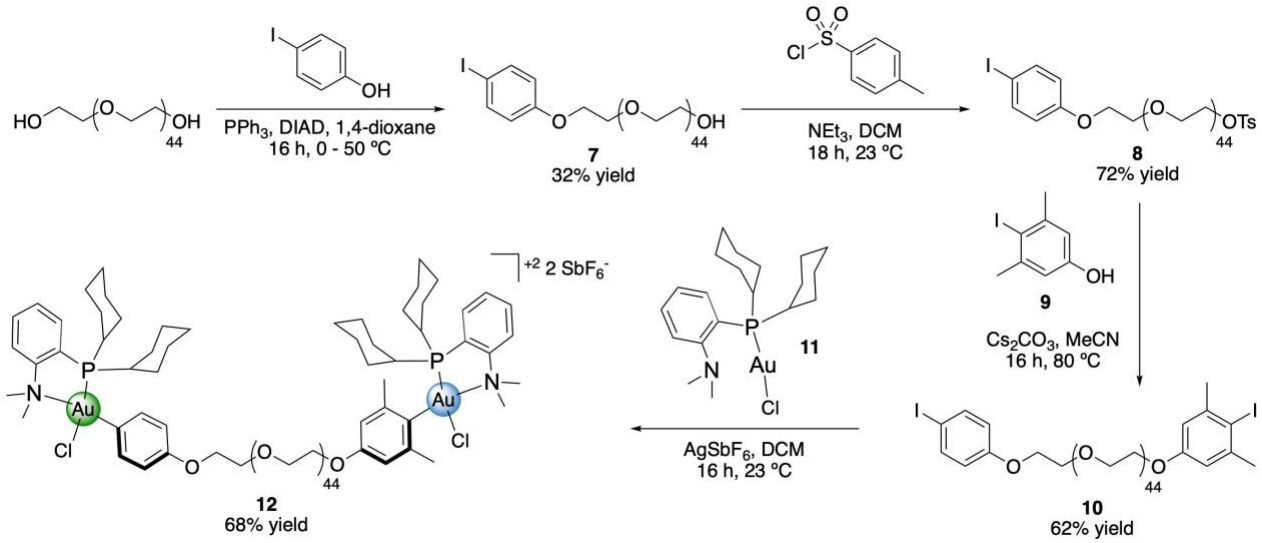


Figure 3. Synthetic scheme for the preparation of the heterotelechelic bis-Au(III) PEG linker **12**. Additional experimental detail can be obtained in the supplemental information.

Unable to achieve sufficient selectivity with electronically differentiated aryl substituents, we sought to leverage the steric properties of the Au(III) aryl substrates to afford higher degrees of selectivity. In these electronically rich substrates, similar to **1**, the equilibrium for thiol coordination is significantly shifted toward the *S*-coordinated intermediate, and we have demonstrated that the rate of thiol coordination is the selectivity-determining step in these cases.[22] In an attempt to achieve selective *S*-arylation, we prepared OACs with *para*-ethoxy (**3**) and *ortho*-methoxy substituted (**4**) aryl reagents to modify the steric profile which can affect the rate of thiol coordination to the Au center. In the competition experiment with **3** and **4**, we observed ~3:1 selectivity for the *para*-substituted *S*-arylated **GSH** conjugate (**Figure 2A**), which was significantly less selective than the PAd_2-based *P*,*N*-ligated congeners (>95:5) – exemplifying the reactivity-selectivity principle.[22] We hypothesized that by using larger aryl substituents, we could slow thiol coordination further and achieve greater selectivity. Accordingly, **5** was prepared with an *ortho*-*O*-benzyl ether, but in the competition experiment, we observed a slightly diminished ~2:1 selectivity for the *para*-substituted *S*-arylated product (**Figure 2A**). To explain these results, the minimum percent buried volume ($\%V_{Bur\text{-}Min}$) of the Au(III) chloride complexes was calculate using geometries obtained by DFT (See SI for details).[25] $\%V_{Bur\text{-}Min}$ has been used to explain catalytic activity of transition metal complexes, and this steric descriptor was recently used to predict the coordination kinetics of a thiol to *P*,*N*-ligated Au(III) OACs.[26–28] We observed 64.7 $\%V_{Bur\text{-}Min}$ for **1**, whereas **4** and **5** had 66.9 and 67.0 $\%V_{Bur\text{-}Min}$, respectively (**Figure 2B**). This small difference in $\%V_{Bur\text{-}Min}$ between **4** and **5** agrees with the observed experimental results. To further increase the steric profile around the Au(III) functionalization (**6**). Notably, the oxidative addition of 2-iodo-*m*-xylene does not proceed with the PAd_2-based *P*,*N*-ligand, likely due to the additional steric hindrance imparted by the bulky and conformationally rigid 1-adamantyl groups. With a calculated $\%V_{Bur\text{-}Min}$ of 71.0 for **6**, it was expected that **6** would give more selectivity than both reagents containing *ortho*-substitution (**4** and **5**). In the competition experiment with **1** and **6**, >99.9% *S*-arylation of **GSH** by OAC **1** was observed. Additionally, when **6** was used in the absence of a second Au(III) reagent, complete functionalization of **GSH** occurred in 15 minutes, demonstrating its competency as a cysteine *S*-arylation reagent (Figure S32).

It was hypothesized that the observed selectivity between **1** and **6** would allow for incorporation of these end groups into a heterotelechelic polymer linker that would be capable of mediating sequential and selective *S*-arylation reactions (**Figure 3**). Therefore, a Mitsunobu reaction with substoichiometric 4-iodophenol and commercial 2 kDa PEG was performed to achieve mono-arylation (**7**). Unreacted PEG starting material and di-arylated byproducts were removed *via* preparative high-performance liquid chromatography to produce **7** in high purity. Next, the remaining terminal PEG alcohol was tosylated to produce **8** and

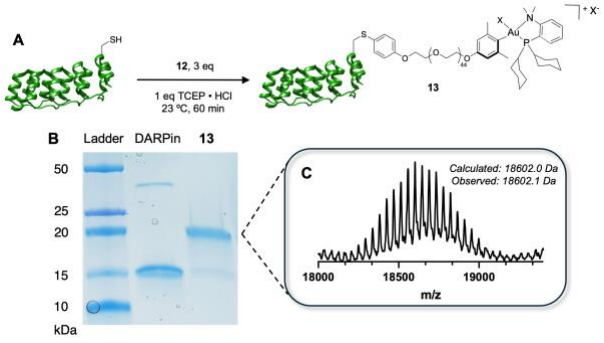


Figure 4. **A**. Synthetic scheme of regioselective PEGylation of DARPin with **12**. X = chloride or formate due to formic acid in the LC-MS mobile phase. **B.** SDS-PAGE gel of *S*-arylation of **12** with DARPin to produce **13** - not purified. Lane 1 - protein ladder. Lane 2 - DARPin (non-reducing conditions). Lane 3 - **13**. **C**. Deconvoluted total ion chromatogram of **13**. Observed and calculated masses are with X = formate.

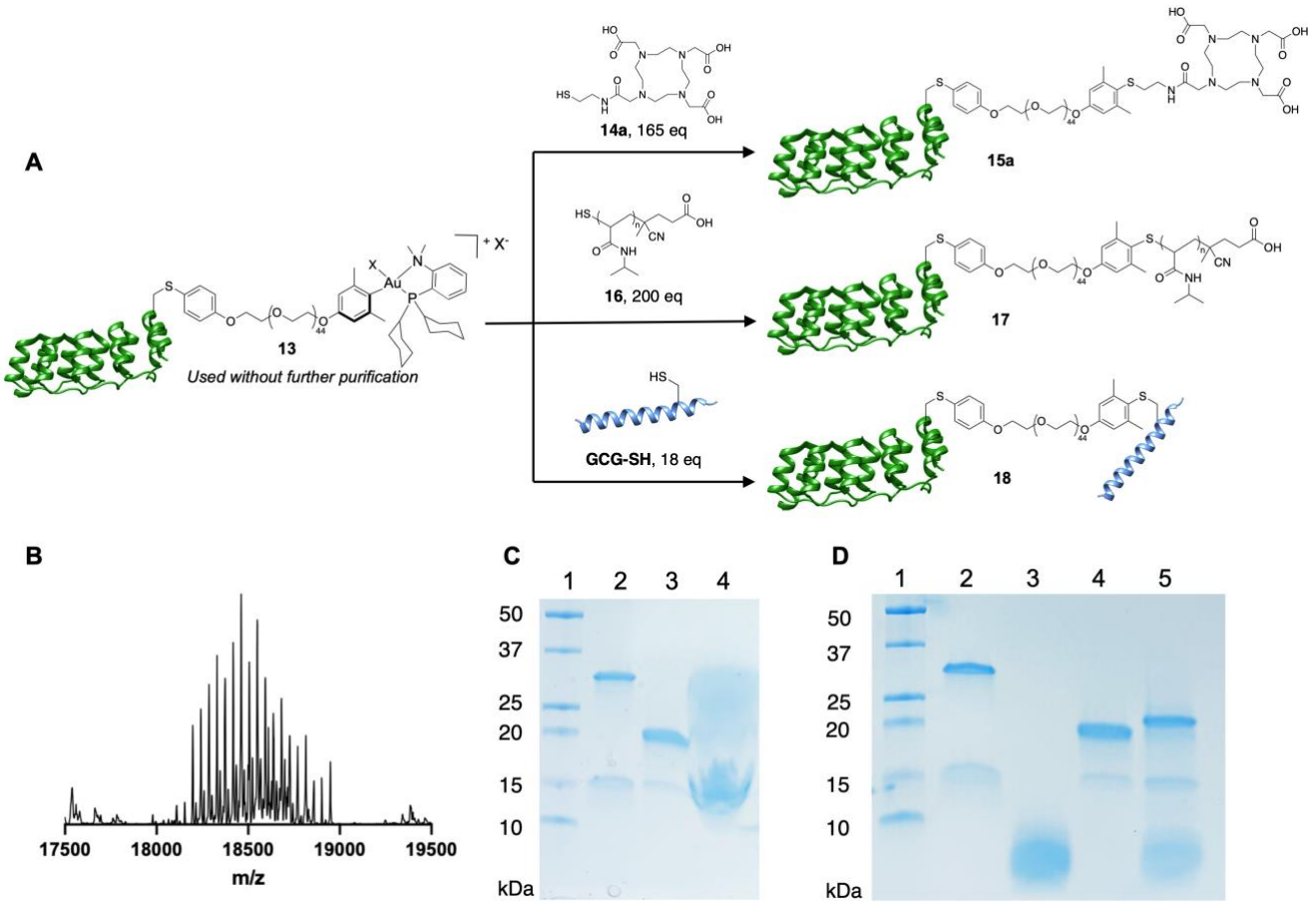


Figure 5. **A**. Synthetic scheme of *S*-arylation of DOTA-SH (**14a**), pNIPAM (**16**), and thiolated glucagon (**GCG-SH**) with the PEGylated DARPin OAC (**13**). X is ambiguous due to counterions in the reaction buffer. **B**. Deconvoluted total ion chromatogram of **15a**. Calculated mass is 18505.3 Da. Observed mass is 18506.3 Da. **C**. SDS-PAGE gel for the *S*-arylation of **13** with **16** to produce a DARPin block copolymer conjugate (**17**) - not purified. Lane 1 - protein ladder. Lane 2 - DARPin (non-reducing conditions). Lane 3 - **13**. Lane 4 - **17**. Conjugate **17** appears from 20–35 kDa and excess **16** is present at ca. 15 kDa. **D**. SDS-PAGE gel for the *S*-arylation of **13** with **GCG-SH** to produce a DARPin-GCG heterodimer (**18**) - not purified. Lane 1 - protein ladder. Lane 2 - DARPin (non-reducing conditions). Lane 3 - **GCG-SH**. Lane 4 - **13**. Lane 5 - **18**. **18** appears at ca. 22 kDa. Unless otherwise noted, all gel lanes were prepared under reducing conditions (5% mercaptoethanol v/v). See supplementary information for additional details.

subsequently arylated with 4-iodo-3,5-dimethylphenol (**9**) to yield **10** (See SI pages S52-S60). Finally, oxidative addition of **10** with [(PCy_2)Me-DalPhos]AuCl (**11**) and silver hexafluoroantimonate as a halide scavenger resulted in the heterotelechelic bis-Au(III) PEG OAC (**12**).

Next, it was investigated if regioselectivity could be achieved with these sterically distinct aryl substituents with a polymeric linker and protein (**Figure 4A**). In previous work, our group has performed PEGylation of a designed ankyrin repeat protein (DARPin) containing a single surface exposed cysteine residue at 70 μM with 1.3 equivalents of Au(III) PEG OAC.[21] Continuing with DARPin as the model protein, *S*-arylation of **12** was conducted using these conditions (see SI for details). Formation of DARPin-PEG-DARPin homodimer was observed as a significant byproduct along with incomplete conversion of DARPin monomer by SDS-PAGE (SI Figure S42). Therefore, the reaction conditions were optimized such that conversion to mono-PEGylated product **13** was maximized, while the formation of a DARPin homodimer, which would indicate a lack of regioselectivity, was minimized. As we propose that the bimolecular coordination is the selectivity-determining step at low concentrations, we hypothesized that decreasing the concentration of the reaction would improve the regioselectivity of the reaction. By diluting the conjugation to 35 μM and using 3 equivalents of **12**, 95% conversion of DARPin to **13** was observed with no observable formation of homodimer product after one hour by SDS-PAGE (**Figure 4B**, conversion determined by ImageJ optical densitometry). This confirmed that the selectivity in the small molecule experiments could be translated to the macromolecular context. The formation of **13** was also verified *via* LC-MS (**Figure 4C**), indicating that the *m*-xylene Au(III) portion of **12** remained intact and available for further conjugation following the completion of the first *S*-arylation reaction. Therefore, **13** was used in the following experiments without purification, highlighting the practicality of this one-pot method.

We first sought to demonstrate that the DARPin-PEG OAC (**13**) could undergo a second *S*-arylation event with thiolated small molecules, thereby providing a modular method for PEG end-group functionalization. Therefore, a small library of thiolated derivatives of biologically relevant small molecules including DOTA (**14a**, metal chelator), glucose (**14b**, saccharide), and coumarin (**14c**, fluorophore) were reacted with **13**. The resulting heteroconjugates (**15a-c**) were characterized by LC-MS, wherein the corresponding masses of each product were observed (**Figure 5B**, Figures S43-S45).

The addition of a second, distinct polymer block to an existing protein-polymer conjugate can allow for the generation of additional structures with unique properties. Therefore, to obtain a second macromolecular block of interest, p(NIPAM)-SH (**16**) was prepared *via* reversible addition fragmentation chain transfer

(RAFT) polymerization and subsequent aminolysis (See SI for details). p(NIPAM)-SH is a promising candidate to influence conjugate behavior, as its thermal responsivity in aqueous solution is well-characterized.[29] Polymer **16** (200 eq) was added directly to **13** to yield **17** (**Figure 5A**). Conjugate formation was observed by SDS-PAGE, showing good conversion to the heteroconjugate (**Figure 5C**). The modular graft-to preparation of discrete protein-block copolymer conjugates such as **17** may be amenable to many polymer classes and sizes, with many potential applications in drug delivery and biomedicine.[30,31]

As an alternative target, we aimed to prepare a polymerically-linked protein heterodimer. This type of macromolecular scaffold is known to be influential for improving the pharmacokinetics and *in vivo* activity of protein dimers by allowing flexibility between receptors to improve receptor targeting and binding.[32,33,34] Therefore, we added thiolated glucagon (**GCG-SH**) (18 eq) as a model therapeutic hormone to **13** to produce **18**, wherein the corresponding molecular weight could again be observed by SDS-PAGE (**Figure 5D**). The second *S*-arylation occurred at 11 μM, demonstrating the robustness of this transformation even at low concentrations. This one-pot formation of a protein heterodimer results in *S*-aryl linkages that are known to be resistant to hydrolysis and reversibility, which could be useful *in vivo*.[35]

In summary, we used computational and experimental studies to determine the factors governing PCy_2-based *P*,*N*-ligated Au(III)-mediated *S*-arylation and subsequently leveraged them to inform substrate design. These substrates were incorporated as end groups on a heterotelechelic PEG linker for sequential functionalization. Control of kinetics on these end groups allowed for the one-pot construction of protein heteroconjugates with excellent regioselectivity and good kinetics. This report showcases the utility of tunable, air-stable organometallic polymer reagents in the modification and construction of complex biomolecular conjugates. Furthermore, the generation of protein-polymer-Au(III) complexes demonstrated herein is expected to enable rapid developments in the growing fields of biological therapeutics, protein assembly, and nanomedicine.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details, NMR spectra, characterization, and computational details (PDF).

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Author Contributions

G. E. K. and J. W. T. contributed equally to this work. G. E. K., J. W. T., M. F. P., H. R. M., and E. A. D. performed experiments. J. W. T. performed the computational studies. G. E. K. and J. W. T. wrote the original manuscript with contributions from all authors. G. E. K., J. W. T., A. M. S., and H. D. M. conceived and designed the experiments. K. N. H., A. M. S, and H. D. M. supervised the research and edited all versions.

‡These authors contributed equally.

Conflicts of Interest

H. R. M., E. A. D., A. M. S., and H. D. M. are co-inventors on several patent applications from UCLA associated with the Au(III)-based bioconjugation technology.

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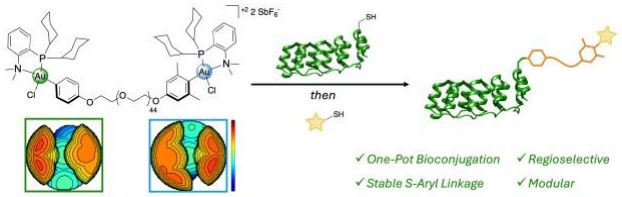
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✓ Stable S-Aryl Linkage
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