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Ligand-Free Palladium-Mediated Site-Specific Protein Labeling Inside Gram-Negative Bacterial Pathogens

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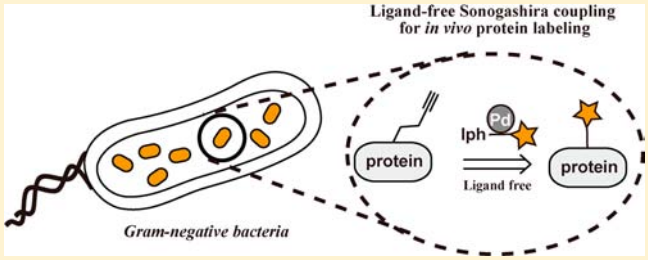
Supporting Information

ABSTRACT: Palladium, a key transition metal in advancing modern organic synthesis, mediates diverse chemical conversions including many carbon−carbon bond formation reactions between organic compounds. However, expanding palladium chemistry for conjugation of biomolecules such as proteins, particularly within their native cellular context, is still in its infancy. Here we report the site-specific protein labeling inside pathogenic Gram-negative bacterial cells via a ligand-free palladium-mediated cross-coupling reaction. Two rationally designed pyrrolysine analogues bearing an aliphatic alkyne or an iodophenyl handle were first encoded in different enteric bacteria, which offered two facial handles for palladium-mediated Sonogashira coupling reaction on proteins within these pathogens. A GFP-based bioorthogonal reaction screening system was then developed, allowing evaluation of both the efficiency and the biocompatibilty of various palladium reagents in promoting protein−small molecule conjugation. The identified simple compound−$Pd(NO_3)_2$ exhibited high efficiency and biocompatibility for site-specific labeling of proteins in vitro and inside living *E. coli* cells. This Pd-mediated protein coupling method was further utilized to label and visualize a Type-III Secretion (T3S) toxin-OspF in *Shigella* cells. Our strategy may be generally applicable for imaging and tracking various virulence proteins within Gram-negative bacterial pathogens.



INTRODUCTION

Bioorthogonal reactions, many of which are derived from or inspired by the Cu(I)-promoted azide−alkyne cycloaddition (CuAAC), revolutionized our ability to label and manipulate biomolecules under living conditions.[1−18] However, despite a series of newly developed Cu(I)-stabilizing ligands that have now facilitated its applications particularly on cell surface,[19−25] the CuAAC reaction itself is still largely hindered from being used inside living cells due to the toxicity of Cu(I) ions.[13,26−28] Indeed, labile copper is known to be poisonous to almost all forms of life, and thus, copper ions are tightly controlled within both prokaryotic and eukaryotic cells.[29−31] Besides the oxidative damage of biomolecules by the Cu(I)/Cu(II) pair via a Fenton-like reaction,[31] a recent elegant study also showed that the highly thiolphilic Cu(I) ions can directly impair iron−sulfur cluster containing enzymes inside bacterial cells, which may count for the primary lethal effect of copper in microorganisms and potentially within various eukaryotic species.[32] To avoid the use of Cu(I), many valuable "metal-free" bioorthogonal reactions including 1,3-dipolar cycloaddition between azides and cyclooctynes and the inverse-electron-demand Diels−Alder cycloaddition between *trans*-cyclooctene and tetrazine have been developed and applied to diverse living organisms with high biocompatibility and efficiency.[33−48] Meanwhile, the repertoire of transition-metal-mediated ligation reactions has also been increasingly explored as alternative choices.[49−55]

An emerging example is the palladium-mediated cross-coupling reactions that have been successfully demonstrated on exogenously delivered small molecules inside cells,[56] paving the way for applying similar chemistry to label proteins,[53] the most abundant biomolecules within a cell. Notably, microorganisms have long been utilized for reductive deposition of Pd(II) to Pd(0) nanoparticles, application of which ranges from metal recovery to synthesis of nanocatalysts for bioremediation.[57] These interesting works, termed "bio-Pd" technology, further confirmed the compatibility of Pd species with bacterial cells.[58] Indeed, Pd-mediated labeling reactions have recently been performed on purified proteins as well as on proteins displayed on *E. coli* cell surface without apparent toxicity.[59−62] However, although attempts have been made for residue-specific labeling of internal proteins from a methionine auxotroph *E. coli* strain,[63] the biocompatibility as well as reaction procedure

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adopted for live-cell labeling require further verification. In particular, a high amount of preactivated Pd reagent (1 mM) from in vitro study was directly applied to living bacterial cells without further optimization in an in vivo setting, which rendered the modification process rather complicated and potentially toxic to the cell. Moreover, the membrane permeability of the reported negatively charged Pd ligands was not demonstrated. Nevertheless, expanding the Pd-triggered chemistry for site-specific protein labeling within a native cellular context remains a challenge.

Enteric pathogens, many of which are Gram-negative bacteria species such as *Shigella flexneri*, *Salmonella typhimurium*, *enteropathogenic E. coli* (*EPEC*), and *enterohemorrhagic E. coli* (*EHEC*), can produce a plethora of virulent proteins (e.g., type III effectors) that cause severe infections in humans.[64] Direct labeling and manipulation of proteins within these pathogenic microorganisms would substantially facilitate our understanding of the virulence mechanism of such toxins. We recently expanded the genetic code of several enteric bacterial pathogens by the pyrrolysine(Pyl)-based system, which enabled us to install functional handles for CuAAC-mediated protein labeling in these species.[65] However, the toxic Cu(I) ions prevented us from using CuAAC for labeling internal proteins in living microorganisms.

Herein we present the development of a ligand-free and biocompatible Pd-mediated reaction for protein conjugation with a self-liganding cross-coupling partner (Figure 1a). This method, in conjunction with two rationally designed unnatural amino acids (UAAs) carrying bioorthogonal handles (Figure 1b), allowed site-specific and highly efficient labeling of proteins within bacterial cells. Such a ligand-free reaction system is simpler and more efficient and biocompatible than the previous approach with a ligand. Furthermore, the identified palladium reagent in the absence of a ligand can be transferred from an in vitro screening process directly into an intracellular setting without the concern of ligands' cell permeability issues. Taken together, we show that the ligand-free Pd-mediated protein conjugation method offers a facial and generally applicable approach for bioorthogonal labeling of proteins inside different Gram-negative bacterial pathogens.

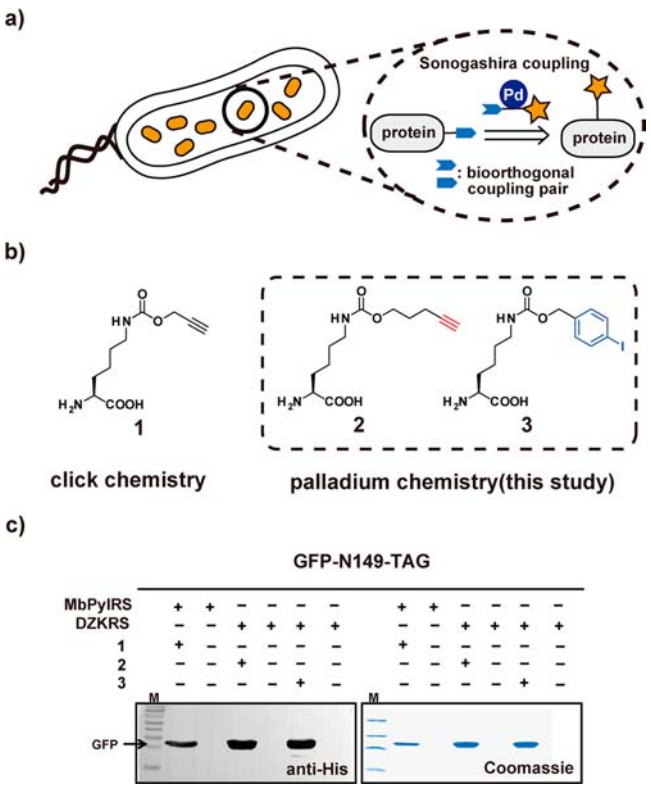

Figure 1. Pd-mediated bioorthogonal coupling for site-specific protein labeling inside bacterial cells. (a) Labeling scheme. (b) Structures of two rationally designed Pyl analogues (**2**, **3**) for Pd-mediated cross-coupling reactions on proteins. Previously reported Pyl analogue for protein click labeling (**1**) is also shown. (c) Immunoblotting analysis confirming incorporation of **1**–**3** into the GFP-N149-TAG template with a C-term His-tag.

RESULTS AND DISCUSSION

We started by introducing functional groups capable of participating in Pd-mediated chemistry into proteins via Genetic-code expansion with UAAs carrying the desired handles. The Pyl-based system was chosen because its Pyl-tRNA synthetase (PylRS)-$tRNA^{Pyl}_{CUA}$ pair has recently become an attractive "one-stop shop" for people who wish to site specifically incorporate unnatural functionalities into proteins in diverse prokaryotic and eukaryotic cells[66−75] and even in multicellular organisms.[76,77] For example, a series of Pyl analogues containing bioorthogonal reaction handles (e.g., alkene, alkyne, azide, tetrazine) has been developed by many laboratories for diverse bioconjugation applications.[14−18,40−48,78−84] We designed and synthesized two Pyl analogues carrying a terminal alkynyl group (**2**, alk-) and an iodophenyl group (**3**, Iph-) (Figure 1b, Supporting Information). These two Pyl analogues offer a pair of reaction partners capable of conducting the aqueous copper-free Sonogashira cross-coupling reaction triggered by Pd species. Although an alkyne-containing Pyl analogue (**1**, Figure 1b) was previously developed by Chin et al. for protein click labeling,[15] it is not suitable for Pd-mediated coupling reactions due to elimination of its propargyl group by Pd ions via a "depropagylation" reaction.[85−87] To solve this problem, **2** was utilized that contains a longer aliphatic linker inert to Pd-mediated cleavage. In order to effectively introduce **2** and **3** into proteins, we used green fluorescent protein bearing an amber codon at residue Asp149 (GFP-N149-TAG) as the template to screen wild-type PylRS from *M. barkeri* (wt-MbPylRS) as well as two MbPylRS mutants with an expanded binding pocket (Figures S1 and S2, Supporting Information). The DZKRS mutant (L274A, C313S) that was previously evolved by us for recognition of a diazirine-containing Pyl analogue[80] showed excellent incorporation efficiency for both **2** and **3**, whereas the rest of the MbPylRS variants exhibited either low or no incorporation of **2** or **3** (Figure 1c and Figures S1a and S2a, Supporting Information). These results further demonstrated the excellent flexibility of the enlarged Pyl-binding pocket in DZKRS for accommodating long linear as well as bulky aromatic side chains. Electrospray ionization mass spectrometry (ESI-MS) of purified full-length GFP-N149-**2** (expected 27 847 Da, found 27 847 Da) and GFP-N149-**3** (expected 27 997 Da, found 27 997 Da) confirmed that the incorporated **2** and **3** were not modified within *E. coli* cells (Figures S1c and S2b, Supporting Information). Yields of GFP-N149-**2** and GFP-N149-**3** proteins produced in *E. coli* cells by the DZKRS-$tRNA^{Pyl}_{CUA}$ pair were estimated to be ∼30 and ∼20 mg/L, respectively. Finally, in addition to *E. coli*, **2** and **3** were also shown to be effectively encoded in *Shigella* and *Salmonela* cells using our recently

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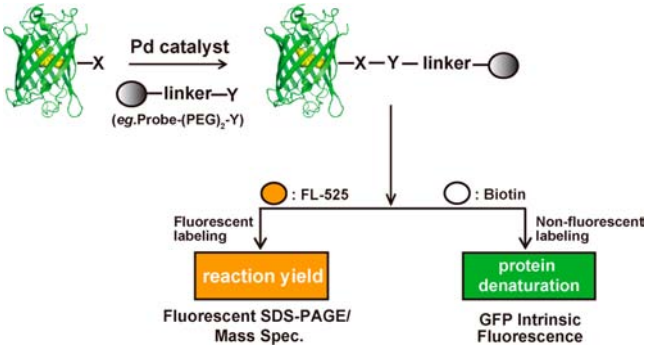
developed Pyl-based incorporation system in these pathogens[65] (Figure S3, Supporting Information).

Next, we aimed to identify biocompatible and efficient Pd reagents for protein labeling. Because Pd ions may denature proteins, a major challenge is to find a suitable Pd reagent that can overcome the potential denaturation effects on proteins without compromising its catalytic activity. Most current catalyst-discovery strategies relied on examining the product yield from robust small molecules or model peptide substrates. Alternatively, the more fragile intact proteins have been used only as the template for searching optimal ligands or optimizing reaction conditions rather than for direct catalyst screening. Therefore, the identified reagents or reaction conditions may not meet the more stringent requirement for labeling native proteins, which often contain various reactive residues with a delicate tertiary structure prone to denaturation. Furthermore, the congestion of residues on the protein surface may also pose a significant steric hindrance, which may largely affect the results from the small-molecule-based catalyst screening. We aimed to solve these problems using a GFP-based system capable of investigating both the conjugations efficiency and the potential denaturation effects of different reagents used for protein labeling (Scheme 1).

Scheme 1. GFP-Based System Capable of Investigating Both the Conjugation Efficiency and the Denaturation Effects of Different Reagents for Protein Labeling[a]



[a]Conjugation efficiency can be determined by in-gel fluorescence from protein samples labeled by a fluorophore (e.g., FL525). Conjugation yield can be further quantified by mass spectrometry. Potential denaturation effects can be determined by intrinsic fluorescence of GFP during the conjugation process with a nonfluorescent tag (e.g., Biotin).

We first examined the conjugation efficiency between our GFP templates (GFP-N149-**2** or GFP-N149-**3**) and the labeling fluorophore Fluor 525 (FL525) bearing a pair of complementary reaction groups (Iph- and alk-) for Pd-mediated Sonogashira cross-coupling (Figure S4, Supporting Information). A total of 12 representative commercially available Pd reagents with or without ligands (Figure 2a) was surveyed which can be divided into five groups (Figure 2b): entries 1−3 contain previously reported ligands for Pd-mediated protein cross-coupling in vitro and on bacteria surface,[61,63] entries 4−7 might undergo a similar mechanism as Pd nanoparticles[88,89] upon reduction by sodium ascorbate without additional ligands, entries 8 and 9 both possess a water-soluble phosphine ligand, entry 10 is a commonly used Pd(0) catalyst in Sonogashira reaction,[88,90] and entries 11 and 12 have thiourea ligands. Reagent loadings were all kept at 100 μM, which are 10 equiv of GFP templates, and reaction yields determined by relative fluorescence from the FL525-labeled protein samples on SDS-PAGE gel are listed in Figure 2b and Table S1, Supporting Information (raw data are included as Figure S5, Supporting Information). To our surprise, two simple Pd sources, Na_2PdCl_4 and $Pd(NO_3)_2$, were found to exhibit the highest catalytic activity (pH 7.6, room temperature) without the need for preactivation or extra ligands (Figure 2b, entries 5 and 7, Figure S5, Supporting Information). Further investigation indicated that the PEG linker in Iph-FL525 may enhance reaction efficiency by stabilizing the catalytic intermediates (Figure S6, Supporting Information), which is consistent with a very recent report using self-liganded, Pd-mediated Suzuki−Miyaura coupling for protein PEGylation.[91] In addition, reaction yields in entries 4, 6, and 7 were increased in the order of $OAc^- < Cl^- < NO_3^-$, which is consistent with previous reports showing a similar reactivity trend among palladium nanoparticles generated from these different Pd(II) sources.[88,92] Noteworthy, the labeling efficiencies were generally higher between an alk-bearing GFP and an Iph-tethered FL525 (Figure 2b) than between an Iph-bearing GFP and an alk-tethered FL525 (Table S1, Supporting Information) in the presence of different Pd reagents. This is likely because the first step of Sonogashira coupling is oxidative adduction of a Pd compound to the Iph- moiety,[92] which might be hindered when it is displayed on proteins. In contrast, when the terminal alkyne is installed on proteins, an excess amount of Iph-bearing small molecules may couple with Pd first, leading to an accelerated conjugation reaction with alk-containing proteins.

We next focused on the reagent $Pd(NO_3)_2$ for further investigation due to its high efficiency as well as the aforementioned unique advantages of ligand-free reagents for live-cell labeling. The ligation product and yield of $Pd(NO_3)_2$-mediated Sonogashira coupling on GFP-N149-**2** were confirmed by LC-ESI-MS with a >95% conversion rate (Figure S7, Supporting Information; expected 28 538 Da, found 28 540 Da, Figure 2c). The labeling reaction was further monitored by in-gel fluorescence from FL525-labeled protein samples as a function of time, which demonstrated that the reaction (mediated by 100 μM $Pd(NO_3)_2$ with 1 mM sodium ascorbate) reached completion in approximately 40 min, a rate comparable with that of the CuAAC-mediated protein labeling in the presence of a newly developed acceleration ligand-BTTAA[93] (100 μM $CuSO_4$/500 μM BTTAA, 5 mM sodium ascorbate; Figure 2d, Figure S8, Supporting Information). In addition, we also used the similar in-gel fluorescence approach to optimize $Pd(NO_3)_2$ concentration (Figure 2e, Figure S9, Supporting Information). A $Pd(NO_3)_2$ loading as low as 20 μM, which is only 2 equiv of the substrate GFP-N149-**2** protein (10 μM), was found to efficiently promote the labeling reaction with 100 μM Iph-FL525 (yield > 95%). In contrast, the loading of the $Pd(OAc)_2$/**L2** reagent from a previous study was 1 mM at 37 °C, which is 50 equiv of the protein substrate.[63] Together, our ligand-free $Pd(NO_3)_2$ reagent features high efficiency and low reagent loading for labeling purified proteins. Noteworthy, to our knowledge, our system is the first example of Pd-mediated protein labeling at room temperature (25 °C) with high efficiency, which may better avoid side reactions or damaging effects from Pd-mediated reactions performed at 37 °C.

Since denatured GFP will lose its intrinsic fluorescence, we then monitored GFP fluorescence to assess the potential denaturation effects on protein substrates during the Pd-

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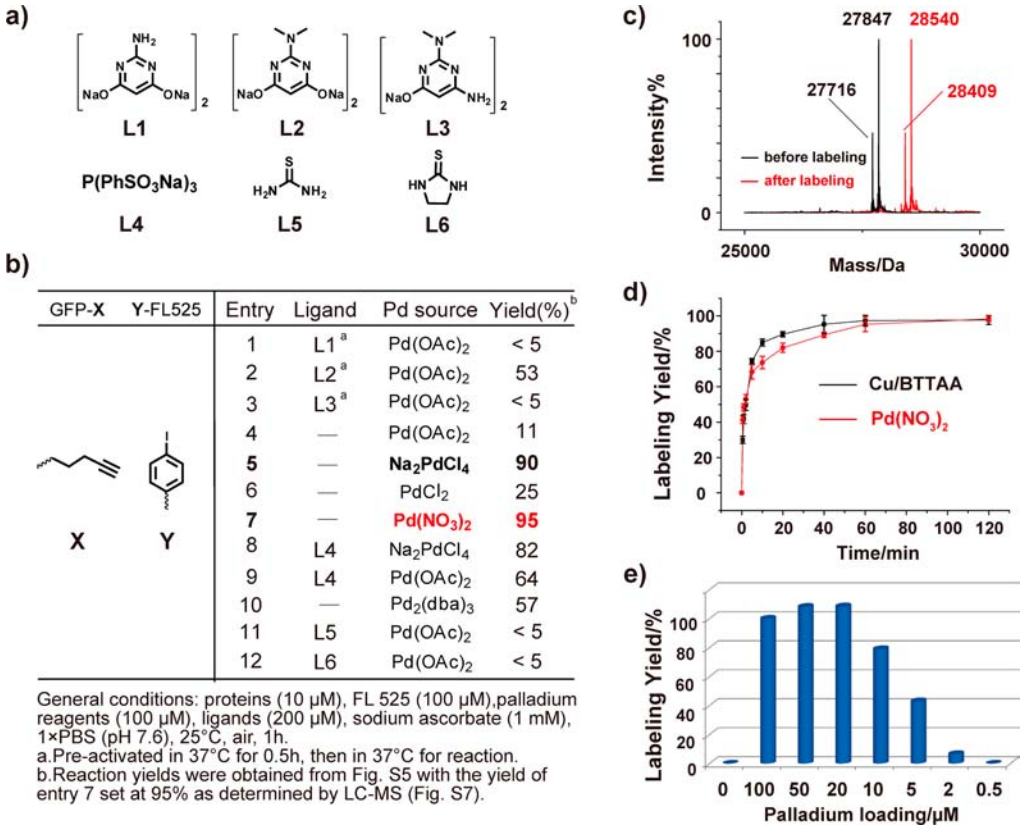

Figure 2. Searching for an efficient Pd-mediated protein-labeling reaction. (a) Structures of Pd ligands used in this study. (b) Efficiency of Pd reagents in assisting the cross-coupling reaction between GFP-N149-**2** and Iph-FL525. (c) ESI-TOF-MS analysis of GFP-N149-**2** before and after Pd-mediated Sonogashira coupling with Iph-FL525. Before reaction (black): full length (major peak, found mass 27 847 Da, expected mass 27 847 Da) and N′-Met cleaved GFP-N149-2 (minor peak, found mass 27 716 Da, expected mass 27 716 Da). After reaction (red): full length (major peak, found mass 28 540 Da, expected mass 28 538 Da) and N′-Met cleaved GFP-N149-2-FL525 (minor peak, found mass 28 409 Da, expected mass 28 407 Da). (d) Comparison of reaction dynamics between Cu/BTTAA-mediated and $Pd(NO_3)_2$-mediated protein labeling (protein 10 μM, Cu/BTTAA 100 μM, $Pd(NO_3)_2$ 100 μM). (e) Protein-labeling efficiency at indicated concentrations of $Pd(NO_3)_2$. Labeling yields in d and e were both calculated based on the relative fluorescence from each FL525-labeled protein sample on SDS-PAGE gel deducted by the amount of protein loaded (see Supporting Information for more details).

$(NO_3)_2$-mediated labeling process. An Iph-bearing biotin was used as a nonfluorescent conjugation partner for this study, which can be effectively conjugated onto GFP-N149-**2** as verified by immunoblotting analysis using streptavidin-HRP (Figure S10, Supporting Information). Notably, the fluorescence of GFP-N149-**2** did not show a noticeable difference with and without the $Pd(NO_3)_2$-mediated labeling reaction (100 μM loading) within the time we tested (0−60 min; Figure 3a). In contrast, a significant fluorescence decrease was observed during a click labeling reaction between GFP-N149-**2** and azide-biotin in the presence of 100 μM Cu(I) ions (Figure 3a). Therefore, $Pd(NO_3)_2$ exhibits a lower denaturation effect than Cu(I) ions on GFP substrate. In addition, to further examine the compatibility of Pd reagents with more fragile proteins such as enzymes, we used both $Pd(OAc)_2$/**L2** complex and $Pd(NO_3)_2$ to treat the wild-type Luciferase (Luc) enzyme for 1 h followed by luminescence measurement. A similar strategy has been previously employed to ascertain the compatibility of an ruthenium-mediated azide-reduction reaction with a protein enzyme-RNaseA.[94] Interestingly, the $Pd(OAc)_2$/**L2** labeling condition (preactivation of 100 μM palladium reagent followed by labeling at 37 °C) caused complete loss of Luc activity, whereas a negligible decrease of Luc activity was detected under our $Pd(NO_3)_2$-labeling condition (100 μM reagent loading at 25 °C; Figure 3b). This observation further demonstrated the excellent compatibility of the ligand-free $Pd(NO_3)_2$ reagent with protein substrates.

To further characterize the compatibility of $Pd(NO_3)_2$ within living bacterial cells, the viability of *E. coli* cells after 200 μM $Pd(NO_3)_2$ treatment (with 1 mM sodium ascorbate, 1 h at 25 °C) was stained with the *BacLight* Cell Viability Kit (Invitrogen). No obvious cell death was observed from the propidium iodide (PI) staining channel (red channel; Figure 3c), as judged by confocal fluorescence microscopy. Meanwhile, live bacterial cells were clearly visible from the SYTO 9 staining channel (green channel; Figure 3c). The permeability of the cell membrane was next examined by trypan blue staining. Flow cytometric analysis showed negligible membrane damage (<3%) after 200 μM $Pd(NO_3)_2$ treatment for 1 h (Figure S11, Supporting Information). Finally, the proliferation rate of $Pd(NO_3)_2$-treated *E. coli* cells were also measured for a period of 3 h starting from a cell density of $OD_{600} \approx 0.2$, which verified that $Pd(NO_3)_2$ did not affect the proliferation of *E. coli* cells (Figure 3d). These biocompatibility analyses were also conducted on *Shigella* cells, yielding similar results (Figure S12, Supporting Information). Taken together, our data confirmed that $Pd(NO_3)_2$ at a concentration equal to or below 200 μM exhibited negligible toxicity to bacterial cells and little damaging effects to the cell membrane. These observations are consistent with the previously reported toxicity

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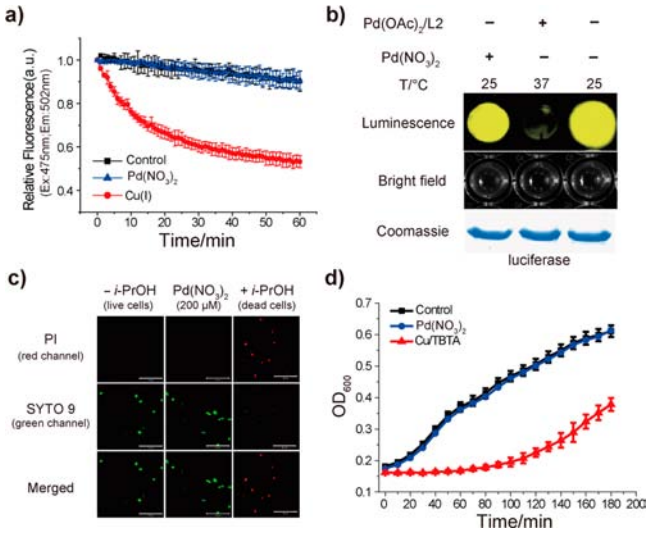

Figure 3. Compatibility of $Pd(NO_3)_2$ with native proteins and live cells. (a) Relative intrinsic fluorescence change of GFP (ex = 475 nm; em = 502 nm) upon $Pd(NO_3)_2$-mediated Sonogashira coupling with Iph-biotin. Control reaction was run in the absence of $Pd(NO_3)_2$ and sodium ascorbate. CuAAC-mediated labeling was used for comparison (GFP 10 μM, Cu(I) 100 μM, $Pd(NO_3)_2$ 100 μM). (b) Biocompatibility of $Pd(OAc)_2$/**L2** and $Pd(NO_3)_2$ with Luciferase. Luminescence was taken from the chemiluminescent channel in ChemiDoc (Bio-Rad) with bright-field images taken as a control. SDS-PAGE gel below verifies that equal amounts of Luc protein were used (Luc 10 μM, $Pd(OAc)_2$/**L2** 100 μM, $Pd(NO_3)_2$ 100 μM). (c) Confocal fluorescence imaging for determining *E. coli* cells viability by PI (red channel) and SYTO9 (green channel) staining after 200 μM $Pd(NO_3)_2$ treatment. *E. coli* cells treated without or with 70% isopropanol (*i*-PrOH) were used as "live" or "dead" control cells, respectively. Scale bars: 10 μm. (d) Change of cell density with and without (control) 200 μM $Pd(NO_3)_2$ or in the presence of 200 μM Cu(I)/TBTA was monitored by OD_{600}.

study when using bacteria for production of palladium nanoparticles.[58]

We went on to employ $Pd(NO_3)_2$ for protein labeling within living enteric bacteria. ICP-MS (inductively coupled plasma mass spectrometry) analysis was first conducted on *E. coli* cells treated by 200 μM $Pd(NO_3)_2$, which showed a ~50-fold increase of intracellular Pd concentration within 1 h than the nontreated blank cells (Figure S13, Supporting Information). This result confirmed that Pd species can effectively enter bacterial cells. Notably, as expected, the negatively charged **L2** ligand significantly decreased bacteria uptake of palladium when treated with the same concentration of palladium complex (Figure S13, Supporting Information). To optimize the loading of $Pd(NO_3)_2$ for intracellular protein labeling, *E. coli* cells expressing GFP-N149-**2** was next incubated with different concentrations of $Pd(NO_3)_2$ and Iph-FL525 for 1 h at 25 °C. After extensive washing, cells were lysed and subjected for SDS-PAGE analysis (Figure S14, Supporting Information). Fluorometric assessment of the SDS-PAGE gel indicated that the best labeling efficiency can be achieved in the presence of 200 μM extracellular $Pd(NO_3)_2$ with the same concentration of labeling probe (Figures 4a and S14, Supporting Information). The specificity of the $Pd(NO_3)_2$-mediated intracellular labeling of GFP-N149-**2** with Iph-FL525 was further confirmed inside *E. coli* cells (Figure 4b, lanes 3, 6, 9). As a control, *E. coli* cells expressing GFP bearing an azido-Pyl analogue (Figure S4, Supporting Information) or without UAA supplementation showed only background fluorescence after the labeling reaction (Figure 4b).

Finally, to extend this strategy for intracellular protein labeling in enteric bacteria other than *E. coli*, we demonstrated the labeling of virulence proteins inside living *Shigella* cells. A bacterial Type-III secretion (T3S) effector-OspF was used which is conserved in all these aforementioned enteric pathogens.[95,96] OspF is known to be secreted into host cells through a bacterial T3S system. Upon entering host cells, OspF acts as an epigenetic modulator by irreversibly dephosphorylating MAPKs (mitogen-activated protein kinases) such as Erk, resulting in altered host inflammatory transcriptional responses.[95,97] We incorporated **2** into OspF at residue K102 inside *Shigella*, followed by Pd-mediated labeling of the resulting protein (OspF-K102-**2**) upon addition of 200 μM $Pd(NO_3)_2$ and 200 μM Iph-FL525. SDS-PAGE analysis of *Shigella* cell extracts after the labeling reaction showed a single fluorescent band corresponding to the labeled OspF protein, which further verified the high specificity of our Pd-mediated Sonogashira coupling on target proteins inside enteric microorganisms(Figure 4c, lane 4). Notably, unlike the GFP model protein in *E. coli* cells, the expression level of recombinant OspF protein was less than that of many endogenous proteins in *Shigella* cells (Figure 4c, lanes 1−3; lanes 7−8, full-length OspF protein expressed in *Shigella* was only detectable using an anti-OspF antibody). Therefore, this observation excludes the possibility that the specific labeling of a target protein versus endogenous proteins was an artifact due to the higher expression level of the target protein within cells. Moreover, the fluorescent-labeled OspF protein within living *Shigella* bacteria was also clearly visible by confocal fluorescence microscopy (Figure 4d). Critically, when cells expressing OspF bearing the azido-Pyl analogue were treated under the same condition, no labeling was observed. Nor did we detect a fluorescent signal when *Shigalla* cells were treated by the fluorophore Iph-FL525 alone, which further confirmed that our observed protein labeling depends on the Sonogashira reaction promoted by $Pd(NO_3)_2$ (Figure 4d).

■ CONCLUSIONS AND OUTLOOK

Considerable efforts have recently been devoted to expanding the repertoire of bioorthogonal reactions with improved biocompatibility. Transition metals represent a powerful toolkit for selective construction of chemical bonds, which offers diverse chemical transformations that were hardly feasible previously. Therefore, one direction focused on searching for alternative transition metals that are capable of promoting bioorthogonal conjugation reactions with attenuated toxicity than Cu(I) ions utilized in CuAAC. Encouraged by the emerging biofriendly applications of the powerful palladium reagents in biological systems, we decided to develop a facile approach for intracellular labeling of proteins with the assistance of palladium compounds. To this end, we first genetically and site-specifically incorporated two pyrrolysine analogues into proteins, which offered facile handles for palladium-mediated cross-coupling reactions on proteins. Our subsequent GFP-based catalyst screening process offered the advantage of evaluating both the efficiency and the denaturation effects of Pd reagents. This strategy led to the discovery of $Pd(NO_3)_2$ as a simple and highly efficient and biocompatible reagent for fluorescent labeling of target proteins via the copper-free Sonogashira coupling reaction. Additional in vivo analysis demonstrated effective uptake and negligible cytotox-

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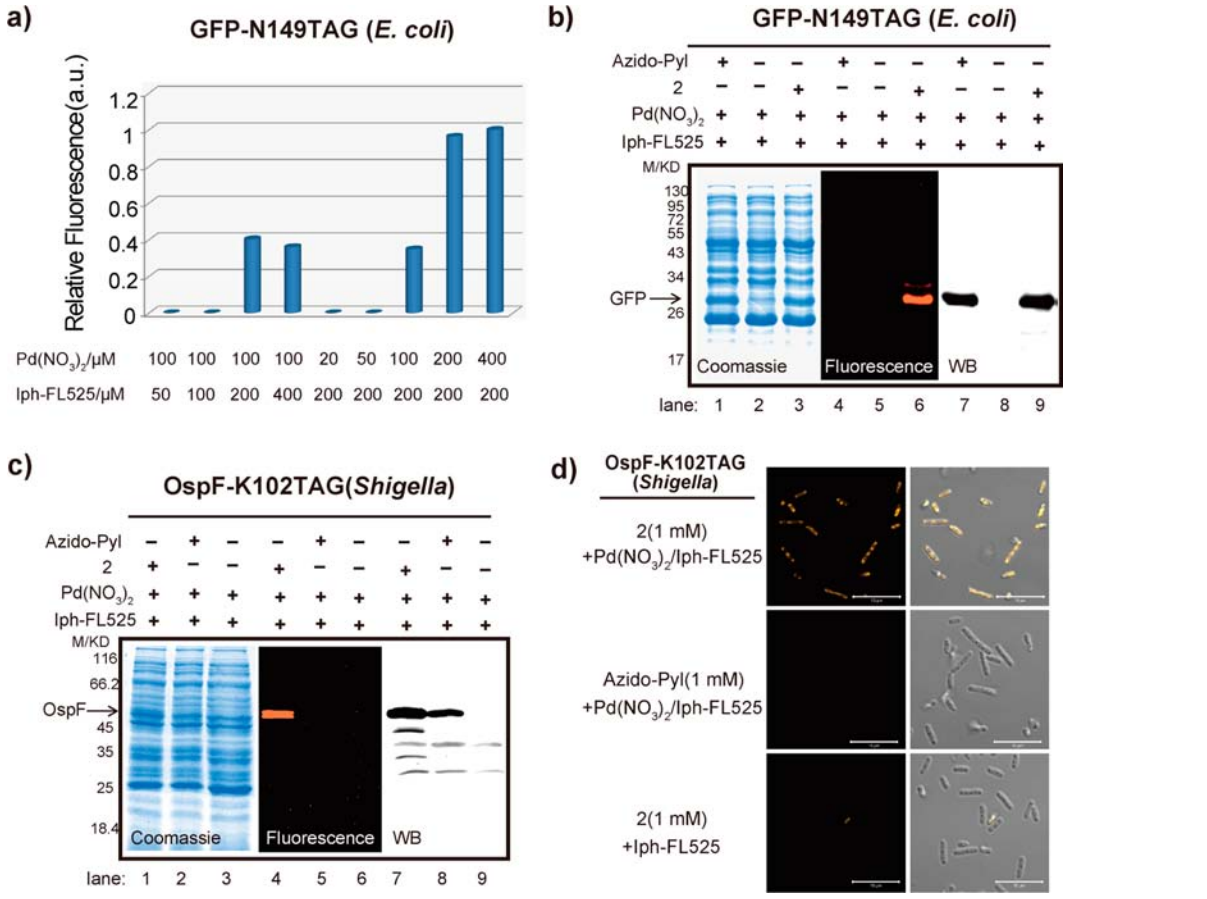

Figure 4. Pd-mediated intracellular protein labeling in live bacteria. (a) Optimizing the concentrations of $Pd(NO_3)_2$ and Iph-FL525 for labeling GFP-N149-**2** in *E. coli* cytoplasm. (b and c) Confirming the specificity of the $Pd(NO_3)_2$-mediated labeling of target proteins: GFP-N149-**2** inside *E. coli* (b) and OspF-K102-**2** inside *Shigella* (c). Bacterial cell lysate after the labeling reactions was examined by SDS-PAGE (left), fluorescence-gel imaging (middle), and Western Blotting analysis (anti-GFP and anti-OspF antibodies) (right). (d) Confocal microscopic imaging of $Pd(NO_3)_2$-triggered Sonogashira coupling for site-specific labeling of OspF-K102-**2** protein inside *Shigella* cells. Scale bars: 10 μm. Loadings of $Pd(NO_3)_2$ and Iph-FL525 were 200 μM in b–d.

icity of $Pd(NO_3)_2$, which was successfully applied by us for labeling of GFP as well as virulence proteins inside living enteric pathogens.

Consistent with the previously reported activities of palladium nanoparticles generated from different Pd(II) sources, our study revealed a similar reactivity trend among these Pd(II) compounds in promoting cross-coupling between proteins and small molecules. This seems to suggest that our $Pd(NO_3)_2$-triggered reaction, in the presence of an electron donor-sodium ascorbate, might go through a nanoparticle mechanism. Indeed, the recent "bio-Pd" study demonstrated that Pd(0) nanoparticles can be generated from Pd(II) in the presence of electron donors (e.g., H_2, formate)[98] on the cell surface as well as inside living bacterial cells.[99,100] The reaction mechanism of the $Pd(NO_3)_2$-mediated protein–small-molecule conjugation is currently under investigation in our laboratory. Finally, with many newly developed biocompatible fluorophores for live-cell imaging,[101−103] our palladium-mediated intracellular protein-labeling method presented here may find broad applications in visualization and tracking of diverse virulence proteins within Gram-negative bacterial pathogens.

EXPERIMENTAL SECTION

Compounds Synthesis. *1. UAA Synthesis.* Newly developed pyrrolysine analogues **2** and **3** were synthesized using a simple procedure. Pent-4-yn-1-ol or 4-iodobenzyl alcohol (10 mmol) was added to a solution of triphosgene (1.49 g, 5 mmol) in dry CH_2Cl_2 (DCM, 50 mL). The reaction was stirred for 8–12 h at room temperature. Then the solvent was evaporated to get the related carbonochloridate. Boc-Lys-OH (2.46 g, 10 mmol) was dissolved in a mixture of 1 M NaOH (5 mL) and THF (5 mL) and then cooled to 0 °C. Chloroformate diluted in 10 mL of THF was added dropwise over 5 min, and the reaction was allowed to stir for 12–18 h at room temperature. The solution was then cooled to 0 °C again and washed with ice-cold Et_2O (50 mL). The water layer was subsequently acidified with ice-cold 1 N HCl to pH 1–2 and extracted with ice-cold EtOAc (2 × 30 mL). Combined organic layers were dried over Na_2SO_4, and solvents were evaporated to give the product. The product was then dissolved in DCM (15 mL), and TFA (15 mL) was slowly added to the solution. The reaction was stirred at room temperature for 60 min, while excess DCM was evaporated under vacuum. The residue was redissolved in MeOH (5 mL) and precipitated into 300 mL of Et_2O. Precipitates were collected and dried under vacuum, affording pure white final product **2** or **3** in a total yield of 50–65%.

2. Labeling Reagents (Iodophenyl Group). *N*-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)-4-iodobenzamide (the Iph-$(PEG)_2$ linker) was first synthesized. NHS (1.13 g, 10 mmol), DMAP (0.122 g, 1.0 mmol), and 4-iodobenzoic acid (2.47 g, 10 mmol) were mixed in 50 mL of anhydrous DCM. Five minutes later, DCC (2.06 g, 10 mmol) was added to the solution. The reaction was cooled for the first hour at 0 °C and left to react at room temperature overnight. The solution was diluted with DCM, filtered to remove dicyclohexylurea, and

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evaporated to yield the active ester. Then this active ester was dissolved in 50 mL of DCM and added to 2,2′-(ethylenedioxy)bis-(ethylamine) (32 g, 212 mmol) over a period of 90 min under vigorous magnetic stirring. The reaction was left at room temperature for 12 h. The solution was filtered, washed with water (3 × 200 mL), dried over anhydrous $MgSO_4$, and concentrated to yield the Iph-$(PEG)_2$ linker. Then it was coupled with FL 525 or biotin using standard EDC coupling protocol to give the labeling reagents. Experimental details and compound characterization are included in the Supporting Information.

Expressions of Proteins Bearing UAA in Pathogen Bacteria. For nonvirulent *E. coli* cells (DH10B), plasmids encoding DZKRS-$tRNA^{Pyl}_{CUA}$ pair and GFP-N149-TAG genes were cotransformed using heat shock protocol. For virulent cells, like *Shigella*, *Salmonella*, and EPEC, the plasmid pSupAR-Mb was cotransformed with a plasmid carrying the target genes with an amber mutation by electro-transformation. After 1:100 dilution, overnight bacterial cultures were grown at 37 °C in LB medium till OD_{600} reached 0.6, at which point 1 mM (final concentration) **2** or **3** was added to the culture. Bacteria were continuously grown at 37 °C for another 30 min before being transferred to 30 °C for induction in the presence of 0.02% arabinose overnight. Purified UAA-containing proteins were characterized by ESI-MS as the followed procedures.

Labeling of Purified Proteins by Sonogashira Coupling. Stock solutions of labeling probes (2.5 μL, 20 mM) and palladium complex (5 μL, 10 mM) were mixed in a 0.75 mL Eppendorf tube. For reagents prepared in water, 7.5 μL of DMSO was added to the mixture. For reagents prepared in DMSO, 2.5 μL of DMSO and 5 μL of ddH_2O were added. The cocktail solution was then initiated by sodium ascorbate (1 μL, 500 mM) before being added to the reaction system (3.6 μL of cocktail was needed for a 120 μL reaction). For reagents $Pd(OAc)_2$/**L1**, $Pd(OAc)_2$/**L2**, and $Pd(OAc)_2$/**L3**, the cocktail after initiation by sodium ascorbate was preactivated at 37 °C for 0.5 h. Then reactions were all performed in room temperature (25 °C, 300 rpm in Eppendorf Thermomixer) for 1 h. All reactions were then quenched by adding 0.5% 3-mercaptopropanoic acid solution in water (20−120 μL reaction system) before LC-MS or SDS-PAGE analysis. Final concentrations in reaction system: palladium reagents, 100 μM; sodium ascorbate, 1 mM; labeling probes, 100 μM; GFP-N149-2/-3, 10 μM; buffer, 1 × PBS buffer, pH 7.6. Reagent loadings were changed as indicated.

Mass Spectrometry. Mass spectrometry analysis of purified GFP proteins carrying **2** or **3** and their labeling products were performed using an Agilent 1200 LC system and 6510 Q-TOF MS spectrometer with electrospray ionization (ESI-LC-MS). Formic acid (0.1%) in H_2O as buffer A and 0.1% formic acid in acetonitrile as buffer B were taken as the solvent system. LC separation for GFP and its variants were carried out with a Poroshell 300SB-C18 column (analytical 2.1 × 75 mm, 5 μm, Agilent technologies), and positive mode was chosen for ESI-MS to analyze all samples. The protein charge envelope from the raw spectrum was deconvoluted into noncharged form by the Agilent Mass Hunter Qualitative Analysis software (Agilent Technologies). Theoretical masses of wild-type proteins were calculated using PROTEIN CALCULATOR v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html), and theoretical masses for modified proteins were adjusted manually.

Toxicity of $Pd(NO_3)_2$ to Bacterial Cells. Bacterial cells were harvested by centrifugation (4 °C, 5 min, 5000 rpm) and resuspended in PBS buffer. To remove the LB medium and free unnatural amino acid completely, bacteria cells were washed three times. Culture cells were diluted with 1 × PBS buffer (pH 7.6) to give OD_{600} = 1.0. A 360 μL aliquot was then added to a 1.5 mL Eppendorf tube followed by a 10.8 μL aliquot of cocktail (the cocktail solution of Iph-biotin and palladium complex was prepared as described in Supplementary Method 6, Supporting Information). Cells were then gently shaken at 25 °C for 1 h. Labeled cells were collected by centrifugation (4 °C, 5 min, 10000 rpm) and resuspended in 0.85% NaCl solution (500 μL). This process was repeated 3 times to remove the unreacted reagents completely. Cells were then stained with a BacLight Cell Viability Kit (Invitrogen) and left for 15 min in the dark according to the manual or previous report. Confocal microscope images for BacLight cell viability were taken on a Zeiss LSM 700 laser scanning microscope with a Plan Apochromat 100 × oil-immersion objective in a scan zoom (averaging 8) after the cells were trapped between a slide and an 18 mm square coverslip using 50% glycerin.

Labeling Proteins in Living Bacterial Cells. Induced bacterial cells were harvested by centrifugation (4 °C, 5 min, 5000 rpm) and resuspended in PBS buffer. To completely remove the LB medium and free unnatural amino acids, induced cells were washed three times followed by dilution with 1 × PBS buffer to give OD_{600} = 1.0. A 360 μL aliquot was then added to a 1.5 mL Eppendorf tube followed by a 10.8 μL aliquot of cocktail. (The cocktail of Iph-FL525 and palladium complex was prepared as Supplementary Method 6, Supporting Information. Different concentrations of $Pd(NO_3)_2$ and Iph-FL525 were used as indicated.) Cells were then shaken gently at 25 °C for 1 h. Labeled cells were collected by centrifugation (4 °C, 5 min, 10 000 rpm) and resuspended in 0.85% NaCl solution. This process was repeated several times to completely remove unreacted reagents. Cells were finally resuspended in 200 μL of PBS solution and analyzed by confocal fluorescence imaging. Images shown in Figure 4d were taken from a Zeiss LSM 700 laser scanning microscope with a Plan Apochromat 100 × oil-immersion objective in a scan zoom (averaging 16). Alternatively, the centrifuged bacterial cells after the labeling process were directly lysed with SDS loading buffer and then subjected to SDS-PAGE gel for coomassie blue, fluorescence, and Western Blot analysis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, supplemental data, and complete ref. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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