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Palladium-Mediated Cell-Surface Labeling

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Supporting Information

ABSTRACT: Benign C−C bond formation at various sites in cell-surface channels has been achieved through Suzuki−Miyaura coupling of genetically positioned unnatural amino acids containing aryl halide side chains. This enabled site-selective cell surface manipulation of *Escherichia coli*; the phosphine-free catalyst caused no cell death at required Pd loadings, suggesting future in vivo application of catalytic metal-mediated bond formation in more complex organisms.

Palladium-mediated reactions have revolutionized modern synthetic chemistry but have yet to find broad applications in biology.[1−4] However, the absence of Pd from all known biological systems provides an intriguing opportunity for covalent manipulation of in vivo systems. The selective and catalytic nature of such reactions may allow modification to be undertaken with exquisite precision, even in complex biological environments.[5] Yusop et al.[6] recently reported the use of heterogeneous catalyst particles to mediate small-molecule couplings within a cell. While the applications of such systems are limited by endosomal compartmentalization, that work nevertheless represents a key example of the potential of such reactions for eventual in vivo applications.

A more general strategy for biology would involve the use of a small-molecule catalyst to modify and manipulate the biomacromolecules that are the "workhorses" of the cell. In this context, the molecules of the central dogma[7] appear to be vital targets for cellular modification.[8] We recently reported the development of the phosphine-free, water-soluble system $Pd(OAc)_2(ADHP)_2$ (**1**) (Figure 1b)[9] and demonstrated its use in efficient Suzuki−Miyaura cross-couplings at a genetically incorporated amino acid[10] on a protein substrate via a "tag-and-modify" approach.[8,11] This catalytic system and approach has since been used not only for Suzuki−Miyaura couplings[12] but also for Sonogashira couplings[13] (using an *N,N*-dimethyl-ADHP variant) applied to other proteins[12] and peptides.[13] This provides encouraging evidence for the biocompatibility of such reactions. Here we describe translation of this Suzuki−Miyaura chemistry into a cellular context, demonstrate low toxicity at effective palladium loadings, and confirm sufficient compatibility to mediate alteration of cell-surface functionality.

Channels and pores at cell surfaces play a key role in modulating ion and small-molecule passage through membranes and are targets in therapy.[14] The OmpC protein homotrimer forms a β-barrel pore in bacteria[15] and is a site for receptor binding during phage infection.[16] Antiporin antibodies are induced during pathogenic infections such as typhoid,[17] so OmpC has become a target for vaccine platforms and for

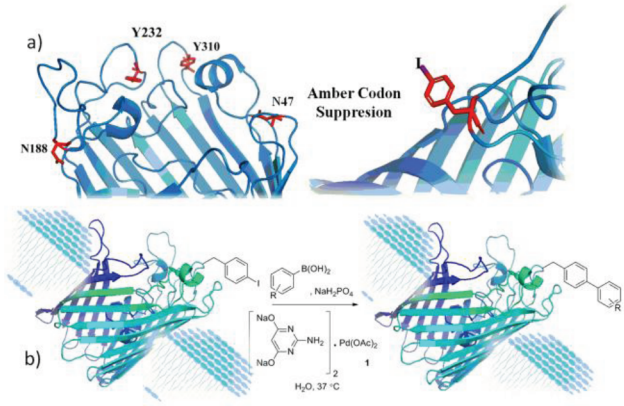

Figure 1. (a) OmpC sites targeted for *p*IPhe via amber codon suppression. (b) Modification mediated by catalyst **1** at a cell surface.

further study.[14] It is therefore a useful and abundant protein on the *Escherichia coli* surface that has been elegantly examined in methionine auxotrophic cells using azide−alkyne chemistry.[18]

OmpC contains 16 transmembrane, antiparallel strands connected by seven internal and eight external loops.[15] We identified four "tag" sites on separate loops chosen to limit disruption of both cell and protein structure and function. These sites combined an unhindered environment with good accessibility (Figure 1a); notably, these loops of OmpC provide a strategy for possible epitope display.[14] Four mutant plasmids [pOmpC-(N47·), (N188·), (Y232·), and (Y310·)] under T7 promoter control in a pET-11a vector were generated. Each was cotransformed into *E. coli* strain JW2203-1[19] with pEVOL(*p*IPhe), a plasmid coding for a mutant aaRS/$tRNA_{CUA}$ pair for incorporation of *p*IPhe via amber stop codon suppression;[20] this produced four different cell types displaying OmpC-N47X, N188X, Y232X, and Y310X, where X denotes the unnatural amino acid *p*-iodophenylalanine (*p*IPhe) (**2**).[21] JW2203-1 is deficient in host expression of OmpC,[19] and its use ensured that only plasmid-generated proteins would be present in the membrane; their presence was confirmed by SDS-PAGE after induction in the presence of *p*IPhe [Supporting Information (SI) Figure 2].[22,23] In the absence of *p*IPhe, no protein production was observed (SI Figure 3). The presence of *p*IPhe was further confirmed by "in-gel" tryptic digestion and subsequent LC−MS analysis (SI Table 1 and SI Figure 4). Misfunctional OmpC is toxic;[24] steady growth at the same rates with good cell viability as for wild-type (WT) OmpC was observed under these conditions, suggesting correct

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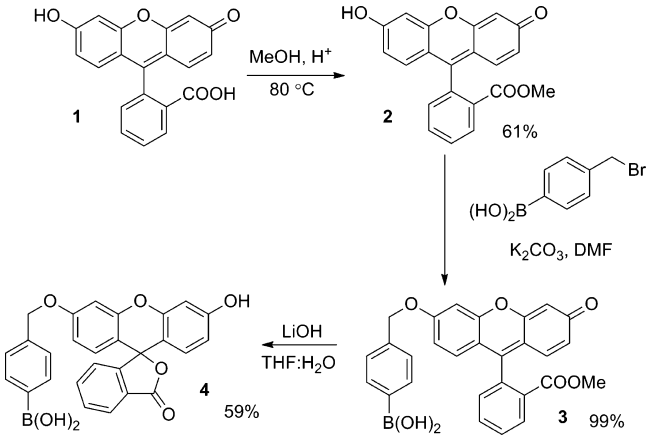
porin cellular function. Single-channel recording confirmed correct porin activity, with observed characteristic trimeric closing and spermidine-induced blockade in all cases (see SI Figures 5 and 6).[25]

With these four "tagged" cell types in hand, we tested the effectiveness of $Pd(OAc)_2{\cdot}(ADHP)_2$ **1** for its efficiency in modifying OmpC-N47X in a cellular environment with model fluorescent boronic acid **3**, which was readily synthesized from fluorescein (Scheme 1; see SI Figure 1 for fluorescence

Scheme 1. Synthesis of Fluorescent Boronic Acid 3 (Also See the SI)



spectra). Cells were treated with 1.6 mM boronic acid **3** and 0.35 mM **1** for 1 h at 37 °C in pH 8 phosphate buffer. Gratifyingly, clear fluorescent labeling was observed under these conditions. Next, the "tag" position was investigated: of the mutant strains, labeling of *E. coli* OmpC(Y232X), displaying *p*IPhe at site 232 on loop 5, was shown to be most effective, with a minimum 55% increase in fluorescence intensity over the others (Figure 2). Although it cannot be discounted that this

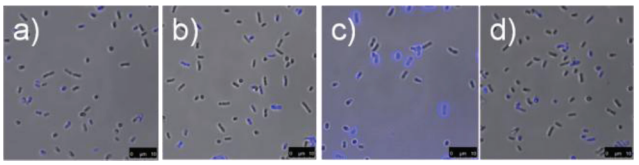


Figure 2. Labeling of OmpC mutants: (a) N47X, (b) N188X, (c) Y232X, (d) Y310X. Scale bar 100 μm.

greater level of fluorescence may be due to greater OmpC production or *p*IPhe incorporation in this cell variant, it is also consistent with greater accessibility of the "tag" at this site. Critically, when cells displaying wild-type (WT) OmpC protein were treated under identical conditions, no labeling was observed, even when the cells were grown in the presence of *p*IPhe **2** (see SI Figure 5). Moreover, control reactions run in the absence of any one of the key reaction components (boronic acid, Pd-catalyst **1** or *p*IPhe) led to no observed fluorescence, consistent with a Suzuki–Miyaura Pd(0)-mediated process (Figure 3). It also discounted the possibility of nonspecific uptake or binding of **3**; this was particularly important to rule out alteration of the cell surface via other mechanisms.[26,27] Furthermore, when the coupling reaction was conducted in the presence of an excess of reducing sugar D-glucose (2 equiv relative to **3**), no alteration in labeling

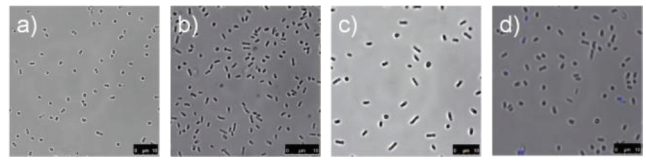


Figure 3. (a–c) Control reactions run in the absence of (a) boronic acid, (b) **1**, or (c) *p*IPhe. (d) Control with WT OmpC cells.

efficiency was observed, suggesting that similarly the presence of soluble sugars does not affect the reaction (SI Figure 9).

The membrane fraction of labeled cells was analyzed by SDS-PAGE, with a fluorescent band being clearly observed for labeled OmpC (SI Figure 10). Significantly, no other proteins were seen to be labeled. Notably, although the amber stop codon is rarely used in *E. coli*, Johnson et al.[28] recently reported that unnatural amino acids are not incorporated in response to "natural" UAG codons.

With these promising results in hand, we began to investigate the parameters of this cellular alteration reaction. The reaction progress was initially monitored over the course of 1 h; a steady increase in labeling efficiency was observed. Interestingly, over prolonged periods, a slight discontinuous increase in labeling consistent with effects from cell division was seen at later time points (SI Figure 7), raising the possibility of using this reaction in dynamic process monitoring. When the reaction temperature was lowered to 30 °C, a significant drop in labeling was observed. At room temperature, no labeling was achieved, even in the presence of high boronic acid and palladium loadings (7 and 1.5 mM, respectively; see SI Figure 8).

To optimize the boronic acid concentration, Pd levels were kept constant at 0.35 mM. Labeling could be observed at concentrations as low as 0.3 mM boronic acid, albeit at lower levels (Figure 4a). An increase in labeling was observed with

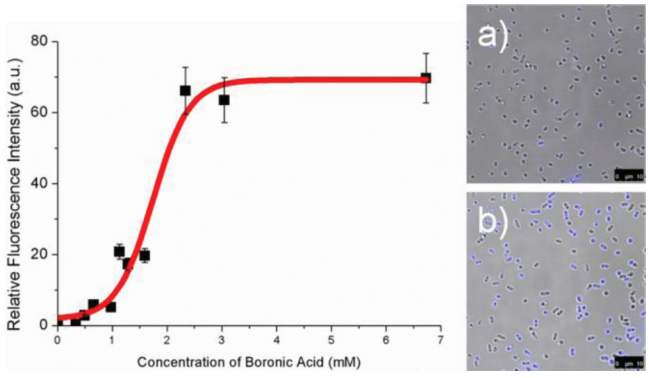


Figure 4. Variation of fluorescence intensity with increasing boronic acid concentration: (a) 0.3 mM, (b) 2 mM.

increasing boronic acid concentration until ~2 mM boronic acid (5.7 equiv relative to Pd) was reached (Figure 4b), above which little improvement was observed, suggesting that either the Pd concentration had become the limiting factor or the reaction was complete.

Optimization of the Pd concentration was undertaken at this boronic acid concentration of 2 mM. Unlike the case of boronic acid optimization (where a sigmoidal logarithmic dose–response was observed; Figure 4), no low-level labeling was observed at low Pd loadings (Figure 5a). Instead, at 330 μM a critical threshold was reached, and a dramatic increase in labeling was observed. No further increase in labeling efficiency

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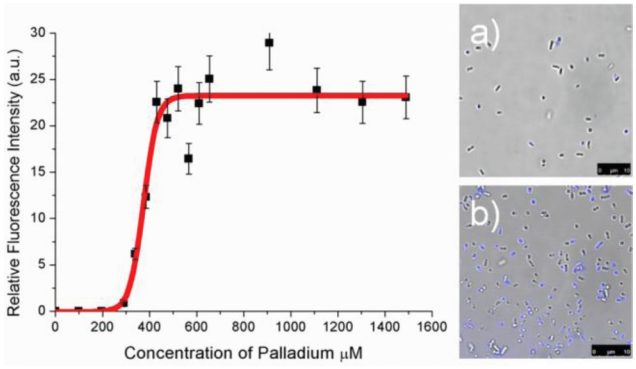

Figure 5. Variation of fluorescence intensity with increasing Pd concentration: (a) 0.3 mM, (b) 0.45 mM.

was obtained with Pd loadings beyond 450 μM (Figure 5b). Increasing the density of cells present led to no change in this threshold level, indicating that such an effect is not due to sequestering of the catalyst by cellular proteins (SI Figure 15). This dramatic observed change in labeling and hence reaction efficiency is consistent with a threshold or even hormetic response[29] and so opens up the possibility of a responsive "off/on", Pd-switchable titrated system enabled by this effect.

To realize the potential of such a stimulated system, we were aware of the necessity for the corresponding "stimulus" to be biocompatible, not only through bio-orthogonal reactivity/selectivity but also in regard to sufficiently low toxicity to living organisms. For example, while copper-mediated "click" chemistry is undoubtedly a useful tool for synthetic biology and biological labeling, its applications have been limited by the suggested toxicity associated with the metal catalyst.[30] This has led to the development of copper-free variants,[31−35] which while useful as stoichiometric processes, lose some of the strategic flexibility that catalytic variants might allow in biology (such as threshold effects). Though other transition metals, including palladium, have been suggested to be toxic at high doses, little work has been undertaken to determine their toxicity at the low levels described in this work, particularly in the bound form of an activated catalytic species.[36,37]

To probe the toxicity of our $Pd(OAc)_2{\cdot}(ADHP)_2$ catalyst, cells were incubated in the presence of varying concentrations for 1 h at 37 °C. Samples were then analyzed by flow cytometry, using the common viability stain trypan blue to measure the percentage of dead cells. To our delight, cell death at the optimized concentration of 0.45 mM Pd was <3% (Figure 6b), the same level observed in the positive live control (Figure 6a), indicating very low or insignificant toxicity associated with such levels. Limited toxicity was observed at a concentration of 0.7 mM, while at concentrations as high as 1.9 mM only 36% of cells were observed to be dead (Figure 6c). Similar results were obtained when boronic acid was added to the system to probe whether any toxic side products of the Suzuki−Miyaura coupling may have been formed. Comparison of this result (LD_{50} > 2 mM) with that of the Pd optimization experiments (threshold 330 μM), clearly shows that essentially nontoxic levels of Pd loading can be utilized while still exhibiting good labeling efficacy (Figure 6). While this low toxicity may at first seem slightly surprising, comparison to the literature showed that the optimized concentration of 53 ppm of Pd is also below the LD_{50} of 200 ppm previously estimated for oral administration of uncomplexed Pd(II) in rats.[38] While no direct inference can be made between these two very

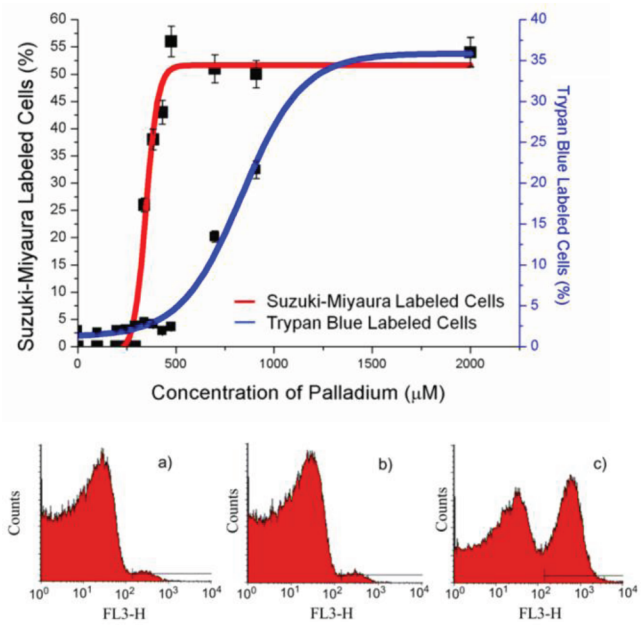

Figure 6. (top) Percentage of labeled cells with variation of palladium, with fluorescein boronic acid **3** and viability stain trypan blue. (bottom) Fluorescence-activated cell sorting (FACS) traces at (a) 0 mM, (b) 0.45 mM, and (c) 2 mM palladium catalyst **1**.

different biological systems, or indeed the two test conditions, it still suggests that low, nontoxic levels of Pd will prove effective in biological Suzuki−Miyaura reactions. It should also be noted that the reactions disclosed here appear to be confined to the cell surface; there is no evidence for **1** being cell permeable. The toxic effects in an intracellular context may therefore be very different from those observed in this report.

In conclusion, we have demonstrated Pd-mediated Suzuki−Miyaura cross-coupling on a living cell surface using genetically "tagged" aryl halide-containing porin channels created through the incorporation of the unnatural amino acid *p*IPhe into OmpC protein monomers. Coupling was observed with a fluorescent boronic acid **3** mediated by the $Pd(OAc)_2{\cdot}(ADHP)_2$ catalyst system **1**. In addition, catalysis was shown to be effective at a critical Pd loading (with apparent threshold/hormetic behavior) at which no significant cell toxicity was observed under the described reaction conditions. The potential applications of the Suzuki−Miyaura cross-coupling in threshold control of biomolecules on cell surfaces (e.g., protein couplings or covalent multimerizations) and in vivo are particularly intriguing. Moreover, the excellent aqueous solubility of boronic acids may allow better site-selective control and precise attachment of, for example, hydrophobic alterations at cell surfaces in a manner previously inaccessible because of nonspecific sequestering. The fluorescence of biaryls (and typical low fluorescence in aryl precursors) also raises the potential for in vivo fluorogenic labeling. Such synthetic Pd-mediated cell-surface presentation may also enable strategies for epitope and ligand display on membrane scaffolds as alternatives to those explored genetically.[14,39] More broadly, we also suggest that the recent confluence of key developments in the field leading toward efficient[28] and whole-organism amber codon suppression[40] and intracellular Pd-mediated processes,[6] coupled with the low toxicity and cellular manipulation described here, may eventually allow Pd-mediated

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control of in vivo function. Work toward these ends is currently underway.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details, compound characterization, and fluorescence microscopy images. This material is available free of charge via the Internet at http://pubs.acs.org.

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