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Palladium-mediated *in situ* synthesis of an anticancer agent

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As a novel prodrug activation strategy Pd(0) nanoparticles entrapped within a modular polymeric support were used, in a cell culture system, to synthesise the anticancer agent PP-121 from two non-toxic precursors, thereby inducing cell death in the first example of *in situ* mediated drug synthesis.

Bioorthogonal chemistries that can be carried out within a biological system without affecting normal cellular function have revolutionized the analysis of biological processes in their native environment.[1] Classical examples include the Staudinger ligation,[2] strain-promoted azide–alkyne cycloaddition,[3] and the inverse-electron demand Diels Alder reaction of tetrazines.[4] Recently, bioorthogonal reactions using transition metals (Rh, Au and Pd) have begun to be successfully applied in a biological setting.[5–9] Modifications of proteins using genetically encoded halogenated phenylalanines have for example enabled *in vitro* labelling of proteins *via* palladium-mediated coupling to boronic acid tags,[10] thereby allowing the non-intrusive and real-time study of proteins[11,12] and carbohydrates[13] in bacteria. Another approach has been the application of palladium nanoparticle catalysts with allylcarbamate cleavage of both caged fluorophores and pro-drugs (e.g. allylcarbamate-amsacrine), as well as a Suzuki-Miyaura cross-coupling reaction inside mammalian cells.[14] Palladium-mediated transformations have since been used to selectively activate proteins and other prodrugs. Chen showed the activation of the enzyme phosphothreonine lyase (Ospf) based on decaging of a propargyloxycarbonyl (Proc) protected catalytic lysine residues with homogeneous palladium catalysts,[15] while Weiss demonstrated that the anticancer drug 5-fluorouracil could be generated by the *in situ* (extracellular) decaging of a propargyl protected prodrug.[16]

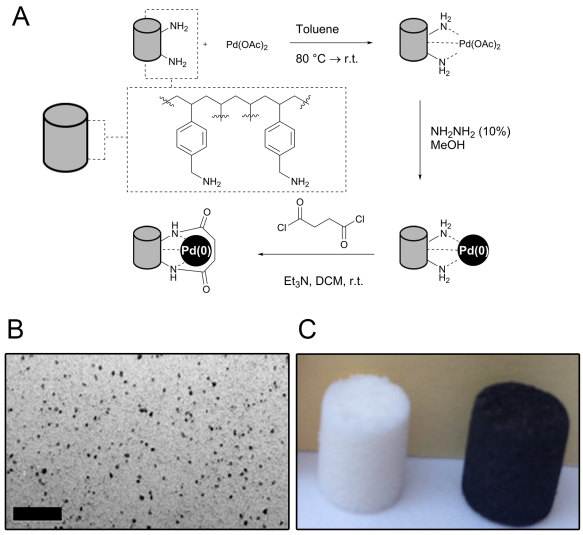

Fig. 1. Synthesis and characterization of a modular support functionalized with Pd(0) nanoparticles. (a) Synthesis of the supported Pd catalyst; (b) TEM analysis showing the homogeneous distribution of palladium nanoparticles throughout the support, with an average particle size of 9.2 ± 1.5 nm (scale bar 100 nm) and a Pd content of 0.24 ± 0.08 µmol/mg (ICP-OES, n = 9); (c) The modular supports (9.0 × 7.5 mm) before (left) and after (right) functionalization.[17]

Here, the scope of palladium-mediated chemistry in a biological environment was extended to *in situ* drug synthesis, with C–C bond formation *via* a Suzuki-Miyaura cross-coupling demonstrated with the activation of a quenched *bis*-iodo-BODIPY scaffold and the synthesis of the anticancer agent PP-121 from two coupling partners.[18]

Loading an active metal onto a solid support is a common method to generate heterogeneous catalysts.[19,20] We have previously reported the entrapment of catalytically active, biologically compatible palladium(0) nanoparticles into polymers[14,21–23] with the generation of modular sintered aminomethyl polystyrene resin beads[17,24] in which nanoparticles of palladium are trapped within a physical polymer framework (Fig. 1).

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Here, the catalytic activity of these modular Pd catalysts was confirmed by the decaging of *bis*-propargyloxycarbonyl (Proc) rhodamine 110 **1** (Fig. 2a) in phosphate buffered saline (PBS), 5% fetal bovine serum (FBS), and PC-3 (prostate adenocarcinoma) cell lysate.[5,14,25,26] The addition of the Pd catalyst to a solution of **1** (20 μM) resulted in the generation of **2** with a >500-fold increase in fluorescence in PBS, and a 115-fold and 46-fold increase in cell lysate and in 5 % FBS, respectively (Supporting Fig. S1). The catalyst also decaged **1** (20 μM, 18 h incubation) in a cell-based assay, resulting in labelling of PC-3 cells (Supporting Fig. S2 and S3). To investigate the catalytic activity in a Suzuki-Miyaura cross-coupling reaction, *bis*-iodo-1,3,5,7,8-pentamethyl-BODIPY **3** was reacted with 2-thienyl and 4-phenyl boronic acids (Fig. 2b). *Bis*-iodo BODIPY **3** is non-fluorescent due to the heavy atom quenching effect,[27] but becomes fluorescently unquenched following cross-coupling chemistry with 2-thienyl or 4-phenyl boronic acids, which gives the *bis*-thienyl BODIPY **4** ($\lambda_{Ex/Em}$ 520/574 nm) and *bis*-phenyl BODIPY **5** ($\lambda_{Ex/Em}$ 518/552 nm) (Supporting Fig. S4) with 14 and 31-fold increases in fluorescence, respectively. The coupling reaction between 2-thienyl and 4-phenyl boronic acid with *bis*-iodo BODIPY **3** in the presence of the Pd catalyst in cell lysate and in 5% FBS resulted in a 5.6 and 1.2-fold increase in fluorescence for **4** and a 2.5 and 3.3-fold increase for **5**, respectively (pure samples of **4** and **5** in cell lysate and 5 % FBS gave 14 and 15-fold, and 3 and 20-fold increase, respectively) (Supporting Fig. S5), with the cell lysate influencing the fluorescence of **5**, but not **4**. A modest increase in fluorescence (< 1.5-fold) was also observed in the absence of a boronic acid due to a partial de-iodination of the BODIPY **3**.[28] However, the emission maximum of the de-iodinated product (1,3,5,7,8-pentamethyl-BODIPY) is 520 nm compared to the >540 nm for both cross-coupling products **4** and **5** (Supporting Fig. S6), thus allowing spectral resolution. PC-3 cells were incubated with *bis*-iodinated BODIPY **3** and 2-thienyl boronic acid or 4-phenyl boronic in the presence of catalyst and analyzed by flow cytometry and fluorescence microscopy (Supporting Fig. S7 and S8). A shift of the cell population towards higher fluorescence intensity (42 %) was observed when incubating cells with **3**, 2-thienyl boronic acid and catalyst compared to control cells incubated without Pd (8 %), indicating the *in situ* formation of **4** (Fig. 2c). Fluorescence microscopy verified the presence of intracellular **4** with an increase in fluorescence compared to cells treated only with **3** and 2-thienyl boronic acid (Fig. 2d).

The Pd mediated cross-coupling reaction was applied to the *in situ* synthesis of the cytotoxic agent PP-121 **10**. PP-121[18,29] is known to suppress anaplastic thyroid carcinoma tumor growth by inhibition of mTOR (a member of the phosphatidylinositol-3-OH kinase (PI(3)K) family)[30] and tyrosine kinases (VEGF receptor).[18] Retrosynthetically, **10** can be formed from iodopyrazole **8**[18] and boronic ester **9** (Fig. 3a), and incubating **8** and **9** in the presence of Pd catalyst under aqueous conditions gave a 62 % yield of **10** in 72 h (Fig. 3b).

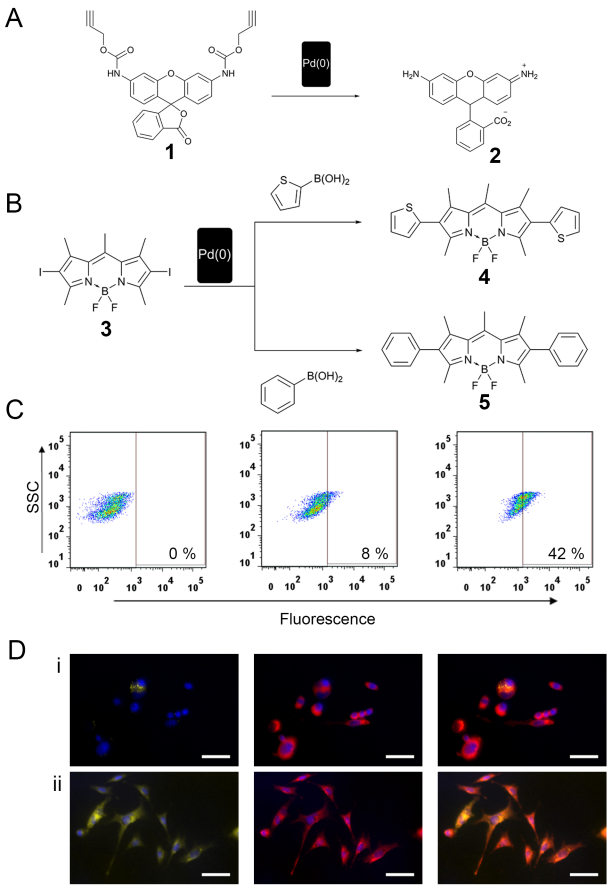

Fig. 2. Fluorescent "switch-on" *via* Pd mediated synthesis. (a) Cleavage of Proc-rhodamine 110 **1** to give fluorescent rhodamine 110 **2**. (b) Suzuki-Miyaura cross-coupling of the *bis*-aryl BODIPY dyes (**4** and **5**): Pd mediated synthesis of *bis*-thienyl BODIPY with PC-3 cells. PC-3 cells incubated with compound **3** in the presence of the Pd catalyst and 2-thienyl boronic acid. (c) Flow cytometry analysis: Left panel: untreated cells; middle panel: control cells treated with **3** and 2-thienyl boronic acid; right panel: cell treated with **3**, 2-thienyl boronic acid and Pd catalyst. (D) Cells were stained with CellMask™ Deep Red (plasma membrane stain), fixed with paraformaldehyde, incubated with DAPI (nuclei stain) and imaged by fluorescence microscopy: (i) without Pd, (ii) with Pd. Panels show from left to right: cell nucleus (blue) and synthesized compound **4** (yellow; cell nucleus (blue) and cell membrane (red); and merged images (orange indicates co-localization of synthesized compound **4** within cells). Scale bar 20 μm.

PP-121 exhibits high cytotoxicity on PC-3 cells, which express high levels of the VEGF receptor and are susceptible to kinase inhibitors, with <50 % cell viability at 0.4 μM (Fig. 3c and Supporting Fig. S9). The toxicity of the PP-121 precursors **8** and **9** were evaluated on PC-3 cells to establish the ideal concentration range that could be used in the *in situ* cross-coupling reactions. Azaindole boronic ester **9** showed negligible toxicity up to 10 μM, while iodo-pyrazole **8** showed no toxicity below 4 μM (Fig. 3c). The cross-coupling reaction (0.5 μmol Pd) was performed in the extracellular space of PC-3 cells by incubating **8** (2 μM) and **9** (10 μM) for 5 days in cell culture, with cell viability decreasing by 50 % under these conditions (Fig. 3d). Since PP-121 induces apoptosis,[18] the extent of apoptosis upon Pd mediated *in situ* synthesis of PP-121 on PC-3 cells after 24 h was evaluated *via* double staining with the apoptosis marker annexin V-FITC and propidium iodide (PI).

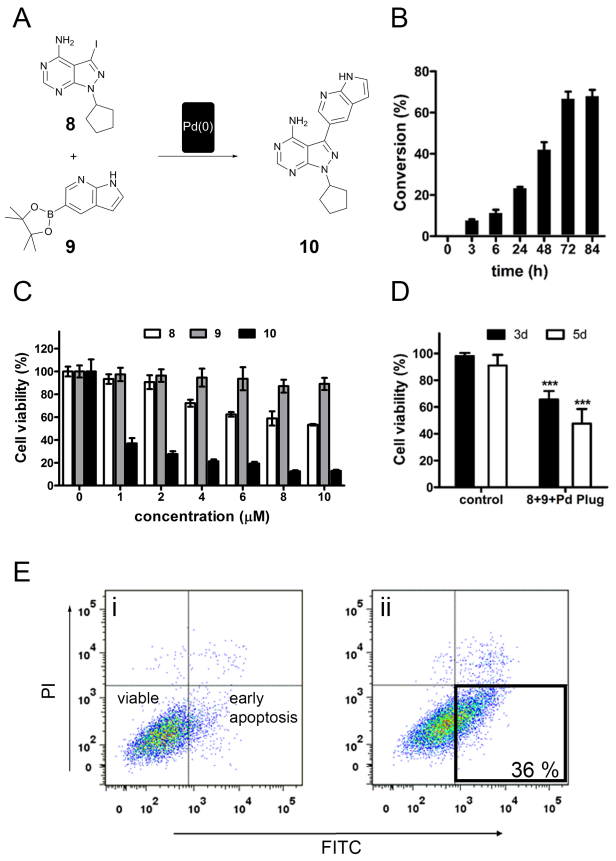

Fig. 3. Bioorthogonal *in situ* synthesis and evaluation of the anticancer agent PP-121 **10** mediated by Pd(0): (a) Cross-coupling of iodopyrazole **8** (2 μM) with boronic ester **9** (10 μM); (b) Formation of **10** monitored by HPLC with detection at 254 nm (conversion is calculated based on the integration of the peaks of **8** and **10**); (c) Cytotoxicity of **8**, **9**, and **10** on PC-3 cells. PC-3 cells were incubated with 0–10 μM of **8**, **9**, and **10** for 72 h after which cell viability was measured (MTT assay, n = 3). (d) PC-3 cells were incubated with **8** (2 μM) and **9** (10 μM) in the presence of Pd (0.5 μmol) for 3 and 5 days, after which cell viability was measured (MTT assay, n = 3). The data represent the mean ± S.D. *** P<0.001 by one-way ANOVA with Dunnett post-test, compared with the control group treated without the Pd. (e): PC-3 cells were incubated with **8** (2 μM) and **9** (10 μM) in absence (i) and in presence (ii) of Pd (0.5 μmol) for 24 h and stained with Annexin-V/FITC (X-axis) and PI (Y-axis), followed by flow cytometry analysis. Early apoptotic cells are located in the bottom right quadrant.

Treatment of PC-3 cells with precursors **8** and **9** or the Pd catalyst (Supporting Fig. S11) did not show any increase in annexin-V labelling, compared to untreated cells. However, early apoptosis was evident in cells treated with the two precursors in the presence of the catalyst, as indicated by a 36 % shift in the cell population towards higher FITC fluorescence intensity (Fig. 3e).

In conclusion, we have demonstrated the ability of a biologically inert catalyst to mediate cross-coupling reactions in a biological cell culture setting. This was established by the *in situ* synthesis of a BODIPY dye *via* Suzuki-Miyaura cross-coupling with two aryl boronic acids, resulting in fluorescent labelling of mammalian cells. The concept was successfully applied to the *in situ* synthesis of the anticancer agent PP-121 through Suzuki-Miyaura cross-coupling of two non-toxic precursors, which induced localized cytotoxicity and early apoptosis on PC-3 cancer cells. The range of therapeutic agents containing diaryl bonds provides a good basis for the applicability of this novel strategy of forming a cytotoxic compound *in situ via* cross-coupling of two inactive precursors and opens doors to new methods of prodrug activation.

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