In-cell dual drug synthesis by cancer-targeting palladium catalysts

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Abstract: Transition metals have been successfully applied to catalyze non-natural chemical transformations within living cells, with the highly efficient labeling of subcellular components and the activation of prodrugs. In vivo applications, however, have been scarce, with a need for the specific cellular targeting of the active transition metals. Here, we show the design and application of cancer-targeting palladium catalysts, with their specific uptake in brain cancer (glioblastoma) cells, while maintaining their catalytic activity. In these cells, for the first time, two different anticancer agents were synthesized simultaneously intracellularly, by two totally different mechanisms (in situ synthesis and decaging), significantly enhancing the therapeutic effect of the drugs. Tumor specificity of the catalysts together with their ability to perform simultaneous multiple bioorthogonal transformations will empower the application of in vivo transition metals for drug activation strategies.

Bioorthogonal reactions have been explored and tuned over the past 20 years to allow them to be successively applied in an array of cellular manipulations.1-10 However, reaction conditions within the biological milieu are challenging and only a handful of such chemical reactions are viable under these demanding conditions. Although the use of transition metal catalysis within living systems is non-trivial, due to stability, efficiency and potential poisoning of the catalysts, it has gained importance with many successes over the past few years.11-13 Palladium mediated transformations, especially, have been successfully used in a number of biological settings, including protein modifications and activations.14,15 These transformations typically use common Pd salts such as Pd(OAc)₂ or allyl₂PdCl₂ in high concentrations, which are not practical for in vivo applications. Encapsulating Pd catalysts has been shown to improve biocompatibility, thus Rotello, for example, showed the encapsulation of a homogeneous Pd catalyst on the surface of gold nanoparticles and their ability to intracellularly activate a prodrug of the anticancer drug 5-fluorouracil.16 Heterogeneous Pd nanoparticles embedded in polymers have been used successfully in the intra- and extracellular activation of caged fluorophores, as well as Pd catalyzed prodrug activation via depopropargylation reactions and anticancer drug synthesis via Suzuki–Miyaura cross-coupling.17-20 Intracellular activation of anticancer prodrugs via Pd catalyzed chemistries requires the catalysts to be actively targeted. There are a number of possible targeting scenarios, such as the addition of targeting ligands to the catalyst, thereby enhancing catalyst uptake and tumor specificity,21 which in combination with a dual and orthogonal Pd catalyzed prodrug activation would be a step toward potential in vivo applicability.

Herein, we report the synthesis of a targeted, multifunctional Pd catalyst, composed of palladium nanoparticle functionalized fluorescent microspheres (PdNP) decorated with the cyclic-RGD cancer targeting functionality (cRGDfE-PdNP). For validation, we show their catalytic activity by the intracellular activation of a profluorophore, but then excitingly the simultaneous generation of two anticancer drugs via two different Pd catalyzed reactions (decaging and synthesis) inside mammalian cancer cells, thus fully exploiting the potential of these Pd catalysts.

We have demonstrated that Pd nanoparticles entrapped within polystyrene microspheres are able to enter cells and perform Pd mediated reactions.20,24 Here, we used fluorescent microspheres (207.7 ± 5.9 nm) loaded with Pd nanoparticles (8.3 ± 1.7 nm, see Fig. 1), conjugated to a targeting ligand. (Fig. 1)

Supporting information for this article is given via a link at the end of the document.
The cyclic peptide cRGD is a potent antagonist of the αβ₃ receptor [26,27] that plays an important role in angiogenesis and is overexpressed in many tumors and tumor vasculature, and has been used extensively as a targeting ligand [28–32]. Here, the optimized cyclic RGDF moiety, functionalized with a dioxaotacnido acid (Doc) spacer, was used as the targeting vector (Supporting Scheme S1). This was introduced via coupling of the side-chain protected peptide c(RPf/Pd)?(Bu)E)-Doc-OH (see Supporting Scheme S2) to amino groups on the surface of the fluorescent Pd-loaded particles, with subsequent side-chain protecting group removal with 0.1 M HCl in HFIP, to give the targeting Pd-functionalized particles cRGDE-PdNP (Fig. 1, Supporting Scheme S2). Cellular uptake of the fluorescent particles (cRGDE-PdNP) was evaluated on the αβ₃-positive human glioblastoma cell line U87-MG and the αβ₃-negative human breast adenocarcinoma cell line MCF-7. The receptor-positive cell line showed >95% uptake after 1 h, whereas <4% of receptor-negative cells showed uptake (as analyzed by flow cytometry, Fig. 2). The intracellular location of the particles in U87-MG cells was determined by incubating with cRGDE-PdNP for 30 min, with fluorescence microscopy imaging showing that the particles had localized to early endosomes (Fig. 2). A competition assay with the peptide cRGDE-OH corroborated that uptake was due to receptor-mediated endocytosis, thus, when U87-MG cells were preincubated with cRGDE-OH (0.1–10 μM for 30 min), flow cytometry analysis showed a decrease in cRGDE-PdNP uptake (Supporting Fig. S2). The catalytic activity of the cRGDE-PdNP was explored by the deprotection of non-fluorescent propargylcarbamate-protected cresyl-violet 1 (Fig. 3) to form 2 (λ_{ex/em} = 583/622 nm, Supporting Fig. S3), with >6-fold increase in fluorescence at 37 °C after 18 h (Supporting Fig. S4). cRGDE-PdNP were able to activate 1 in a cell-based assay, when U87-MG cells were incubated with the catalysts for 60 min, and subsequently incubated with 1, flow cytometry analysis showed an uptake of cRGDE-PdNP (FITC emission filter 530/30 nm) by >95% of the cell population, as well as fluorescence emission due to the formation of 2 (emission filter 660/20 nm) after 1 h, with a maximum increase in fluorescence observed after 6 h (Fig. 3). The synthesis of 2 was also verified by live cell fluorescence microscopy. These results show that sufficient particles had escaped the endosomes and were catalytically competent. The targeted Pd catalyst was then used to synthesize two different anticancer agents inside cells. 5-Fluourouracil (5FU) 3 is used in clinic and exhibits its anticancer activity by inhibition of thymidylate synthase and incorporation of its metabolites into RNA and DNA. 5FU is generally given in combination with other chemotherapies, enhancing the therapeutic effect greatly [33–35].
Palladium-labile prodrugs of 5FU, for example 5-fluoro-1-propargyl uracil (Pro-5FU) 4 (Fig. 4), have been used successfully in Pd mediated prodrug activation. The cytotoxic agent PP-121 binds to tyrosine kinases (VEGF receptor) and inhibits phosphatidylinositol-3-OH kinases (mTOR), resulting in anticancer activity towards anaplastic thyroid carcinoma and can be prepared synthetically from two building blocks via Suzuki–Miyaura cross-coupling chemistry (Fig. 3). Here, we employed targeted cRGDfE-PdNP to allow localized and site-specific intracellular activation; enabling the simultaneous decaging of Pro-5FU and a Suzuki-Miyaura cross-coupling reaction to form PP-121, thus giving an increased cytotoxicity profile (Fig. 4).

Under physiological conditions and in presence of cRGDfE-PdNP, Pro-5FU 3 was converted to toxic 5FU 4 by Pd catalyzed cleavage of the propargyl group with full conversion to 4 after 24 h (see Supporting Fig. S5), while the anticancer agent PP-121 7 was synthesized from the non-toxic precursors 5 and 6 via a Pd catalyzed Suzuki–Miyaura cross-coupling with 59 ± 2% conversion to 7 after 24 h (see Supporting Fig. S6). Simultaneous decaging of 3, 5, and 6 in the presence of cRGDfE-PdNP in PBS at 37 °C generated 4 and 7 (74 ± 6% and 37 ± 3% conversion, respectively, by HPLC analysis) after 24 h (see Supporting Fig. S7), with the protodeboronation of boronic ester 6 observed as a minor side product (15% of 6, Supporting Fig. S7). This dual prodrug activation/drug synthesis was evaluated in a cell-based assay. Prodrug 3 showed no cytotoxicity up to 50 μM and PP-121 precursors 5 and 6 were non cytotoxic up to 10 μM (Fig. 4). U87-MG cells were incubated with cRGDfE-PdNP for 1 h, washed to remove any extracellular Pd, and incubated with a solution of either prodrug 3, precursors 5 and 6, or a mixture of 3, 5, and 6. Control cells incubated only with cRGDfE-PdNP, the individual precursors, or the combined precursors showed no decrease in cell viability. When combining the catalysts and the precursors, cell viability decreased to 66 % after 5 d when incubated only with 3, and to 44 % when incubated with precursors 5 and 6, whereas in the dual reaction (incubating with 3, 5, and 6) cell viability decreased to 22 % after 5 d (Fig. 5 and Supporting Fig. S8), demonstrating an increase in therapeutic effect when combining the deprotection with the Suzuki-Miyaura cross-coupling reaction.

In conclusion, cRGDfE-functionalized Pd microspheres demonstrated rapid and selective uptake in glioblastoma cells, while demonstrating the ability to activate a profluorophore intracellularly via Pd catalyzed deprotection. The targeted catalyst successfully catalyzed the synthesis of the anticancer agent PP-121 via a Suzuki-Miyaura cross-coupling of two benign precursors, while simultaneously activating a prodrug of 5FU inside glioblastoma cells, resulting in an increase in cell death compared to the individual treatments. In this therapeutic approach healthy cells accumulating the non-toxic drug precursors will not experience any effect of the drugs, whereas only in cancerous cells, in the presence of the Pd catalysts, will the active drugs be formed. This platform, combining dual drug synthesis with tumor specificity of the catalysts, is a step forward to potential in vivo transition metal catalyzed prodrug therapy.

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Keywords: Bioorthogonal chemistry • Palladium • Nanoparticles • Prodrugs

COMMUNICATION

**Targeted catalysts**: Biocompatible Pd catalysts were actively targeted to brain cancer cells and, upon internalisation, catalyzed the synthesis of two anticancer drugs simultaneously via a Suzuki-Miyaura cross-coupling reaction of two benign components and via the decaging of a protected prodrug, leading to cell death.

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