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COMMUNICATION

Gold-Triggered Uncaging Chemistry in Living Systems

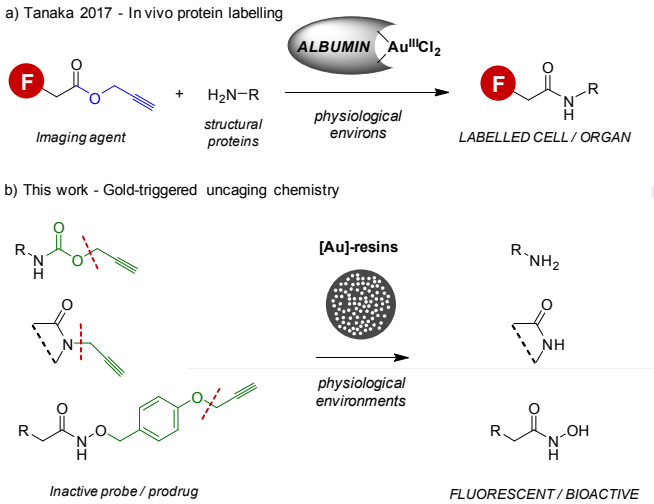
Ana M. Pérez-López, Belén Rubio-Ruiz, Víctor Sebastián, Lloyd Hamilton, Catherine Adam, Thomas L. Bray, Silvia Irusta, Paul M. Brennan, Guy C. Lloyd-Jones, Dirk Sieger, Jesús Santamaría* and Asier Unciti-Broceta*

Abstract: *Recent advances in bioorthogonal catalysis are increasing our capacity to manipulate the fate of molecules in complex biological systems. Herein we report a novel bioorthogonal uncaging strategy that is triggered by heterogeneous gold catalysis and facilitates the activation of a structurally-diverse range of therapeutics in cancer cell culture. Furthermore, this solid supported catalytic system enabled — for the first time— the locally-controlled release of a fluorescent dye in the brain of a zebrafish, offering a novel way to modulate the activity of bioorthogonal reagents in the most fragile and complex organs.*

Since the seminal works that showcased the capabilities of foreign transition metal catalysts to mediate chemoselective transformations in cells,[1] the emerging field of bioorthogonal catalysis[2] has witnessed a wealth of creativity towards a variety of applications ranging from biomolecule labelling,[3a-c] metabolite detection[3d] and intra/subcellular probe release[3e-h] to in situ enzyme[3i-j] and prodrug activation.[3k-o] Substoichiometric activity and access to a greater diversity of chemical processes and functionalities are some of the added benefits provided by abiotic transition metals to the current bioorthogonal toolbox, thus expanding the boundaries of this central field of research.[4] One of the latest additions to this area was recently reported by Tanaka and coworkers,[5] who developed a novel strategy for in vivo protein labeling mediated by glycoalbumin-bound gold(III) complexes (Scheme 1). Despite recent advances in the field, many challenges lie ahead as transition metal catalysts show limited biocompatibility in living systems both in terms of catalytic versatility and inherent toxicity.

Gold catalysis has received enormous attention in organic synthesis over the last decades.[6] Among the chemical properties of gold stand out its preference to coordinate with alkynes in the presence of other functional groups including alkenes.[6,7] Solid supported gold nanoparticles have also attracted the interest of chemists searching for greener catalysts due to their recyclability, the safety of gold and its remarkable catalytic properties to mediate oxidative reactions at or even below ambient temperature.[7] In the chemical biology field, however, the chemistry of gold is dominated by the near-covalent S-Au bonding.[8] This spontaneous bond formation provides the basis for the bottom-up self-assembling of monolayers functionalized with a multitude of small molecules and biomolecules at the surface of the metal, a highly-reliable process that has found widespread application in nanotechnology, biotechnology and theranostics.[8] Because of the high affinity of thiols for gold and their ubiquitous presence in peptides and proteins, the attractive alkynophile properties of gold nanoparticles (Au-NP) pass unnoticed in the biological milieu. We envisioned that embedding Au-NP in a solid support would serve to protect the metal nanostructures from large thiol-rich biomolecules, while allowing the free entry of alkyne-functionalized small molecules to undergo gold-mediated chemistries even in biological systems. Importantly, based on the high biocompatibility of metallic gold, such a device would be optimal to catalyze bioorthogonal transformations in vivo. In a suitable shape and size, this functional device could potentially be implanted by a surgeon at the anatomical site of a localized cancer (e.g. brain) and enable the local "manufacture" of different drugs —in a catalytic fashion— from systemically-administered innocuous starting materials. This unique delivery method would offer the benefits of drug release systems[9] (i.e. focal treatment, reduced general side effects) with fewer limitations (e.g. extended lifetime, access to multiple therapies).

Towards this goal, herein we report the first example of bond cleavage chemistry mediated by heterogeneous gold catalysis in living systems (Scheme 1), a previously-overlooked chemical reactivity of gold that facilitates the efficient bioorthogonal uncaging of various clinically-approved anticancer drugs in cancer cell culture and the first intracranial activation of a bioorthogonal probe in zebrafish.



Scheme 1. a) Au^{III}-mediated bioorthogonal amidation reported by Tanaka and coworkers.[5] b) The solid supported gold catalyzed uncaging strategy developed in this work.

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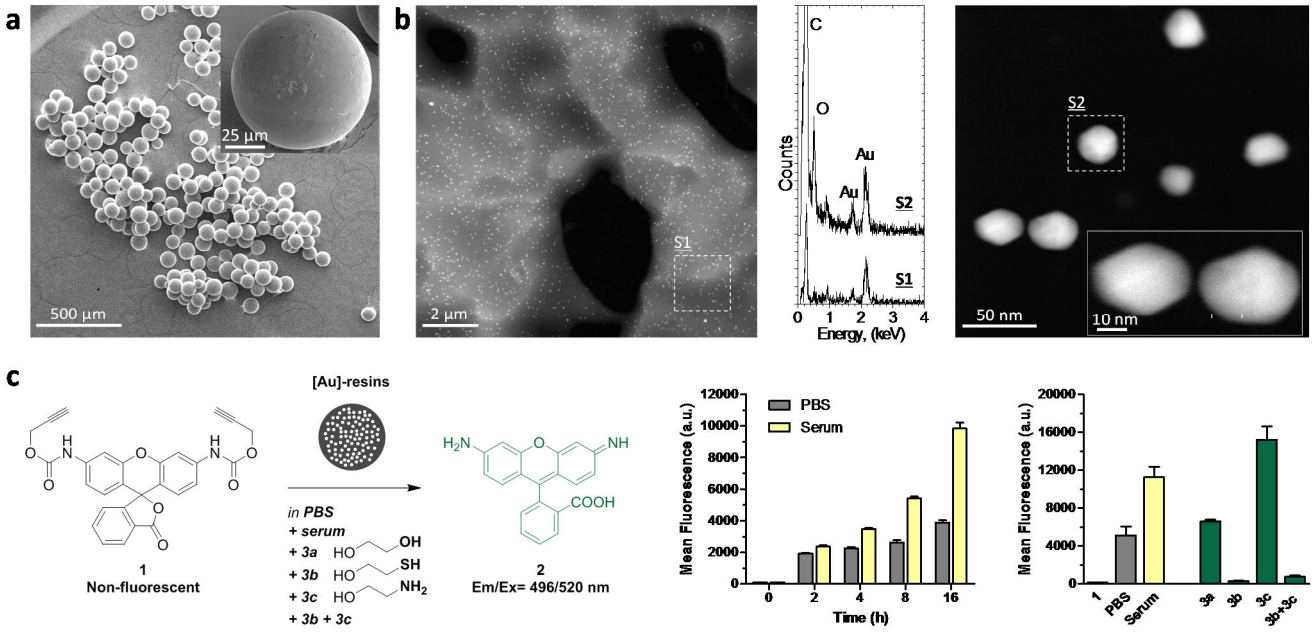


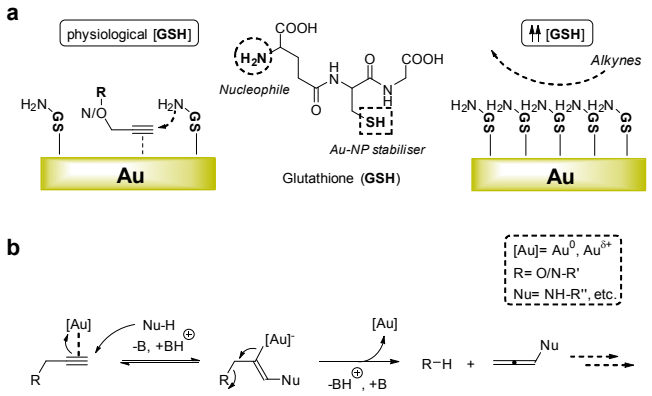
Figure 1. (a) Scanning electron microscopy images of [Au]-resins. (b) HAADF-STEM images of a cross-section of a [Au]-resin at different magnifications and energy-dispersive X-ray spectra of highlighted areas. (c) Cleavage of *N*-Poc-protected prodye **1** (20 µM) in the presence of [Au]-resins (1 mg/mL) at physiological conditions (pH= 7.4, 37 °C). Central panel: reaction kinetics in PBS or serum. Right panel: fluorescence analysis at 24 h in different conditions. Negative control: reagent **1** without [Au]-resins.

Solid supported gold catalysts (Fig. 1a,b) were prepared by in situ generation of Au-NP within a PEG-grafted low-crosslinked polystyrene matrix. In short, amino-functionalized TentaGel® HL resins of 75 microns in diameter (Rapp Polymere GmbH) were treated with tetrachloroauric(III) acid and NaOH in water:THF followed by reduction with tetrakis(hydroxymethyl)phosphonium chloride (THPC; see full synthesis in the Supp. Inf.). THPC was used due to its relatively mild reducing properties to control particle growth and distribution.[10] High angle annular dark field (HAADF) scanning transmission electron microscopy (STEM) images of ultramicrotome cross-sections of the resins showed the presence of polyhedral nanocrystals of 30 nm (average diameter) uniformly dispersed across the polymer framework (Fig. 1b and Supp. Fig. 1). X-ray photoelectron spectroscopy analysis (Supp. Inf.) detected an incremental gradient of gold concentration from the surface to the core of the resins with an $Au^0/Au^{\delta+}$ ratio ranging from 7 (periphery) to 19 (interior).

To investigate the properties of the [Au]-resins in physiological conditions, the devices were incubated with nonfluorescent compound **1**, which upon *O*-depropargylation releases strongly fluorescent rhodamine 110, **2** (Fig. 1c). Reactions were performed in PBS (pH= 7.4) at 37 °C either with or without serum and fluorescence measured with a spectrophotometer. Analysis revealed the rapid generation of fluorescence under both conditions, with the presence of serum achieving a much greater fluorogenic effect (~25% yield), demonstrating the feasibility of performing Au-NP-mediated chemistry beyond the S-Au bonding in biocompatible environments. Time-course and reusability studies (Supp. Fig. 3-4) provided proof of the durability of the devices and their capacity to activate multiple doses of masked reagent, although a gradual decay in activity was observed over time.

To better understand the enhanced catalytic activity of the [Au]-resins in the presence of serum, we investigated the influence of OH, SH and NH_2 groups (nucleophilic groups found in serum proteins) in the conversion of **1** into **2** by adding (in excess) ethylene glycol (**3a**), 2-mercaptoethanol (**3b**) and ethanolamine (**3c**), respectively. These low molecular weight chemicals were used to facilitate diffusion throughout the resins, thereby maximizing their interaction with internal Au-NP. All the reactions were carried out in PBS at 37 °C. Although **3a** had a relatively minor effect on the catalytic capacity of the devices, substantial variations in activity were observed in the presence of **3b** and **3c** (Fig. 1c). Thiol **3b** suppressed the reactivity of the resins almost completely, whereas addition of amine **3c** significantly boosted catalytic activity. The combined presence of an excess of both **3b** and **3c** resulted in low levels of fluorescence intensity. This prompted us to investigate the influence of glutathione in the reaction, a natural reductant that contains a SH and an NH_2 group in its structure. Since glutathione is ubiquitously found in the human plasma and the interstitial fluid at a concentration of 2-20 µM,[11] we studied the reaction of **1** and [Au]-resins at concentrations ranging from 10 to 400 µM. As shown in the Suppl. Fig. 5, increasing glutathione levels up to 50 µM promoted the reaction, whereas greater concentrations led to a substantial reduction in fluorescence intensity. Notably, a large regain in catalytic activity was achieved upon addition of an extra mg of [Au]-resins to the inhibited reactions. In contrast, if the concentration of probe **1** was augmented, no significant increment of fluorescence generation was observed. These results indicate that S-Au bonding of glutathione molecules on the surface of the Au-NP promote the dealkylation reaction until a saturation threshold is reached (see rationale in Scheme 2a). Over the saturation limit, gold-bound biomolecules will coat most active sites on the Au-NP surface thus hindering gold-alkyne coordination. A series of tests carried out to monitor and analyze the reaction (see Supp. Fig. 6-7) corroborated that rhodamine **2** was the main product of the reaction and found intermediates that could correspond to organogold species. However, no reaction byproducts could be either isolated or identified, which points to

the production of short-lived compounds. Based on these experimental observations, we tentatively propose a dealkylation pathway whereby gold acts as a pi-acid to activate the nucleophilic addition of biomolecules onto the terminal alkyne group, leading to release of the leaving group (e.g. a dye or drug) and the generation of reactive allenyl byproducts (Scheme 2b) that isomerise or hydrolyse under the reaction conditions.



Scheme 2. (a) Rationale for the assisting-inhibiting roles of glutathione in [Au]-catalysed *O*/*N*-propargyl cleavage reactions. (b) Tentative reaction mechanism for the [Au]-triggered depropargylation of **1** and **4a-c** in the biological milieu.

Prior to testing the catalytic properties of the devices in cell culture, viability assays (PrestoBlue® reagent) were performed to determine the tolerability of cells to the presence of solid supported gold. As anticipated, [Au]-resins were found to be fully biocompatible at the concentrations tested (Supp. Fig. 8).

Next, the bioorthogonal [Au]-triggered release of a structurally-diverse selection of clinically-used anticancer drugs was investigated in culture with human lung cancer A549 cells. Three different drug precursors were tested (see Fig. 2a): Pro-FUdR,[12a] **4a**, POB-SAHA,[12b] **4b**, and *N*-Poc-DOX, **4c** (novel drug precursor inspired on prior designs[3m,13]). Cells were treated with **4a-c** and [Au]-resins separately (negative controls) or in combination (activation assay), and unmodified drugs **5a-c** used as the positive controls. Remarkably, while prodrugs **4a-c** did not elicit any effect on their own, potent anticancer activity was displayed in combination with [Au]-resins (Fig. 2a), unequivocal evidence that the active drugs are released in situ by heterogenous gold chemistry. Reuse of the [Au]-resins in three consecutive prodrug activation cycles confirmed the capacity of the devices to activate multiple drug doses (Suppl. Fig. 9). The synthesis of drugs **5a-c** was also verified in vitro (Suppl. Fig. 10), confirming the capability of gold to cleave both *O*- and *N*-propargyl groups from a range of molecules based on structurally-different scaffolds. These studies support a potential application scenario where gold-functionalized implants could be used in situ to modulate the spatiotemporal generation of chemotherapeutics from inactive precursors in the treatment of localized malignancies such as brain or prostate cancer.

Encouraged by the biocompatibility and catalytic properties of the [Au]-resins, we embarked on an innovative study to evaluate the capacity of the devices to convert nonfluorescent **1** into rhodamine **2** inside the cranium of zebrafish embryos. First, a single [Au]-resin was carefully transplanted into the optic tectum, a small anatomical cavity[14] of the brain of zebrafish larvae. After the surgery, either reagent **1** (activation assay) or just DMSO (negative control) were added to the medium and embryos imaged at 24 h (n=5). Due to its lipophilicity, prodye **1** can enter the zebrafish through the skin and/or by ingestion and distribute systemically, but will only be converted into fluorescent compound **2** upon reaction with the [Au]-bead. As shown in Fig. 2b, a strong green fluorescent signal originating from the [Au]-resin was observed only when incubated with **1**, confirming the local generation of rhodamine **2**. Prolongation of the study by three additional days corroborated previous observations regarding the sustained functionality of the devices (Suppl. Fig 11). This study, which represents the first bioorthogonal organometallic reaction to be locally performed in the brain of a living animal, proves the capacity of heterogeneous gold catalysts to mediate in vivo bioorthogonal release of functional reagents in a spatially controlled manner.

In conclusion, we have developed a heterogeneous catalytic system that enables access to chemical properties of Au-NP that were previously out of our reach in biological environments. Such devices triggered the bioorthogonal uncaging of a structurally-diverse selection of cytotoxic precursors via an unexplored chemical reactivity of gold, providing a novel and safe method to activate therapeutics by nonbiological chemical stimuli.[3k-o,13,15] Furthermore, this solid supported catalyst enabled —for the first time— the locally-controlled release of a fluorescent dye in the brain of a zebrafish, a notable breakthrough that expands our capacity to chemically modulate the activity of bioorthogonal reagents into the most fragile and complex organs.

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Keywords: Gold • Heterogeneous Catalysis • Bioorthogonal • Prodrugs • Fluorescent Probes

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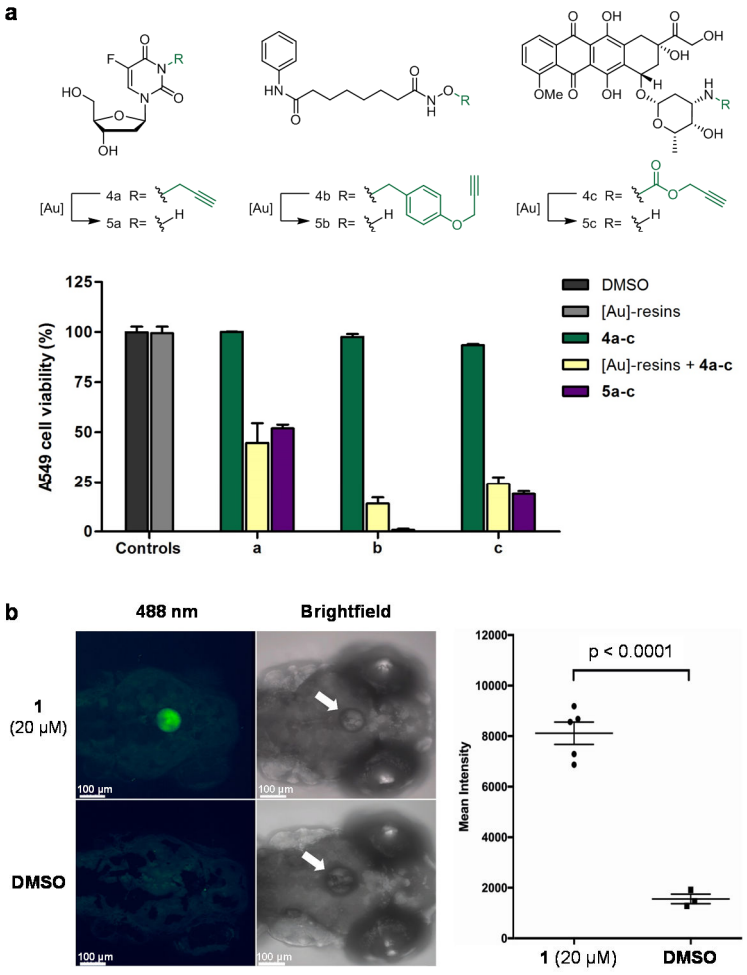

Figure 2. a) Gold-triggered activation of prodrugs **4a-c** in A549 cancer cell culture. Negative controls: [Au]-resins (1mg/mL); **4a-c** (10µM, 100µM and 1µM, respectively). Positive control: **5a-c** (10µM, 100µM and 1µM, respectively). Prodrug activation assay: [Au]-resins + **4a-c** (10µM, 100µM and 1µM, respectively). Cell viability was measured at day 4 using PrestoBlue reagent. Error bars: ± SD from *n* = 3. b) Bioorthogonal gold-mediated release of green fluorescent rhodamine 110 from precursor **1** in the brain of a zebrafish. The presence of the [Au]-resin is indicated with a white arrow. Study of fluorescence intensity shows high statistical significance compared to the negative control (DMSO).

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COMMUNICATION

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Gold-Triggered Uncaging Chemistry in Living Systems

Hidden in the glorious wildness like unmined gold! A novel bioorthogonal uncaging strategy triggered by heterogeneous gold catalysis mediates the activation of probes and therapeutics both in vitro and in vivo.