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COMMUNICATION

Synthesis of heterogeneous enzyme/metal nanoparticles biohybrids in aqueous media and their applications in C-C bond formation and tandem catalysis†

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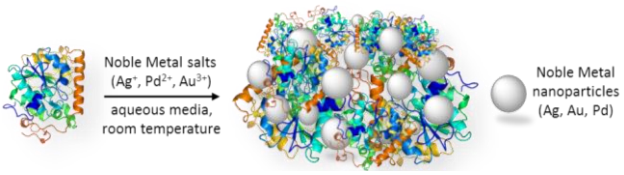
The straightforward synthesis of novel enzyme/metalNPs nanobiohybrids in aqueous medium which combined noble metal and enzyme catalytic activities was developed. These new nanobiohybrids were excellent multivalent catalysts combining both activities in a various set of synthetic reactions in different media and even at ultra-low concentrations (ppb amount).

Nanocatalysis has undergone to an explosive growth during the past decade, both in its homogeneous and heterogeneous forms.[1,2] The large surface-to-volume ratio of nanomaterials compared to bulk materials makes them attractive candidates for their use as catalysts.[3] For example, palladium nanoparticles (PdNPs) have played a pivotal role in a wide variety of C–C coupling reactions.[4] Generally, the most useful approaches for nanoparticles synthesis[5] usually employ harsh conditions (high temperature and pressure and the use of toxic organic solvents). Therefore, biological methods could represent a green alternative. For example, RNA sequences or polypeptides were discovered to aid the formation of nanoparticles by using a reducing agent (typically ascorbic acid or sodium borohydride).[6-7] Amongst all the biomacromolecules, enzymes are natural proteins catalysts (up to 10^{17}-fold rate accelerations) with nanodimensions (eg. lipase from *Candida antarctica* B; 3 nm×4 nm×5 nm) that can catalyse a wide set of reactions with exquisite control of regio- and stereochemistry. They have been also involved as reactants in the synthesis of metal nanoparticles[8] and hybrid nanostructures.[9] Therefore, considering the continuous demand for the development of new catalysts with high-efficiency and broad reaction scope, the creation of heterogeneous hybrid nanocatalysts with an overall catalytic activity resulting from the combination of two different but complementary catalytic activities (noble metal NPs together with enzymes) could represent a straightforward breakthrough in this field.

Here we describe the synthesis of novel enzyme/metalNPs nanobiohybrids, where NPs were generated *in situ* from an aqueous noble metal salt solution (Scheme 1). The enzyme acted, as i) reducing agent for nanoparticle formation, ii) stabilizing and supporting agent (avoiding nanoparticles aggregation) and iii) biocatalyst, all at the same time. These new nanobiohybrids were successfully applied as heterogeneous catalysts in a set of different interesting reactions exploiting selectively the catalytic activity of the metal, the enzyme or both at the same time (domino and tandem reactions). Furthermore these new catalysts were recycled several times keeping intact their catalytic properties. Initially, the synthesis of the nanobiohybrids in aqueous medium was attempted adding the commercial liquid



Scheme 1. Enzyme/MetalNPs biohybrids preparation strategy.

Candida antarctica B lipase (CAL-B) to an aqueous solution of fully water soluble Na_2PdCl_4 salt at room temperature and under gentle stirring. After 24h, the initial clear solution turned into a slight cloudy suspension where only 10% of the enzyme disappeared (by Bradford method[10]) forming a heterogeneous composite (Table 1 and Table S1).

Table 1: Synthesis of enzyme-Pd nanobiohybrids.

[a] 20% (v/v); [b] The amount of precipitated protein was calculated by Bradford assay of supernatant after 24h. [c] the metal amount disappeared from the solution was calculated by ICP-AES analysis of supernatant after 24h.

Catalyst	Co-Solvent[a]	Metal salt (1 mg/mL)	Protein amount (%)[b]	Metal amount (μmol)[c]
CALB/PdNPs-1	--	Na_2PdCl_4	10	nd
CALB/PdNPs-2	DMF	$Pd(OAc)_2$	99	6.45
CALB/PdNPs-4	ACN	$Pd(OAc)_2$	99	7
CALB/PdNPs-5	MeOH	$Pd(OAc)_2$	99	6.68
CALB/PdNPs-6	THF	$Pd(OAc)_2$	99	7.12

Using $Pd(OAc)_2$ in presence of different co-solvents, after 24 h a quantitative precipitation of the protein was observed (Table 1). An ICP-AES analysis of the supernatants revealed that 6.45-7.57 μmol of Pd^{2+} amount were entrapped on the protein. CALB/PdNPs-(2-7) biohybrids were recovered by centrifugation, washed with distilled water and lyophilized.

TGA of the CALB/PdNPs-2 lyophilized powder (Figure S1) showed that 26% (w/w) of the amount of Pd composed the solid material, confirming the value previously obtained by ICP-AES.

As initial representative example, the CALB/PdNPs-2 nanobiohybrid was fully characterized by SEM, EDX, XRD, XPS and TEM (Figure 1, Figure S2). EDX experiment (Figure 1) as well as SEM analysis revealed that the formed precipitate was constituted by an aggregate with a mesoporous amorphous suprastructure composed by palladium atoms dispersed into an organic matrix (CAL-B) (Figure S2a). TEM analysis together with XRD and XPS experiments (Figure S2) confirmed the generation of PdNPs embedded in the protein net (Figure 1). The morphology and the distribution of PdNPs were investigated by TEM and HRTEM microscopy. A dual particle size distribution

was observed (Figure 1, Figure S2). The main fraction was composed by rather spherical particles with an average diameter of 1.3 nm densely deposited throughout the hybrid composite. The minor fraction was constituted by larger NPs with an average diameter of 4.5 nm randomly decorating the enzymatic network (Figure S2). The HRTEM image of the larger NPs clearly showed their atomic lattice confirming the high crystallinity of the NPs (Figure S2). The CALB/PdNPs (3-6) biohybrids were also characterized by EDX and TEM (Figure S3). In all the cases, the bimodal distribution in NPs was maintained although with slight differences in distribution size (1.5-3.5 and 5.5-6.8 nm average size for main smaller NPs and minor larger NPs, respectively (Figure S3)).

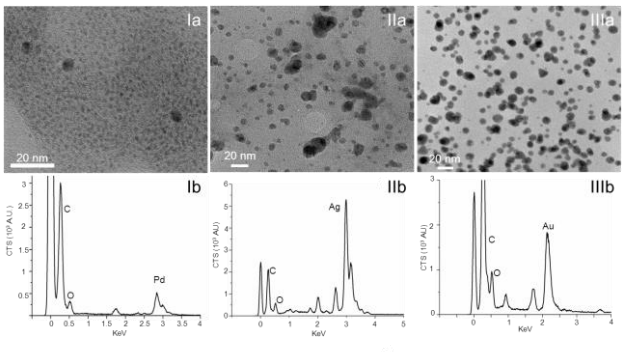


Fig 1. Physicochemical characterization of nanobiohybrids I) CALB/PdNPs-2, II) CALB/AgNPs-1, III) CALB/AuNPs-1 a) TEM images. b) EDX pattern,

To expand the method, $AgNO_3$ and $HAuCl_4$ were tested as precursors with the same experimental procedure previously developed for CALB-PdNPs hybrids. With the best conditions, quantitative (CALB/AgNPs-2) or almost quantitative (CALB/AuNPs-1) yields of both biohybrids were achieved (Table S2). ICP analyses revealed that 37-41 μmol of Ag^+ and about 19 μmol of Au^{3+} were entrapped inside the protein net. EDX and TEM analyses confirmed the presence of silver or gold spherical nanoparticles (Figure 1, Figure S4) with a unique particle size distribution of about 8 nm average diameter for CALB/AgNPs-1 and CALB/AuNPs-1 (Figure S4). When Ag and Au nanobiohybrids were synthesized in presence of cosolvents, the average size distribution was slightly higher (10 nm) for CALB/AgNPs-2 and CALB/AuNPs-2 (Figure S4).

Therefore, considering all these results, a two-step mechanism for the enzyme-noble metal NPs nanobiohybrid formation can be proposed (Scheme S1): i) a first rapid adsorption of soluble Me^{n+} ions on the enzyme, reducing its solubility and acting as a cross-linker between the enzyme's molecules (initial fast precipitation) and afterwards ii) an "*in situ*" reduction of metal ions in the absence of any exogenous reducing agents, finally generating the NPs.[11-12] FTIR experiments together with the pH-dependent zeta potential measure of native CAL-B and the CALB/PdNPs-2, supported this idea (Figure S5)(For a more detailed discussion about nanobiohybrid formation mechanism, see Supporting Information).

The synthesized metal NPs were very stable in aqueous solution without any changes in particle size and morphology within three months (data not shown) providing further evidence that enzyme network acts not only as physical support and reducing agent during the NP synthesis but it also serves as stabilizing agent.

The catalytic properties of CALB/MetalNPs biohybrids were initially tested in the hydrolysis of 4-nitrophenyl butyrate **1** to obtain the chromogenic 4-nitrophenol **2** (by enzyme catalysis), in the reduction of **2** to 4-aminophenol **3** (by metal catalysis) and in the domino one-pot transformation of **1** to **3** (by combo-catalysis) as model reactions (Figure 2, Table S3).

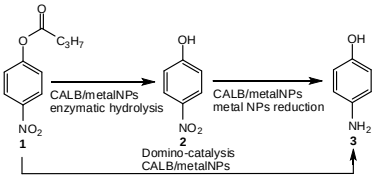
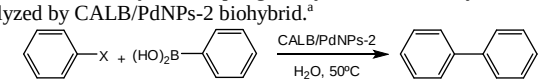


Fig. 2 Domino reaction for the synthesis of aminoarene **3**.

Among all the Pd nanohybrids, CALB/PdNPs-5 and CALB/PdNPs-6 recovered the highest enzymatic activity (around 47%) in the hydrolysis of **1** compared to the starting soluble CALB. Almost no enzymatic activity (<5%) was found in the CALB/AgNPs biohybrids whereas 25% of the initial enzymatic activity was recovered in the CALB/AuNPs biohybrids (Table S3). For the catalytic reduction of **2**, the reaction rate constant (*k*) and the turnover frequency number (TOF) were calculated for the CALB/MetalNPs biohybrids showing the better enzymatic activity (Table S3, Figures S6-8). The CALB/PdNPs-5 biohybrid exhibited the highest *k* and TOF values (0.6 min^{-1} and almost 150 min^{-1}, respectively) with only slight differences compared to CALB/PdNPs-6 (Table S3). As far as we know, the TOF value is *the highest* one described in the literature for this reaction.[13] *k* and TOF values for CALB/AgNPs-1 and CALB/AuNPs-1 were 0.28 min^{-1} and about 10 min^{-1}, and 0.31 min^{-1} and about 28 min^{-1}, respectively, being these values comparable to the best ones reported in literature for these metals.[14] Consequently, CALB/PdNPs-5 was selected for the direct domino transformation of **1** to **3** (Figure 2, Figure S6) confirming the good results previously achieved for separate.

We continued our study evaluating the catalytic performance of the CALB/PdNPs in C-C coupling reactions. The Suzuki-Miyaura cross coupling reaction in aqueous media was attempted using the CALB/PdNPs-2 biohybrid as catalyst (considering its highest NPs superficial area owing to their smallest average diameter and its highest catalytic activity in the reduction of **2**) (Table 2). Different aryl-halides, bases and the presence of phase transfer catalysts (PTC)[15] were evaluated (Table 2 and Table S4).

Table 2: Suzuki-Miyaura coupling of aryl halides with aryl boronic acid catalyzed by CALB/PdNPs-2 biohybrid.[a]



4 **5** **6**

[a] Reaction conditions: **4** (0.5 mmol), **5** (0.55 mmol), H_2O (1 mL), 130 ppb of Pd catalyst, 50°C; [b] Phase transfer catalyst; 0.165 mmol; [c] 1.5 eq.; [d]Calculated by HPLC analysis as described in Supporting Information.

Entry	X	PTC[b]	Base[c]	Time (h)	Yield (%)[d]
1	Cl	--	NaOH	48	2
2	Br	--	NaOH	24	50
3	I	--	NaOH	24	55
4	Br	TBACl	NaOH	2.5	99
5	I	TBACl	NaOH	38	52

Using chlorobenzene, the reaction yield was negligible in all the cases (Table 2, entry 1) whereas 50-55% yield of biphenyl **6** was obtained when aryl-bromide or iodide were used (Table 2, entries 2 and 3). The addition of PTCs showed a positive effect only when the reaction was performed in presence of aryl-bromide achieving almost quantitative yields of **6** in presence of TBACl (Table 2, entry 4), using 0.025% (mol/mol) of Pd amount (about 130 ppb of Pd), with a TON and TOF of 3876 and 1550 h^{-1},

respectively.[16] The nanobiohybrid was used during 5 reaction cycles retrieving similar results (in term of yield and rate (TOF)) in each cycle and without significant activity loss (Table S5).

To further expand the practical application of these biohybrids in organic synthesis, the Heck reaction was studied (Table 3 and Table S6) selecting CALB/PdNPs-4 as representative catalyst.

Table 3. Heck coupling of aryl iodide with ethyl acrylate catalyzed by CALB/PdNPs-4.[a]

7 8 9

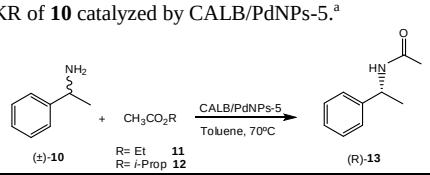
Co-solvent (%v/v, H_2O)	T (ºC)	Time (h)	Yield[b] (%)
--	70	24	0
25	70	18	99
50	70	24	20

[a] Reaction conditions: **7** (0.274 mmol), **8** (0.55 mmol), DMF (1 mL), 1 mg of CALB/PdNPs-4 catalyst, 70°C, triethylamine (TEA) (0.412 mmol); [b] Calculated by HPLC analysis as described in Supporting Information.

Different reaction parameters as temperature or presence of water where evaluated in order to soften the common harsh reaction conditions (Table S6). In the best conditions, quantitative conversion of product **9** in 18 h at 70ºC with 25% (v/v) water (Table 3) was achieved. The biohybrids CALB/PdNPs-2, 5 and 6 exhibited similar results (data not shown).

Finally, the nanocatalysts were tested in a tandem catalysis process (both enzymatic and Pd catalytic activities acting at the same time) as the dynamic kinetic resolution (DKR)[17] of *rac*-phenylethylamine **10** (Table 4 and Table S7).

Table 4. DKR of **10** catalyzed by CALB/PdNPs-5.[a]



Entry	R	Base[b]	Time (h)	*C* (%)[c]	ee (%)[d]
1	$Prop_{iso}$	--	5	88	90
2	Et	--	4	98	>99
3	Et	TEA	4	91	28
4[e]	Et		4	98	>99

[a] Reaction conditions: **10** (0.01 mmol), **11** or **12** (0.06 mmol), toluene (1 mL) and 70°C, 5 mg CALB/PdNPs-5. [b] 0.07 mmol. [c] Calculated by RP-HPLC analysis. [d] Determined by chiral HPLC. [e] CALB/PdNPs-6 was used as catalyst.

Firstly, both reaction (hydrolysis and racemization) were studied separately (Figure S9). Free lyophilized CALB was used to study the enzymatic transesterification of **10**, showing a very high enantioselectivity toward the R enantiomer (ee>99%). The CALB/PdNPs-4 and CALB/PdNPs-5 were used in the Pd-racemization process of the enantiopure S-**10** achieving the *rac*-**10** in both cases (Table S8). Therefore, the tandem enzyme-Pd catalysis was performed using CALB/PdNPs-5 which was the best catalyst in term of enzymatic hydrolytic activity. Different parameters as acyl donors, use of molecular sieves or different bases were evaluated (Table 4, Table S7). In the best case, using ethylacetate as acylating agent, *rac*-**10** was transformed by CALB/PdNPs-5 in *R*-**13** at almost quantitative conversion and excellent enantiopurity (ee>99%) in 4 h. The CALB/PdNPs-5 was reused for three cycles maintaining its activity and selectivity intact (Table S9). In the same conditions, the CALB/PdNPs-6 biohybrid showed the same result (Table 4).

In summary, the straightforward *in situ* synthesis of metal NPs, induced by enzyme, generates a new class of enzyme-metalNPs biohybrids has been achieved at very mild conditions (aqueous medium, room temperature and no reductive agents). These nanobiohybrids have been proved as excellent heterogeneous catalysts in different synthetic reactions such as domino aminoarene synthesis, C-C bond formations and tandem dynamic kinetic resolution of secondary amines. We envision that these new hybrid catalysts that could open a new way to rationally exploit the advantages offered by the combination of organometallic chemistry and biocatalysis.

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Notes and references

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† Electronic supplementary information (ESI) available: Experimental section, characterization data of the different nanobiohybrids, recycling studies and additional Tables and Figures. See DOI: 10.1039/XXXXXXX

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