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Preparation of an Immobilized Lipase-Palladium Artificial Metalloenzyme as Catalyst in the Heck Reaction: Role of the Solid Phase

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Abstract: A *p*-nitrophenylphosphonate palladium pincer was synthesized and selectively inserted by irreversible attachment on the catalytic serine of different commercial lipases with good to excellent yields in most cases. Among all, lipase from *Candida antarctica* B (CAL-B) was the best modified enzyme. The artificial metalloenzyme CAL-B-palladium (Pd) catalyst was subsequently immobilized on different supports and by different orienting strategies. The catalytic properties of the immobilized hybrid catalysts were then evaluated in two sets of Heck cross-coupling reactions under different conditions. In the first reaction between iodobenzene and ethyl acrylate, the covalent immobilized CAL-B-Pd catalyst resulted to be the best one exhibiting quantitative production of the Heck product at 70 °C in dimethylformamide (DMF) with 25% water and particularly in pure DMF, where the soluble Pd pincer was completely inactive. A post-immobilization engineering of catalyst surface by its hydrophobization enhanced the activity. The selectivity properties of the best hybrid catalyst were then assessed in the asymmetric Heck cross-coupling reaction between iodobenzene and 2,3-dihydrofuran retrieving excellent results in terms of stereo- and enantioselectivity.

Keywords: immobilization; lipases; metalloenzymes; palladium pincers

Introduction

Artificial metalloenzymes are formed by the combination of a biomolecule (peptide, DNA or protein)[1–3] – as host – and an organometallic compound. Being a second coordination sphere, the protein matrix confers to the metal catalysts better performances especially in terms of stereo- and regioselectivity.[4–8] Sometimes, non-catalytic metals can even be transformed into very interesting catalysts by means of their insertion into a proteic core.[9]

The metal insertion on the protein scaffold has been generally performed by three different strategies:[1–3] (i) supramolecular anchoring (by a strong affinity ligand, such as biotin[10]), (ii) dative anchoring (by direct metal coordination with amino acid residues)[11] or (iii) covalent attachment (by selective anchoring of a metal-binding ligand on an α-amino acid).[12]

Among the three strategies, the covalent protocol presents several advantages, such as a more precise knowledge of the location of the metal complex by a site-selective coupling, no dependence on the binding affinity of the complex to the protein and the possibility to promote the target modification in different sites on the protein structure. Recently, elegant strategies based on the modification of the native protein scaffold[13] or the combination of genetic modification of proteins by insertion of unnatural amino acids, and orthogonal strategies promoting the selective covalent coupling of the organometallic complexes have been developed.[14] Nevertheless, despite their tremendous potential, most of the artificial enzymes described to date have been mainly developed under a more academic point of view. Hence, with a more applied aim, the development of concepts such as versatility or large availability of the used host protein must be strongly considered. For example, the combination of

proteins with high catalytic versatility could generate higher possibilities depending on the transition metal or metal binding ligands inserted. Moreover, the accessibility to a high amount of the protein is mandatory in order to obtain a high amount of the final artificial metalloenzymes.In this context, lipases – acylglycerol hydrolases – fulfill both requirements. These are very versatile enzymes with successful results in different processes using non-natural substrates[15] and they are commercially available in large amounts. Recently, these enzymes have been combined with metals in the preparation of several hybrids catalysts.[16–18] Considering the catalytic mechanism of these enzymes – with a catalytic serine in the active site – *p*-nitrophenol (pnP) phosphonate esters (typically used as serine-protease irreversible inhibitors[19]) can be applied as practice anchoring units on the organometallic complex for its specific incorporation into the catalytic pocket.[19]

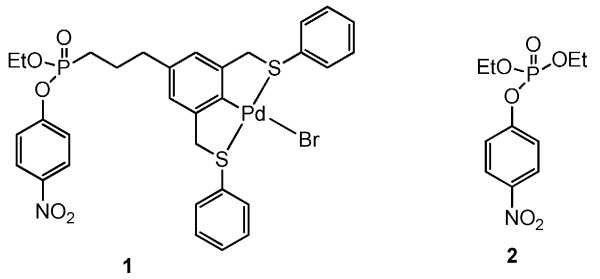
In general, these kinds of artificial metalloenzymes have been created in solution and the host protein showed a critical influence on the catalytic properties of the created artificial metalloenzymes. Indeed, especially the stability of the enzyme clearly results as the bottleneck of the entire strategy mainly considering the drastic reaction conditions expected by the metal transition application. Hence, this kind of catalyst cannot be reused for several cycles.

Therefore in this paper we describe the preparation of an artificial metalloenzyme on a solid support fulfilling all these criteria. Thanks to the use of the solid-phase chemistry, many advantages such as the possibility to use excess of metal complexes, quantitative transformations or easy purification have been generated.[21]

Herein, the design of immobilized Pd-lipase artificial metalloenzymes is described. The *p*-nitrophenylphosphonate palladium pincer **1** (Scheme 1) was synthesized and used as an organometallic complex.

For that purpose, lipases from different sources, different enzyme immobilization protocols (covalent attachment and physical adsorption) and different support materials (Sepharose and Sepabeads©) were combined in order to create different immobilized artificial enzymes.

Finally, a post-immobilization catalyst engineering (based on the specific chemical modification of the supporting matrix surface) was evaluated in order to obtain the best artificial metalloenzyme. All the new artificial metalloenzymes were evaluated as catalysts in C–C coupling Heck reactions under different conditions. The best lipase-Pd artificial metalloenzyme showed higher activity than the soluble Pd catalyst, also maintaining a complete *trans* selectivity and it was recycled several times. Beside the activity, even the stereo- and enantioselectivity of the best hybrid catalyst were evaluated by its application in an asymmetric Heck cross-coupling reaction with excellent results.



Scheme 1. Different phosphonate-based structures. Organopalladium pincer (**1**) and lipase irreversible inhibitor (**2**).

Results and Discussion

Site-Directed Incorporation of the Pd Complex 1 to Different Lipases

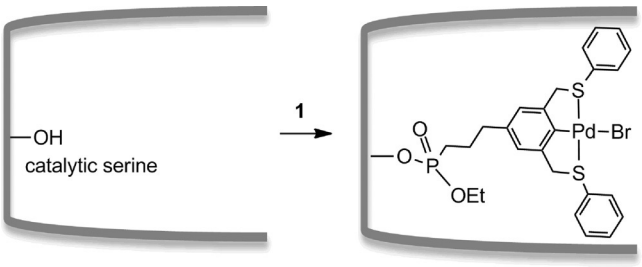
The anchoring of the *p*-nitrophenylphosphonate palladium pincer to the lipase catalytic serine (Scheme 2) was followed by an enzymatic hydrolytic activity drop, determined by monitoring the difference in absorbance increase of the released *p*-nitrophenolate anion by UV spectrophotometry.

The reaction of **1** with different lipases was carried out using five equivalents of **1** with respect to the lipase amount and the lipases were previously covalently immobilized on CNBr-activated Sepharose (Sepha-CNBr) (Scheme 3).

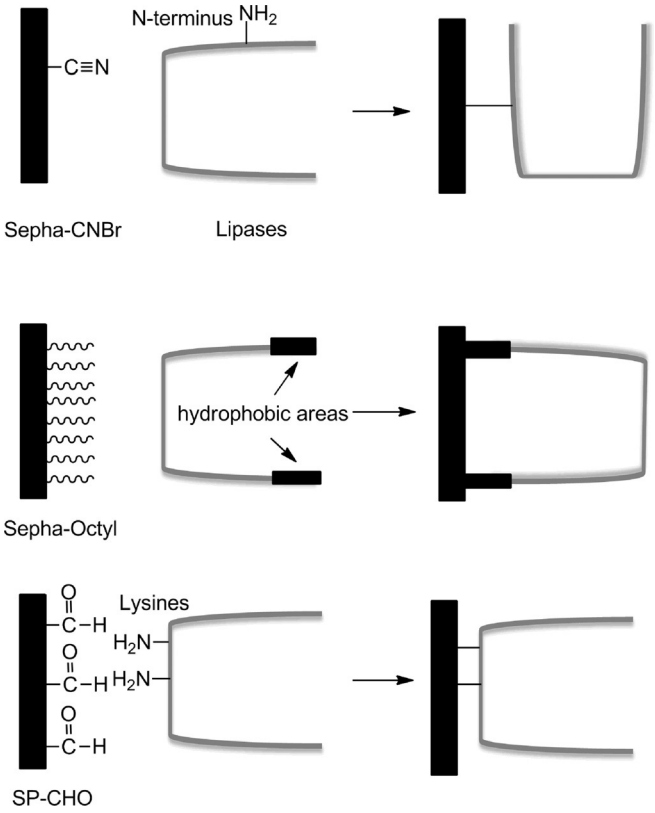
Figure 1 shows the large differences in yield of organometallic insertion depending on the protein. Lipase from *Candida antarctica B* (CAL-B) was rapidly inactivated, and after 5 min the artificial metalloenzyme Sepha-CNBr-CAL-B-**1** was formed (Figure 1).

Mass spectroscopic analysis (MALDI-TOF) corroborated the anchoring of palladium pincer achieving the desired organoPd-CAL-B hybrid (Figure S1, Supporting Infomation).

Lipases from *Rhizomucor miehei* (RML) or *Candida rugosa* (CRL) also achieved an almost complete



Scheme 2. General strategy to prepare lipase metalloproteins.



Scheme 3. Different orienting strategies for lipase immobilization.

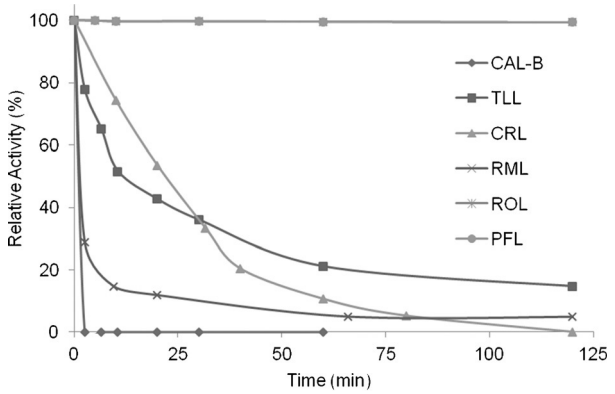


Figure 1. Coupling reaction of organometallic pincer **1** and different lipases immobilized on CNBr-activated Sepharose.

active site modification with **1** but with longer reaction times (65 and 125 min, respectively). Using lipase from *Thermomyces lanuginosus* (TLL), the highest achieved conversion was around 80%. However, lipases from *Pseudomonas fluorescens* and *Rhizopus oryzae* were not modified at all, maintaining the initial activity (Figure 1). The lipase modification has been performed in aqueous media on enzymes immobilized by covalent attachment at pH 7 (through the *N*-terminus), where generally these enzymes mainly exist in their closed conformation (active site blocking by the lid). Thus, in these two unsuccessful cases the metal incorporation was tested on the lipase immobilized by interfacial activation on Sepharose functionalized by octyl groups (Sepha-Octyl) – a hydrophobic support – (Scheme 3b).

In this immobilization strategy the lipase is adsorbed on the support fixing its open conformation[22] and giving to the substrates the highest accessibility to the active site. In the case of ROL immobilized on Sepha-octyl, most of the lipase activity (~80%) was lost by the insertion of **1** in the active site (Figure 2a).

However, no modification was observed for Sepha-Octyl immobilized PFL (Figure 2b). This phenomenon could be explained because PFL shows the longer peptide lid among all these enzymes with a huge hydrophobic oxyanion.[23] When the usual lipase inhibitor *p*-diethyl-*p*-nitrophenyl phosphate (**2**) was used, the catalytic serine of PFL immobilized on Sepha-CNBr was modified at 80% in 125 min.

Therefore, these results demonstrated that **1** can be incorporated in most of the immobilized lipases tested, although the most effective result was achieved using CAL-B as protein scaffold.

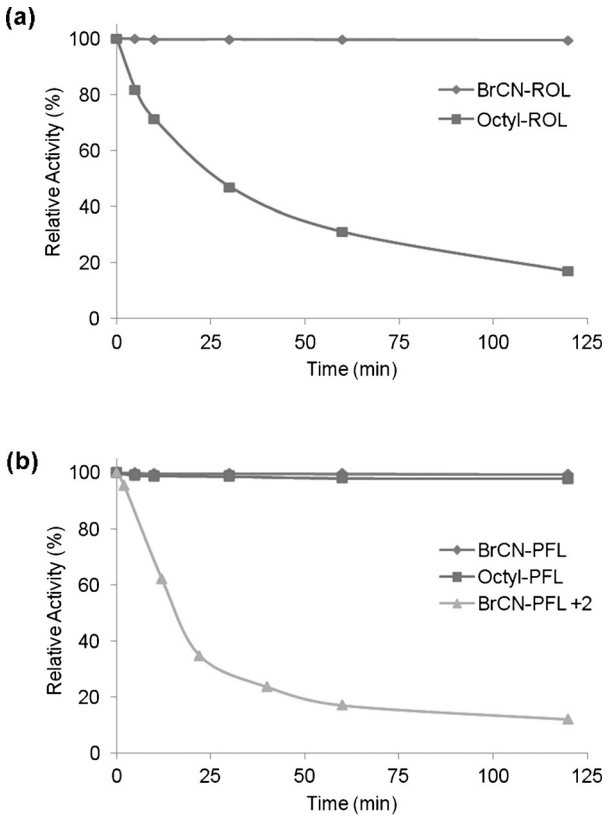


Figure 2. Effect of different orienting immobilization strategies during the coupling reaction between organometallic pincer **1** and a) *Rhizopus oryzae* lipase (ROL) and b) *Pseudomonas fluorescens* lipase (PFL).

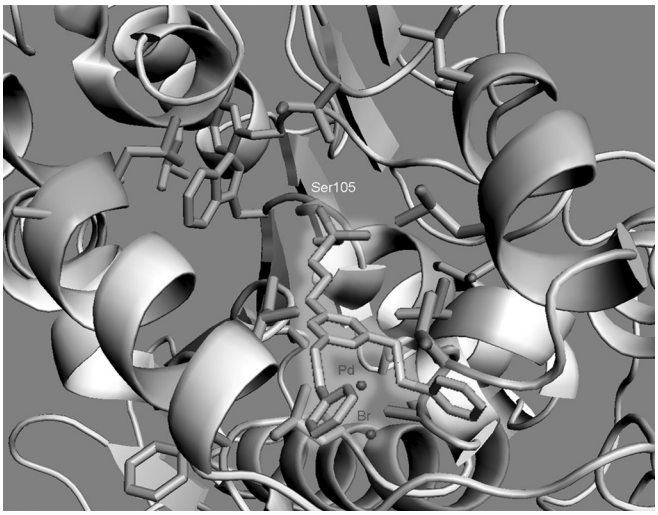


Figure 3. Manual docking of the conjugation of **1** to catalytic serine of *Candida antarctica* lipase (fraction B) (CAL-B) based on the X-ray structure (pdb: 1TCA). Figure was generated using Pymol software.

This result could be explained analyzing the 3D structure of the CAL-B and the size of **1**. In fact, among the tested lipases, this enzyme presents the shorter lid with a small oxyanion around 11 Å width and 17 Å long, similar to the size of **1** (15 Å long), permitting to the phosphonate ester much more accessibility for reacting with the catalytic serine (Figure 3).

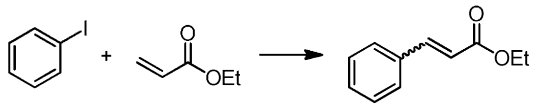
Moreover, also the presence of several hydrophobic groups around the oxyanion could stabilize the presence of the pincer located on the active site.

Assessment of Immobilized CAL-B-Pd-1 Artificial Metalloenzymes in Heck Reactions

First, we evaluated the catalytic capacity of the sole soluble Pd pincer **1** in the Heck reaction (Scheme 4).

Table 1 shows the results of the reaction between iodobenzene and ethyl acrylate in the presence of **1** (Scheme 4) under different experimental conditions.

Under the standard conditions, 120 °C in DMF, **1** catalyzed the Heck reaction with >99% yield in 18 h with a TOF value of $230 \pm 11\ h^{-1}$. In order to use more sustainable conditions, the reaction was tested at 70 °C in DMF but unfortunately **1** did not catalyze the reaction. However, the organometallic complex was active in the presence of 25% of water (v/v) in DMF at 70 °C – *greener condition*s – achieving complete conversion at 29 h with a TOF value of $142 \pm 7\ h^{-1}$. The presence of more water content in the reaction [50% (v/v)] reduced the catalyst activity to a 47% yield and a TOF value of $27 \pm 2\ h^{-1}$ (Table 1).



Scheme 4. Heck cross-coupling reaction between iodobenzene and ethyl acrylate.

The stability of the free and supported pincers has been well characterized demonstrating no leaching of the Pd molecules under the given reaction conditions.[24–25]

Thus the activity of the immobilized Sepha-CNBr-CAL-B-**1** metalloenzyme was tested applying the conditions where Pd pincer **1** was active in the Heck reaction (Table 1, entries 1 and 3). We preliminarily evaluated the immobilized enzyme without the Pd pincer **1** but incorporating the inhibitor **2** on the active site (Table 2, entries 1 and 2).

This Sepha-CNBr-CAL-B-**2** was completely inactive under both reaction conditions. Unfortunately, the immobilized Sepha-CNBr-CAL-B-**1** did not catalyze the reaction under any of the tested conditions (Table 2, entries 3 and 4).

Pursuing our search for optimal immobilized biocatalysts, Novozym435 – the commercial immobilized CAL-B catalyst where the lipase is immobilized mainly by hydrophobic interactions on Lewatit resin – was successfully modified with **1** and the new hybrid Novozym435-**1** was used to catalyze the Heck reaction. This hybrid slightly catalyzed the reaction under the applied conditions, achieving negligible yield (≤5%, Table 2, entries 5 and 6) of the desired product. In this case, the reason for this very low conversion was attributed to the immobilization methodology. In fact, in this case, the immobilization was reversible and the enzyme was leaching from the support

Table 1. Optimization of reaction conditions for Heck cross-coupling.[a]

Entry	Catalyst	Water [%, v/v]	Temp. [°C]	Time [h]	Yield [%]	TOF [h^{-1}]
1	**1**	0	120	18	>99	230±11
2	**1**	0	70	72	0	nd
3	**1**	25	70	29	>99	142±7
4	**1**	50	70	72	47	27±2

[a] *Reaction conditions:* 0.247 mmol of iodobenzene, 0.55 mmol of ethyl acrylate, 0.54 μmol (0.024 mol% of Pd relative to iodobenzene), 0.412 mmol of triethylamine, 1 mL of DMF.

Table 2. Heck reaction catalyzed by different artificial metalloenzymes.[a]

Entry	Catalyst	Water [%, v/v]	Temp. [°C]	Time [h]	Yield [%]	TOF [h^{-1}]
1	Sepha-CNBr-CAL-B-**2**	0	120	72	0	n.d
2	Sepha-CNBr-CAL-B-**2**	25	70	72	0	n.d
3	Sepha-CNBr-CAL-B-**1**	0	120	72	0	n.d
4	Sepha-CNBr-CAL-B-**1**	25	70	72	0	n.d
5	Novozym435-**1**	0	120	72	2	n.d
6	Novozym435-**1**	25	70	72	<5	n.d
8	SP-CHO-CAL-B-**1**	0	120	72	28	16 ± 1
9	SP-CHO-CAL-B-**1**	0	70	55	>99	75 ± 3
10	SP-CHO-CAL-B-**1**	25	70	35.5	>99	116 ± 5

[a] *Reaction conditions:* 0.247 mmol of iodobenzene, 0.55 mmol of ethyl acrylate, 200 mg of each catalyst (0.024 mol% of Pd relative to iodobenzene), 0.412 mmol of triethylamine, 1 mL of DMF.

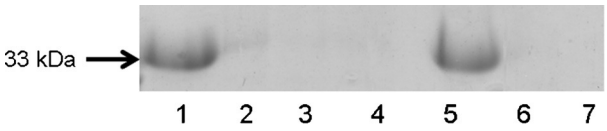


Figure 4. SDS-PAGE analysis of Novozym 435 after incubation in Heck reaction medium at different conditions: 1) H_2O (100%); 2) DMF 75%/H_2O 25% (v/v) at 70°C for 2 h; 3) 6 h; 4) 24 h. DMF 100% (v/v) at 120°C; for 5) 2 h, 6) 6 h, and 7) 24 h.

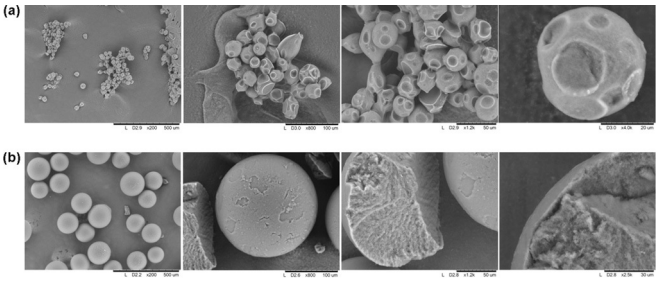


Figure 5. SEM micrographs of the macroporous matrices used as solid support. a) Sepharose. b) Sepabeads.

under the reaction conditions tested. This hypothesis was confirmed by SDS-PAGE of the Novozym435 under the different conditions used (Figure 4).

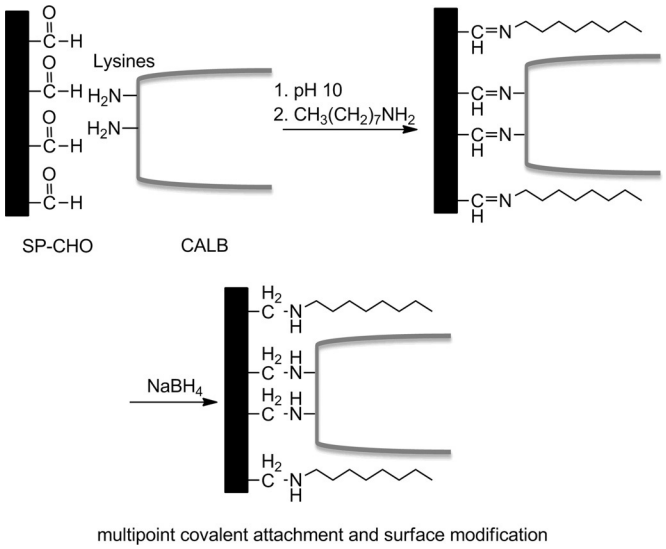
No protein was observed on the support after incubation at 120°C in DMF, and the same occurred in the presence of 25% water and 70°C. This result also demonstrated that the soluble CAL-B-**1** metalloenzyme did not work under these reaction conditions where the homogeneous pincer was active, *the use of the solid-phase thus being mandatory* . This phosphonate system (irreversibly attached to the catalytic serine in the protein) seems to stabilize the palladium complex into the protein avoiding the leaching of the metal in comparison with other chemistries.[26]

Therefore, considering these negative results (the use of a very hydrophilic matrix such as Sepharose and the selection of a reversible immobilization strategy), we decided to use a much more hydrophobic resin such as Sepabeads®, with no swelling effect related to solvent changes. Figure 5 shows the extreme differences between the Sepharose (Figure 5a) and Sepabeads® (Figure 5a) in a low concentration of water.

The Sepharose structure is collapsed under these conditions resulting in a critical impediment for the entrance of the substrates and therefore being a possible additional explanation for the inactivity of Sepha-CNBr-CAL-B-**1** besides the CAL-B conformational changes previously described. On the other hand, Sepabeads are homogeneous and rigid macroporous beads with high superficial surface that maintain unaltered their structure under different reaction conditions (Figure 5b).

Thus, considering the previously described drawbacks, CAL-B was immobilized by a multi-covalent attachment on a Sepabeads resin activated with aldehyde groups (SP-CHO) (Scheme 3, *bottom*). This strategy has been demonstrated to confer a high stability to the enzyme.[27] Furthermore, this immobilization is performed through the richest area of lysine residues of the enzyme. In the case of CAL-B this area is located on the opposite side respect to the lid and the oxyanion location (Scheme 3, *bottom*). CAL-B-SP-CHO preparation was fully modified with **1** in 5 min achieving SP-CHO-CAL-B-**1**. This new immobilized hybrid metalloenzyme catalyzed the Heck reaction, yielding 28% of product at 120°C in pure DMF and >99% yield after 35.5 h at 70°C in DMF with 25% (v/v) water, with a TOF value of 116 ± 5 h^{-1} (Table 2, entries 8 and 10). Surprisingly, the immobilized metalloenzyme catalyzed the Heck reaction with >99% yield at 70°C in pure DMF, conditions where the pincer **1** was not active (Table 2, entry 9). Therefore, this successful result demonstrates the critical role of the enzyme environment together with the support matrix hydrophobicity.

In this direction, a redesign of the support surface focused to increasing its hydrophobicity was performed (Scheme 5).



Scheme 5. Scheme of hydrophobicity modulation of immobilized artificial metalloenzyme by means of chemical engineering of support matrix.

The immobilization of the protein on the aldehyde activated support involves a first step of incubation at pH 10 to generate the imine groups (between aldehydes of support and amines from the protein) which must be reduced to irreversible amine groups in the second step (Scheme 3, *bottom*).

Therefore, in order to generate a more hydrophobic surface surrounding the enzyme environment, before the reducing step, the unreacted aldehyde groups of the support were modified with different alkylamines (methyl, octyl and myristoyl) or ethylenediamine (EDA) as negative control (generation of positive charge). After that, the incorporation of **1** into the different newly engineered CAL-B immobilized preparations was carried out similar to the previous modification on the other CAL-B preparations.

This chemical modification of the support caused an important effect on the catalytic properties of the final artificial metalloenzyme (Table 3).

The SP-CAL-B-EDA-**1** hardly catalyzed the reaction with $<5\%$ of product (Table 3, entry 2), and the SP-CAL-B-C_1-**1** showed a 35% yield in 72 h, five times slower than SP-CAL-B-**1** (Table 3).

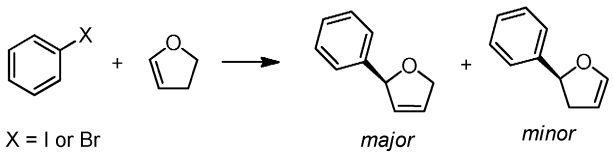
On the other hand, SP-CAL-B-C_8-**1** (hydrophobization effect introduced, Scheme 5) catalyzed the Heck reaction achieving $>99\%$ yield of product in 27 h, with the highest TOF value achieved under these conditions with this Pd complex, $153\pm9\ h^{-1}$ (Table 3, entry 4). When a more hydrophobic chain was introduced, in SP-CAL-B-C_{12}-**1**, no improvement compared with C_8 was observed (Table 3, entry 4). The new SP-CAL-B-C_8-**1** artificial metalloenzyme was reused for two cycles maintaining 70% activity.

In order to demonstrate if this decrease in activity value is due to the loss of protein stability, IR experiments on the catalyst before and after the Heck reaction were performed (Supporting Information, Figure S2). The results show that no significant structural changes on the protein structure occur after the reaction. Therefore, considering that no Pd leaching was observed, the activity decrease maybe due to a possible Pd catalyst deactivation.[24]

Finally, to expand the general scope of the optimized hybrid catalyst and better assess its stereo- and enantioselectivity, we selected an asymmetric Heck cross-coupling reaction between halobenzene and 2,3-dihydrofuran as benchmark reaction (Scheme 6).[28]

This reaction has been recently optimized using soluble phosphine-based ligands in order to impart chirality to a catalytic Pd^{2+} precursor.[28] Hence, as first choice, we used the SP-CAL-B-C_8-**1** catalyst applying the conditions reported in the literature and testing iodo- and bromobenzene. In both cases, no conversion was observed (Table 4).

Subsequently, we carried out the reactions by applying our previously optimized conditions (25%



Scheme 6. Asymmetric Heck cross-coupling reaction between halobenzene and 2,3-dihydrofuran.

Table 3. Effect of the chemical engineering of catalyst surface on the catalytic properties of SP-CAL-B-**1** in the Heck cross-coupling reaction.[a]

Entry	Catalyst	Surface Modification (R)	Yield [%]	Time [h]	TOF [h^{-1}]
1	SP-CAL-B-**1**	–	>99	37.5	110±5
2	SP-CAL-B-EDA-**1**	$CH_2CH_2NH_2$	<5	72	n.d
3	SP-CAL-B-C-**1**	CH_3	35	72	20±1
4	SP-CAL-B-C_8-**1**	$(CH_2)_7CH_3$	>99	27	153±9
5	SP-CAL-B-C_{12}-**1**	$(CH_2)_{11}CH_3$	>99	31	133±7

[a] *Reaction conditions:* 0.247 mmol of iodobenzene, 0.55 mmol of ethyl acrylate, 200 mg of each catalyst (0.024 mol% of Pd relative to iodobenzene), 0.412 mmol of triethylamine, 0.75 mL of DMF and 0.25 mL water and 70°C.

Table 4. Asymmetric Heck reaction of different aryl halides catalyzed by SP-CAL-B-C_8-**1** derivative.[a]

Entry	X	Solvent	Water [%, v/v]	Temp. [°C]	Yield [%]	s ratio[b]	*ee* [%]
1[c]	I	ethylene glycol	0	80	0	nd	nd
2[c]	Br	ethylene glycol	0	80	0	nd	nd
3	I	DMF	25	70	0	nd	nd
4	Br	DMF	25	70	0	nd	nd
5	I	DMF	25	120	95	18	96.6
6	Br	DMF	25	120	0	nd	nd

[a] *Reaction conditions:* 0.247 mmol of halobenzene, 0.55 mmol of 2,3-dihydrofuran, 200 mg of SP-CAL-B-C_8-**1** catalyst (0.024 mol% of Pd relative to halobenzene), 0.412 mmol of triethylamine, 1 mL of solvent mixture, 24 h.
[b] Olefinic selectivity.
[c] Standard conditions reported in ref.[28]

water in DMF at different temperatures). At 70 °C, no conversion was observed in all the cases (Table 4, entries 3 and 4). When the reaction temperature was increased up to 120 °C, the reaction in the presence of iodobenzene showed an almost quantitative conversion and especially a good stereo- (s ratio 18) and enantioselectivity (>96%) (Table 4, entry 5). Under these conditions, the bromobenzene was completely inactive (Table 4, entry 6). These results definitively confirmed the large and powerful applicability of our hybrid catalyst.

Conclusions

In conclusion, we have here presented an effective strategy to produce a heterogeneous immobilized, active and reusable artificial metalloenzyme in relative good amount using a commercial lipase as protein scaffold. A *p*-nitrophenyl phosphonate palladium pincer was selectively inserted by irreversible attachment on the catalytic serine of the lipase. A fine designed solid-phase strategy based on the careful selection of the supporting matrix together with the proper orienting immobilization strategy demonstrated that not only the enzyme scaffold but also the solid-phase chemistry present a crucial role in order to obtain a Pd catalyst with excellent activity, stereo- and enantioselectivity and reusability in Heck cross-coupling reactions. The application of this technology has permitted us to carry out this C–C bond reaction under milder conditions than usual which is of critical importance to industrial applications.

Experimental Section

Materials

Candida antarctica B lipase (CAL-B), *Thermomyces lanuginosus* lipase (TLL) and Novozym 435 solid catalyst were generously donated by Novozymes (Denmark). *Pseudomonas fluorescens* lipase (PFL) was from Amano Pharmaceutical (Japan). Octyl-Sepharose 4BCL and CNBr-activated Sepharose 4BCL were from GE Healthcare (Sweden). Sepabeads® epoxide (SP-EC) was kindly gifted by Resindion-Mitsubishi (Italia). Sepabeads aldehyde support (SP-CHO) was prepared as previously described with minor modifications.[28] Lewatit VO OC 1600 was from Lanxess (Germany). *Candida rugosa* lipase (CRL), *Ryzopus oryzae* lipase (ROL), *Rhizomucor miehei* lipase (RML), *p*-nitrophenol butyrate (pNPB), iodobenzene, ethyl acrylate, ethylenediamine, methylamine, octylamine, dodecylamine, Triton X-100, cetyltrimethylammonium bromide, sodium borohydride and sodium periodate were from Sigma-Aldrich. The commercial analytical standards of isomers of ethyl cinnamate were from Toronto Research Chemicals (Canada) (the *Z-cis* isomer) and Sigma Aldrich (the *E-trans* isomer). Other used reagents were of analytical grade. The scanning electron microscopy (SEM) imaging was performed on a TM-1000 (Hitachi) microscope. The spectrophotometric analyses were run on a V-630 spectrophotometer (JASCO, Japan). HPLC spectrum P100 (Thermo Separation products) was used. Analyses were run at 25 °C using an L-7300 column oven and a UV6000LP detector. The commercial analytical standard of 2-phenyl-2,5-dihydrofuran was from Hong Kong Chemhere Co Limited (China). Column chromatography was carried out on silica gel (silica gel 60, from Merck, Germany). TLC analysis was performed on Merck silica gel 60 F_{254}. The CAL-B structure analysis was performed using the PyMOL (DeLano Scientific) software.

Enzymatic Activity Assay

The activities of the soluble and immobilized enzyme derivatives were analyzed spectrophotometrically by measuring the increment in absorbance at 348 nm produced by the release of *p*-nitrophenol (pNP) ($\varepsilon = 5{,}150\,M^{<M->1}\,cm^{-1}$) in the hydrolysis of 0.4 mM *p*-nitrophenol butyrate (pNPB) in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. Enzymatic activity is given as micromole of hydrolyzed pNPB per minute per milligram of enzyme (IU) under the conditions described above.

Purification of Commercial Lipase Crude Extracts and Immobilization on Sepharose Supports

The purification and immobilization on Sepharose supports of each commercial lipase here used were carried out as reported elsewhere.[22] Briefly, to 10 mL of 5 mM phosphate buffer pH 7 containing 10 mg of each commercial lipase (measured by Bradford assay of crude extract), 1 g of commercial octyl-Sepharose was added. After 3 h, the purified enzyme derivative was recovered by filtration and washed with abundant distilled water. The immobilization yield was almost quantitative in all cases. In order to obtain the solution of pure soluble lipase to be immobilized on the commercial CNBr-activated Sepharose support, 1 g of hydrophobic derivative was added to 10 mL of 25 mM phosphate buffer solution containing 0.5% (v/v) of Triton X100 detergent [only in the case of TLL, 0.6% (v/v) cetyltrimethylammonium bromide (CTAB) was used as detergent]. The resulting desorption mixtures were kept on mechanical stirring for 1 h and subsequently the supernatant containing the soluble enzyme solution was recovered by filtration. The lipase immobilization on CNBr activated Sepharose support – with an enzyme loading of about 10 mg of pure lipase per gram of support – was carried out as reported in the literature.[22]

Immobilization on Sepabeads® Aldehyde Supports

One gram of the aldehyde support (SP-CHO)[29] was added to 10 mL of an enzymatic solution of purified CAL-B (1 mg mL^{-1}) prepared in 100 mM sodium bicarbonate buffer pH 10.1 and the resulting immobilization mixture was maintained under gently stirring for 24 h. In order to reduce the imino and aldehyde groups and stop the immobilization reaction, 10 mg sodium borohydride were added under gentle stirring. After 30 min, the immobilized enzyme was recovered by filtration and washed with abundant distilled water.

Catalyst Engineering by Alkylamine Modification

One gram of the aldehyde support (SP-CHO) was added to 10 mL of an enzymatic solution of purified CAL-B (1 mg mL^{-1}) prepared in 100 mM sodium bicarbonate buffer pH 10.1 and the resulting immobilization mixture was maintained under gently stirring for 24 h. After that, the enzymatic derivative was recovered by filtration and, without any washing, 10 mL of a 100 mM sodium bicarbonate buffer pH 10.1 solution containing 500 mM of methylamine, ethylenediamine, octylamine or dodecylamine was directly added. In the last two cases, 20% and 40% (v/v) dioxane was added, respectively, in order to ensure the full solubility of the modifier compounds. Hence, the resulting mixture was maintained under gentle mechanical stirring for 30 min. After that, 20 mg of $NaBH_4$ were added and the suspension was maintained under gentle mechanical stirring for 30 min. Subsequently, the engineered immobilized enzyme was recovered by filtration and washed with distilled water, 50% (v/v) dioxane aqueous solution and finally abundant distilled water.

Irreversible Inhibition of Lipase Immobilized Preparations by Diethyl *p*-Nitrophenylphosphate (D-pnP)

0.2 g of different immobilized preparations were suspended in 4 mL of 25 mM sodium phosphate buffer solution at pH 7 and 25 °C with or without the presence of 0.5% (w/v) of Triton X-100. Then, 1.5 mM of inhibitor (D-pnP) was added to this solution. The reaction was maintained until the activity of the immobilized enzyme – measured using the assay previously described – was zero or reached a plateau.

Synthesis of Organo-Palladium Complex 1

The synthesis of compound **1** was carried out as described in the literature.[20]

Modification of Lipase Catalysts by the Organo-Palladium Complex 1

0.2 g of each lipase catalyst were added to 4 mL of 25 mM phosphate buffer pH 7. After that, 20 μL of 300 mM acetonitrile solution of **1** (final concentration 1.5 mM) were added and the resulting mixture was maintained under gentle stirring, periodically checking the enzyme activity decrease by the lipase activity assay previously described. The modification yields were calculated as follow: catalyst modification (%) = $(A_r/A_i)\times 100$ where A_r is the catalyst hydrolytic activity after the modification reaction and A_i the catalyst hydrolytic activity before the modification reaction. The insertion of the organometallic **1** into the lipase cavity was analyzed by MADI-TOF mass spectrometry, using native CAL-B as calibration material (Supporting Information, Figure S1). A peak of 35418.53 was found corresponding to a lower mass than the calculated value ([$M_{CAL\text{-}B}$+**1**= 35616.32]. This difference corresponds to the release of the bromine and palladium ions during the measurement in MALDI mass spectra.

In the cases where the modification yields were not quantitative by enzymatic assay measurements, in order to avoid any possible unwanted hydrolytic side reaction of ethyl esters present in the Heck reaction, the residual enzymatic activity was inhibited as previously described.

Desorption Study of Novozym 435 Derivative by SDS-PAGE Analysis

200 mg of Novozym 435-**1** modified derivative were incubated in 1 mL of dimethylformamide (DMF) or an aqueous solution thereof, in order to assess the stability of the catalyst under the reaction conditions expected by the Heck C–C coupling. After different times, the enzyme derivatives were recovered by filtration and analyzed by SDS-PAGE (gel polymerization: 12%).

General Procedure for Heck Cross-Coupling Reaction

In a 1.5-mL screw-cap sealed vessel, 200 mg of each catalyst were added to a solution containing iodobenzene (0.0306 mL, 0.274 mmol) and ethyl acrylate (0.059 mL, 0.55 mmol) in DMF or DMF/distilled water (final volume 1 mL). The mixture was preheated at 70 °C under magnetic

stirring for 5 min. After that, to initialize the reaction, triethylamine (0.057 mL, 0.412 mmol) was added. The final suspension was left under magnetic stirring at 70 °C for the indicated times.

HPLC Monitoring of Heck Cross-Coupling Reaction

The reaction outcome was monitored by HPLC analysis of samples of the reaction withdrawn at different times. The analysis condition determinations were performed with a Kromasil-C8 (150×4.6 mm and 5 μm Ø), at a flow of 1 mL min^{-1}; λ = 267 nm; and a mobile phase: 50% (v/v) ACN in MilliQ water. The *E* configuration of achieved product was confirmed by HPLC identity using the commercial standards (provided as described in Materials) the R_ts being 8.4 min for *Z-cis* isomer and 9.4 min for *E-trans* isomer. The yields were obtained by extrapolating the values through a calibration curve of the *E*-products (R^2 = 0.9975).

To confirm the suitability of the HPLC method, the final reaction mixture was purified by silica gel chromatography. In this case, as well as to perform the recyclability studies, the reaction amounts were scaled up 5 times, maintaining the reactant proportions described above. Hence, the reaction performed under the best conditions [DMF in the presence of 25% (v/v) of distilled water], once it reached its maximum conversion, was extracted with Et_2O. The combined filtrate was dried over anhydrous $NaSO_4$, concentrated under reduced pressure and finally purified by silica-gel chromatography (hexane). The purified yields were in agreement with those obtained by HPLC (215.4 mg of pure *E*-ethyl cinnamate, 99% yield).

Asymmetric Heck Cross-Coupling Reaction

In a 1.5 mL screw-cap sealed vessel, 200 mg of SP-CAL-B-C_8-**1** catalyst were added to a solution containing iodobenzene (0.0306 mL, 0.274 mmol) and 2,3-dihydrofuran (0.041 mL, 0.55 mmol) in 1 mL of DMF/distilled water 75/25 solution. The mixture was preheated at 120 °C under magnetic stirring for 5 min. After that, to initialize the reaction, triethylamine (0.057 mL, 0.412 mmol) was added. The final suspension was left under magnetic stirring at 120 °C for the indicated times.

HPLC Monitoring of Asymmetric Heck Cross-Coupling Reaction

The reaction outcome was monitored by HPLC analysis of samples of the reaction withdrawn after 24 h. The analyses were performed with a Kromasil-C4 (150×4.6 mm and 5 μm Ø), at a flow of 1 mL min^{-1}; λ = 254 nm; and a mobile phase: 50% (v/v) ACN in MilliQ water. The formation of the product was confirmed by HPLC identity using the commercial standards provided as described in Materials. The yields were obtained by extrapolating the values through a calibration curve (R^2 = 0.9912). To analyze the enantioselectivity, the final reaction mixture was purified by silica gel chromatography with pentane/Et_2O (10:1 to 5:1) as a colorless oil. The analysis conditions were: a Daicel Chiralcel OJ-H, 98:2 *n*-hexane/isopropyl alcohol, flow rate = 0.5 mL min^{-1} in agreement with the conditions reported in the literature.[28]

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References

[1] a) M. Durrenberger, T. R. Ward, *Curr. Opin. Chem. Biol.* **2014**, *19*, 99–106; b) M. Genz, V. Kçhler, M. Krauss, D. Singer, R. Hoffmann, T. R. Ward, N. Sträter, *ChemCatChem* **2014**, *6*, 736–740.
[2] J. C. Lewis, *ACS Catal.* **2013**, *3*, 2954–2975.
[3] Y. Lu, N. Yeung, N. Sieracki, N. M. Marshall, *Nature* **2009**, *460*, 855–862.
[4] J. Bos, G. Roelfes, *Curr. Opin. Chem. Biol.* **2014**, *19*, 135–143.
[5] F. W. Monnard, E. S. Nogueira, T. Heinisch, T. Schirmer, T. R. Ward, *Chem. Sci.* **2013**, *4*, 3269–3274.
[6] M. R. Ringenberg, T. R. Ward, *Chem. Commun.* **2011**, *47*, 8470–8476.
[7] C. A. Denard, J. F. Hartwig, H. Zhao, *ACS Catal.* **2013**, *3*, 2856–2864.
[8] M. T. Reetz, *Chem. Rec.* **2012**, *12*, 391–406.
[9] T. Reiner, D. Jantke, A. N. Marziale, A. Raba, J. Eppinger, *Chem. Open* **2013**, *2*, 50–54.
[10] C. M. Thomas, T. R. Ward, *Chem. Soc. Rev.* **2005**, *34*, 337–346, and references cited therein.
[11] a) T. Ueno, H. Tabe, Y. Tanaka, *Chem. Asian J.* **2013**, *8*, 1646–1660; b) H. Inaba, S. Kanamaru, F. Arisaka, S. Kitagawaa, T. Ueno, *Dalton Trans.* **2012**, *41*, 11424–11427.
[12] D. K. Garner, L. Liang, D. A. Barrios, J.-L. Zhang, Y. Lu, *ACS Catal.* **2011**, *1*, 1083–1089.
[13] I. D. Petrik, J. Liu, Y. Lu, *Curr. Opin. Chem. Biol.* **2014**, *19*, 67–75.
[14] H. Yang, P. Srivastava, C. Zhang, J. C. Lewis, *ChemBioChem* **2014**, *15*, 223–227.
[15] a) P. Adlercreutz, *Chem. Soc. Rev.* **2013**, *42*, 6406–6436; b) J. Brabcova, M. Filice, M. Gutarra, J. M. Palomo, *Curr. Biol. Compd.* **2013**, *9*, 113–136.
[16] a) M. Filice, M. Marciello, M. d. P. Morales, J. M. Palomo, *Chem. Commun.* **2013**, *49*, 6876–6878; b) G. Van Koten, *J. Organomet. Chem.* **2013**, *730*, 156–164.
[17] a) M. Filice, J. M. Palomo, *ACS Catal.* **2014**, *4*, 1588–1598; b) K. Engstrom, E. V. Johnston, O. Verho, K. P. Gustafson, M. Shakeri, C. W. Tai, J. E. Bäckvall, *Angew. Chem. Int. Ed.* **2013**, *52*, 14006–14010.
[18] M. Filice, O. Romero, J. Gutierrez-Fernandez, B. de Las Rivas, J. A. Hermoso, J. M. Palomo, *Chem. Commun.* **2015**, *51*, 9324–9327.
[19] J. C. Powers, J. L. Asgian, W. D. Ekici, K. E. James, *Chem. Rev.* **2002**, *102*, 4639–4750.
[20] C. A. Kruithof, M. A. Casado, G. Guillena, M. R. Egmond, A. van der Kerk-van Hoof, A. J. R. Heck, R. J. M. Klein Gebbink, G. van Koten, *Chem. Eur. J.* **2005**, *11*, 6869–6877.

[21] P. J. H. Scott, in: *Linker Strategies in Solid-Phase Organic Synthesis*, John Wiley & Sons, Ltd., Chichester. **2009**.
[22] a) A. Bastida, P. Sabuquillo, P. Armisen, R. Fernandez-Lafuente, J. Huguet, J. M. Guisan, *Biotechnol. Bioeng.* **1998**, *58*, 486–493; b) M. Filice, J M. Guisan, M. Terreni, J. M. Palomo, *Nat. Protoc.* **2012**, *7*, 1783–1796.
[23] J. D. Schrag, Y. Li, M. Cygler, D. Lang, T. Burgdorf, H. J. Hecht, R. Schmid, D. Schomburg, T. J. Rydel, J. D. Oliver, L. C. Strickland, C. M. Dunaway, S. B. Larson, J. Day, A. McPherson, *Structure* **1997**, *5*, 187–202.
[24] N. C. Mehendale, J. R. A. Sietsma, K. P. de Jong, C. A. van Walree, R. J. M. Klein Gebbink, G. van Koten, *Adv. Synth. Catal.* **2007**, *349*, 2619–2630.
[25] D. E. Bergbreiter, P. L. Osburn, J. D. Frels, *Adv. Synth. Catal.* **2005**, *347*, 172–184.
[26] K. Yu, W. Sommer, J. M. Richardson, M. Weck, C. W. Jones, *Adv. Synth. Catal.* **2005**, *347*, 161–171.
[27] C. Mateo, J. M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. Lopez-Gallego, B. C. C. Pessela, A. Hidalgo, G. Fernandez-Lorente, R. Fernandez-Lafuente, J. M. Guisan, *Enzyme Microb. Technol.* **2006**, *39*, 274–280.
[28] C. Wu, J. Zhou, *J. Am. Chem. Soc.* **2014**, *136*, 650–652.
[29] J. M. Guisan, *Enzyme Microb. Technol.* **1988**, *10*, 375–382.

