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Novel nanoparticle/enzyme biosilicified nanohybrids for advanced heterogeneously catalyzed protocols

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Abstract

Novel bio-nanohybrids based on room temperature one-pot synthesized lipase-nanoparticle systems were developed and characterized in this work, with subsequent investigations on their catalytic activities and stability as compared to the free enzymes. Preliminary results pointed to excelling stabilities, solvent tolerance, and activities as compared to free lipases, opening up further scenarios for their utilization under continuous flow conditions as well as in tandem reactions.

Keywords: *biocatalysts, biosilicification, metal nanoparticles, heterogeneous catalysis*

Introduction

Biocatalysis has been consolidated as an important field in recent years in which a series of important advances have paved the way to the utilization of enzymes for a wide range of applications.[1] Enzymes including lipases and phospholipases currently find multiple industrial and biotechnological applications but often easily deactivate, thus being difficult to be reused and/or separated from the reaction system upon reaction completion.[2] Free enzymes in solution can also exhibit issues related to instability (when exposed to certain conditions) in biocatalytic processes.

Enzyme immobilization can overcome many of these problems, leading to reusable and stable systems. The development of new cost effective enzyme immobilization/entrapment techniques has attracted a great deal of attention for the incorporation of such methodologies in industry as a first choice in biocatalyzed processes. The paradigm of enzyme immobilization has remarkably evolved in recent years since efforts have been focused towards innovative immobilization technologies able to render enhanced biocatalytic systems with improved enzyme activity, stability as well as selectivity through a better understanding of the fundamental science behind enzyme immobilization. The synthesis of supported enzymes was traditionally achieved using physical methods, including adsorption and/or entrapping, or chemical methods such as covalent bonding.[3]

Importantly, a recently developed novel methodology was shown to provide a comparatively advantageous enzyme encapsulation by means of biosilicification (silica mineralization).[4] This new methodology could be carried under mild reaction conditions (room temperature, close to neutral pH), in a one-pot reaction by using a silicic acid precursor (e.g. tetramethylorthosilicate – TMOS) and a polycationic

catalyst. The activities and (enantio)selectivites of esterases encapsulated in such silica nanoparticles were very recently reported to be significantly influenced by encapsulation in enantioselective esterifications.[5] This important data supports existing theories that suggested complex and unexpected (even unpredictable) structural modifications of enzymes upon physical and/or chemical immobilization on various solid supports.[6] This innovative and green approach could have a significant impact in future industrial biocatalytic processes.[7]

A step further in the development of biosilicified enzyme systems entails the incorporation of a metal precursor for the preparation of biosilicified nanoparticle/enzyme nanohybrids. The development of artificial metalloenzymes in which a catalytically active organometallic moiety is combined with a macromolecular host is indeed a growing field on biocatalysis. One of the major contributions of combining both structures is the potential to develop cascade-type processes in a "*one-pot*" fashion, eliminating steps related to isolation of intermediates, reducing operation costs and waste minimization. The designed bio-nanohybrid materials could in principle offer advantageous temperature and organic solvent stability as well as comparable/improved activities in biocatalytic processes.

In continuation with recent research endeavors of the group related to the preparation of benign by design advanced nanomaterials for heterogeneous catalysis, this contribution reports a simple, efficient one-pot methodology for the synthesis of biosilicified nanoparticle/enzyme nanohybrids at room temperature which is able to render advanced bio-nanocatalysts featuring excelling solvent tolerance and thermal stability in addition to simple recovery and recycling properties. Preliminary investigations on catalytic activities of the materials were also conducted (e.g. simple

esterification activities) as a first step towards further investigations into flow chemical protocols and tandem reactions currently underway in our laboratories.

Experimental Section

Materials

The free lipase B of Candida antarctica B of *Candida antarctica* (CaL B) was kindly donated by Novozymes®. Heptane, oleic acid, acetone and acetonitrile were purchased from Tedia Co., from Sigma-Aldrich as well as all chromatographic standards. Ethanol was purchased from Vetec Ltda.

Methods

GC-MS Analysis

GC-MS analysis was conducted following a modified EN 14105 method. Free fatty acids were converted into more volatile silylated derivatives in the presence of pyridine and N-methyl-N-trimethysilyltrifluoroacetamide (MSTFA). GC-MS measurements were carried out in duplicate using a DB 5-HT (Agilent, J & W. Scientific, USA) capillary column (10 m x 0.32 mm x 0.1 μm). Quantification was conducted on the basis of calibration curves with internal standards. GC-MS samples were prepared by dissolving 0.1 g of the final product in 1 mL of n-heptane. One hundred microliters of this solution and pyridine solutions of butanetriol (1 mg mL^{-1}) and tricaprine (8 mg/mL), used as internal standards, were added into a flask containing 100 μL of MSTFA. After 15 min, these reactants were dissolved in 8 mL n-heptane. One microliter of this sample was finally injected into Shimadzu CG2010 equipment for analysis.

Lowry-Tinsley Analysis

Esterification measures were performed using a modification of the Lowry and Tinsley assay. The depletion of fatty acid was monitored as follows: 0.30 mL of the reaction solution, including the buffer solutions was added to a tube containing 0.6 mL of *n*-heptane and 1 mL of cupric acetate-pyridine (5% w/v, pH 6.0). The final solutions were vigorously mixed for 30 s in vortex, and the upper organic phase was measured by a UV/visible spectrophotometer at 715 nm. Each reaction was analyzed in triplicate, and content conversion was calculated according to the percentage difference for the absorbance shown by the stock solution.

Protein quantification

The amount of protein from the initial commercial CaL B solutions were quantified following the Bradford method.

Enzyme Biosilicification

In a typical optimized synthetic procedure tetraethoxysilane (TEOS) (4.16g) was added to a stirred solution of n-dodecylamine (1.02g) in 10g of acetonitrile and 10g of buffer solution of Cal-B at room temperature (293K). The solution containing a visible solid precipitate after a few mins was stirred for another 3h, after which the obtained solid formed was filtered off and dried at room temperature for 24 hours.

Preparation of nanoparticle-enzyme nanohybrids

A similar methodology was used for the enzyme-metal nanoparticle biohybrid materials. TEOS (4.16g) was added to a stirred solution of *n*-dodecylamine (1.02g) in acetonitrile (10g), but this time the metal precursor in solution (1% w/w, previously

dissolved in 1-2 mL of solvent to avoid inhomogeneities in the system) was added before the buffer solution of Cal-B (10g). The synthesis was also conducted at room temperature and stirred for 3h. The obtained solid was then filtered off and dried at room temperature for 24 hours. The precursors used were gold (III) chloride, palladium (II) chloride, and ruthenium (III) chloride, respectively. Materials were denoted as IM Metal where IM stands for biosilicified CALB (as in IM CALB) and metals for Au, Pd and Ru, respectively.

Materials characterisation

BET surface area and pore volume measurements were obtained from N2 adsorption/desorption isotherms at 77 K on a Micromeritics ASAP 2000 instrument. Prior to analysis, samples were degassed at 100°C for 12 h.

Transmission Electron Micrographs (TEM) were recorded on a JEOL JEM-2010HR instrument operated at 300 kV. Samples were suspended in ethanol and deposited straightaway on a copper grid prior to analysis. TEM mapping was done on the same samples to find out the homogeneity of C, O and Si as well as metallic elements in the materials. C mapping was found to be very useful to ascertain the presence of the enzyme in the materials.

XPS (aka ESCA) measurements were performed in a ultra-high vacuum (UHV) multipurpose surface analysis system (Specs^{TM} model, Germany) operating at pressures $<10^{-10}$ mbar using a conventional X-Ray source (XR-50, Specs, Mg-Kα, 1253.6 eV) in a "stop-and-go" mode to reduce potential damage due to sample irradiation. The survey and detailed Fe and Cu high-resolution spectra (pass energy 25 and 10 eV, step size 1 and 0.1 eV, respectively) were recorded at room temperature with a Phoibos 150-MCD energy analyzer. Powdered samples were deposited on a

sample holder using double-sided adhesive tape and subsequently evacuated under vacuum (<10^{-6} Torr) overnight. Eventually, the sample holder containing the degassed sample was transferred to the analysis chamber for XPS studies. Binding energies were referenced to the C1s line at 284.6 eV from adventitious carbon.

Esterification Activity Assay

Oleic acid and ethanol were used as substrates for esterification. Every ml of *n*-heptane solution contained an equimolar mixture of substrates (0.1 mol^{-1}) as well as immobilized lipases (10 mg mL^{-1}). Reaction mixture was shaken at 200 rpm at 40°C in vials of 1mL. Aliquots of 300 µL were taken at intervals and the residual fatty acid levels were analyzed using Lowry-Tinsley method, previously described. Specific esterification activity (mmol min^{-1} g) was determined by calculating the transformation of fatty acid to ester, and defined as micromoles per hour per milligram of protein, according to Cao *et al*.[8]

Results and Discussion

The synthesized materials were characterized by means of different methodologies. Starting from simply biosilicified ICALB, biosilicified nano-hybrids were found to possess a relatively high surface area (> 50 m^2 with silica nanospheres covering parts of the enzyme, Figures 1 to 3), while the enzyme was clearly visible as nanotubular arrays and fully accessible in other domains (Figure 1 and Figure 2).

XPS of biosilicified IM proved also the presence of the enzyme (C1s and N1s spectra, respectively, SI) fully accessible at the surface. An important silica content on the surface (Si2p and O1s bands and intensity, SI) was indicative of the

biosilicification of the enzyme. TEM images and mapping further demonstrated the potential, greenness and simplicity of the proposed approach which could render a significantly more stable high surface area material in which the enzyme was clearly accessible (Figure 2), in comparison with recent sophisticated literature reports.[9]

The proposed methodology was subsequently extended to the preparation of nanoparticle-biosilicified nanohybrids utilizing different metals as shown in Figures 4 and 5. A range of different metals were screened in the protocol including Au, Ru and Pd (Figure 4), which provided very interesting and essentially different results.

The presence of the metals was confirmed by XPS and TEM mapping (Figures 3, 4 and SI), which showed trace quantities of Pd species (a mixed contribution of Pd^0 and Pd^{2+}, up to 0.5 wt%) could be clearly detected on the surface of the materials by XPS (Figure 3). XRD experiments could not detect any metals in the materials due to their low metal content and crystallinity. Interestingly, the resulting materials from metal incorporation were remarkably different as depicted in Figure 4. Small and nicely dispersed Pd nanoparticles (<5 nm) could be observed for IM Pd and IM Ru (Figure 4, top left and bottom left), a large number of aggregates with smaller nanoparticles coexisted in IM Au (Figure 4, bottom right and SI). The presence of Pd and Ru was confirmed by EDX and TEM mapping to be in concentrations under 0.5 wt.% (e.g. 0.2-0.3 wt.% for Pd-see Figure 5-and ca. 0.4 w.% for Ru, respectively) and homogeneously distributed in the ICALB materials (Figure 5).

Thermal Stability

Thermal stability studies were subsequently performed for the bio-nanohybrids as compared to the free enzyme counterpart. A simple esterification

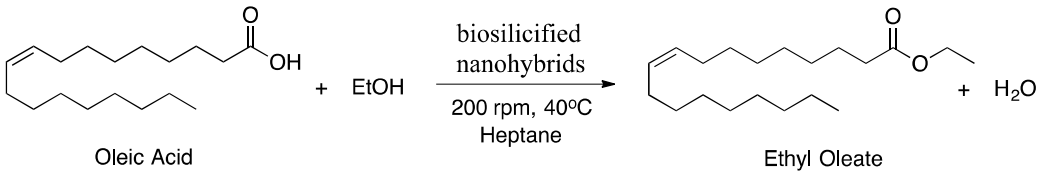
reaction of oleic acid to ethyl oleate (Scheme 1) was selected as model process for enzymatic activity and carried out at different temperatures in the 30-80°C range.

Results, expressed in residual activity, have been depicted in Figure 6 which clearly indicated a remarkable improvement in stability of bio-nanohybrid materials as compared to the free enzyme, particularly at high temperatures (>50ºC). A fully maintained esterification activity was possible at temperatures up to 50°C, with a very remarkable almost fully preserved activity for IMPd and IMAu even at temperatures as high as 65ºC (Figure 6). The immobilization efficiency, measured according to the Bradford method, was further investigated for IM-metal materials where 89 mg of CaLB, in solution, was used for the preparation of the bio-nanohybrid.

Results for immobilization activity summarized in Table 1 clearly indicated good to excellent immobilization efficiencies (68-82 %) for all IM bio-nanohybrids, with particularly good values for IM Au. Supernatant analysis, obtained after filtration, allows the quantification of the amount of protein in the materials, which was in good agreement with immobilization efficiency.

Esterification Activity

Esterification experiments (Scheme 1) were then conducted in conventional organic solvents. The esterification activities of the three novel biosilicified nanohybrids were compared with those of the biosilicified enzyme (IM CaLB) as well as with the parent free enzyme (CaLB).



Scheme 1. Esterification of oleic acid to ethyl oleate catalysed by biosilicified nanohybrids.

Results summarized in Table 2 showcase remarkable improvements in esterification activity of bio-nanohybrid materials in reactions carried out in hydrocarbon solvents (e.g. hexane and heptane) regardless of the type of metal-containing material. In general, the esterification activities were improved in all conducted experiments as compared to the free enzyme.

Blank experiments were also carried out in silicified systems without enzyme/metal and the results show no activity towards esterification. In terms of metals, the esterification activity was found to follow the order:

$$\text{IM Au} > \text{IM Pd} \approx \text{IM CaLB} > \text{IM Ru}$$

which can be simply explained by the mild acidic properties of gold nanoparticles as compared to Ru and Pd (essentially Lewis acidity).[10, 11]

An interesting colour change (from yellow to purple) was observed in the preparation of IM Au nanohybrids, indicative of the growth of nanoparticle size within the material. However, comparable esterification results (not shown) were obtained for freshly prepared IM Au (yellow) with respect to aged IM Au (purple) obtained upon drying for 24 h.

Conclusions

A novel, highly active and stable family of nanoparticle-biocatalyst hybrids have been synthesized using an innovative and simple biosilicification approach. The

proposed methodology maximizes the accessibility of the enzyme to substrates (e.g. for biocatalysis), improving at the same time the stability and activity as compared to free enzyme. We envisaged this protocol can be extended to related continuous flow biocatalysed transformations of biomass and waste derived feedstocks as well as tandem-type of processes currently underway in our laboratories that will be reported in due course.

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References

[1] a) I. Dencic, T. Noël, J. Meuldijk, M.H.J.M. de Croon, V. Hessel, *Eng. Life Sci.* 2013, **13**, 326-343; b) I. Dencic, S.M. de Vaan, T. Noël, J. Meuldijk, M.H.J.M. de Croon, V. Hessel, *Ind. Eng. Chem. Res.* 2013, **52**, 10951-10960.

[2] J.M. Guisan, Immobilization of Enzymes and Cells", 2nd Ed., chapters 1, 2 and 13. Humana Press, 2006.

[3] D.I. Fried, F.J. Brieler, M. Fröba, *ChemCatChem* 2013, **5**, 862-884.

[4] I.I. Junior, F.K. Sutili, K.M. Gonçalves, I.C.R. Leal, L.S.M. Miranda, M. Ojeda, R. Luque, R.O.M.A. De Souza, *Green Chem*. 2013, **15**, 518-524.

[5] S. Emond, D. Guieysse, S. Lechevallier, J. Dexpert-Ghys, P. Monsan, M. Remaud-Simeon, *Chem. Commun*. 2012, **48**, 1314-1316.

[6] C.A. Gasser, G. Hommes, A. Schäffer, P.F.X. Corvini, *Appl. Microbiol. Biotechnol.* 2012, **95**, 115-1134.

[7] C. Jun, B.W. Jeon, J.C. Joo, Q.A.T. Le, S.-A. Gu, S. Byun, D.H. Cho, D. Kim, B.-I. Sang, Y.H. Kim, *Process Biochem.* 2013, **8**, 1181–1187.

[8] X. Cao, J. Yang, L. Shu, B. Yu, Y. Yan, *Process Biochem*. 2009, **44**, 177–182.

[9] a) K. Engström, E.V. Johnston, O. Verho, K.P.J. Gustafson, M. Shakeri, C.-W. Tai, J.E. Bäckvall*, Angew. Chem. Int Ed.* 2014, **53**, 1-6; b) M. Filice, M. Marciello, M. P. Morales, J.M. Palomo, *Chem. Commun.* 2013, **49**, 6876-6878.

[10] S. Letaief, S. Grant, C. Detellier, *Appl. Clay Sci.* 2011, **53**, 236-243.

[11] T. Ntho, J. Aluha, P. Gqogqa, M. Raphulu, G. Pattrick, *React. Kinect. Mechanism. Catal.* 2013, **109**, 133-148.

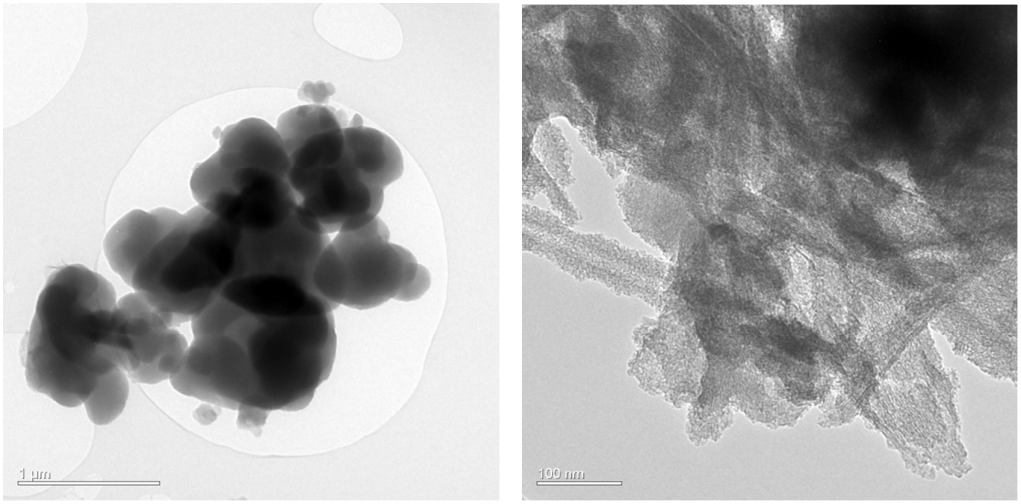


Figure 1. Biosilicified ICALB nanohybrids.

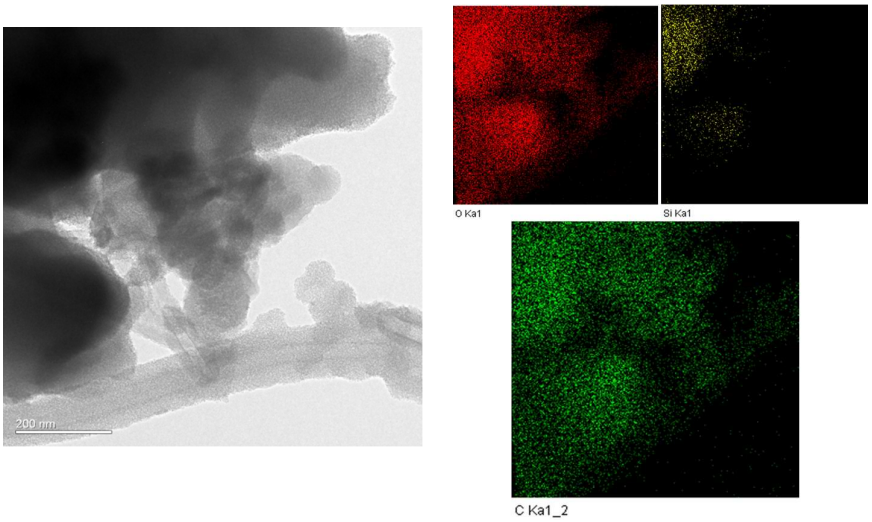


Figure 2. TEM of Biosilicified ICALB nanohybrid. Mapping micrographs (right images) clearly illustrate the parts corresponding to Silicon (Silica from biosilicification) as compared to C (enzyme and organic precursors) and O (both from the silica and the enzyme).

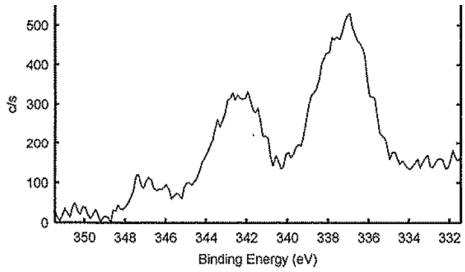


Figure 3. Pd3d XP spectrum of IM Pd material illustrating that a contribution of Pd^0 and Pd^{2+} species (up to 0.5 wt%) could be detected in the biosilicified nanohybrid surface.

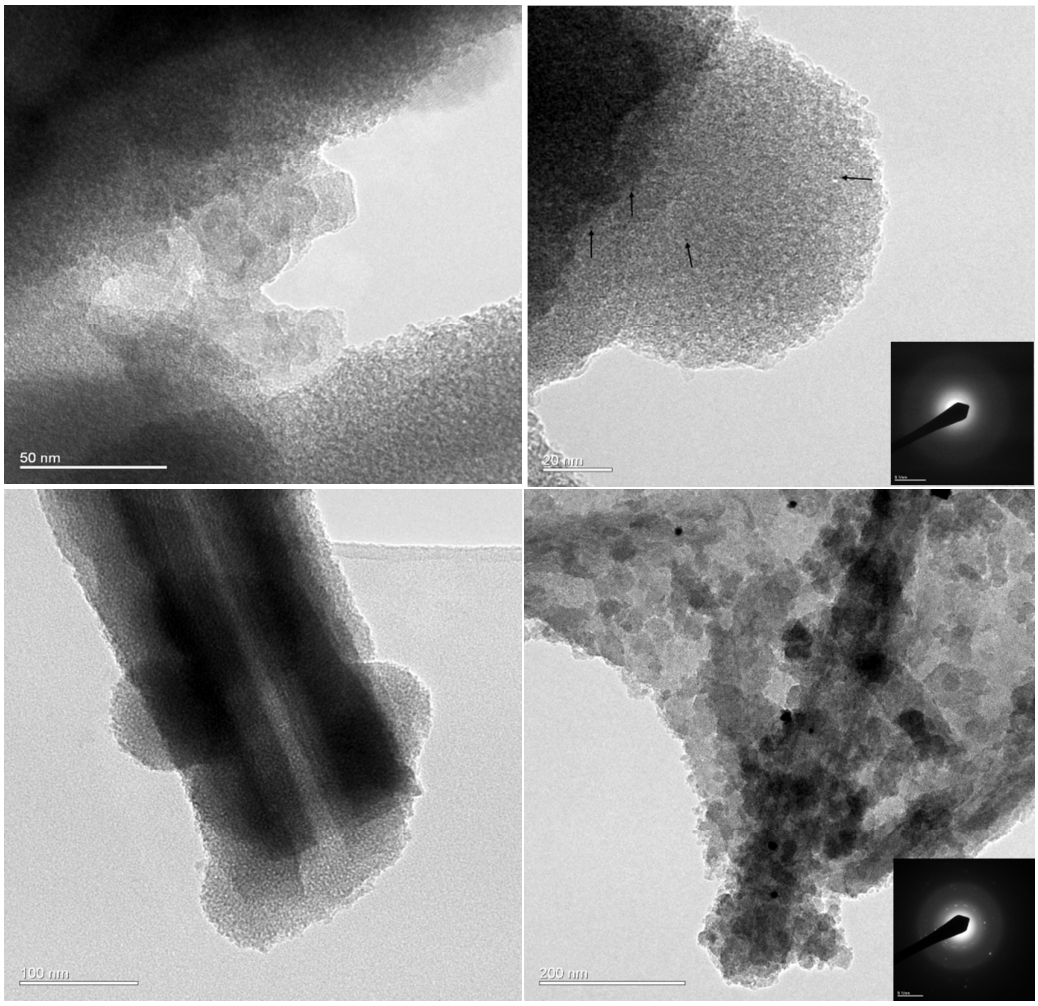
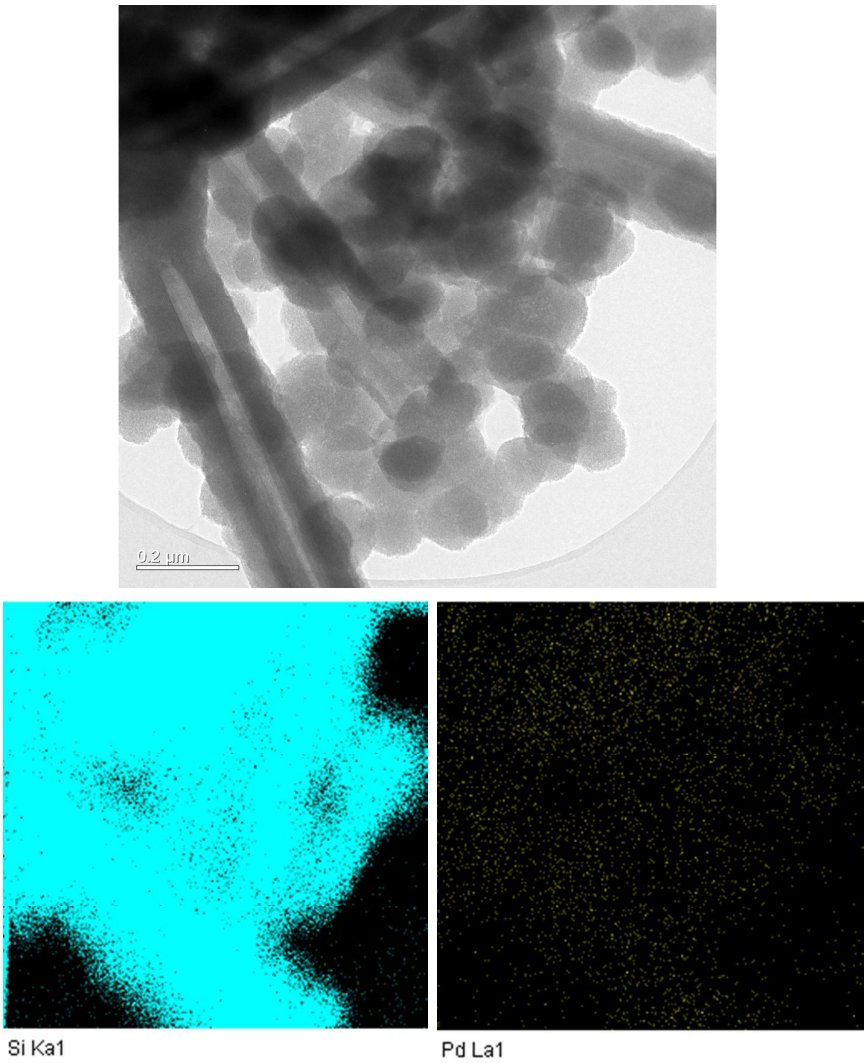


Figure 4. TEM images of A and B) Pd-biosicilified ICALB (top images) as compared to IM (bottom left) and Au-biosicilified ICALB nanohybrids (bottom right). Images depict the significant differences of IM Pd containing an even distribution of small Pd or Ru nanoparticles (<5 nm, see dark arrows pointing to nanoparticles in the materials, also SI for detailed images) with respect to the combination of small NPs and large Au clusters observed in the organic-inorganic Au-ICALB nanohybrid (see SI for detailed images). Insets on right images correspond to Electron Diffraction (ED) patterns of IM Pd and Au, respectively.



Element quantification (average of three measurements) by TEM/EDX

Element	Weight%
C K	13.15
O K	30.65
Si K	56.01
Pd L	0.19

Figure 5. TEM of IM Pd with their corresponding mapping micrographs (bottom coloured images), showing a homogeneous Pd distribution in the material. The Si mapping (bottom left) clearly supports the biosilicification of the enzyme.

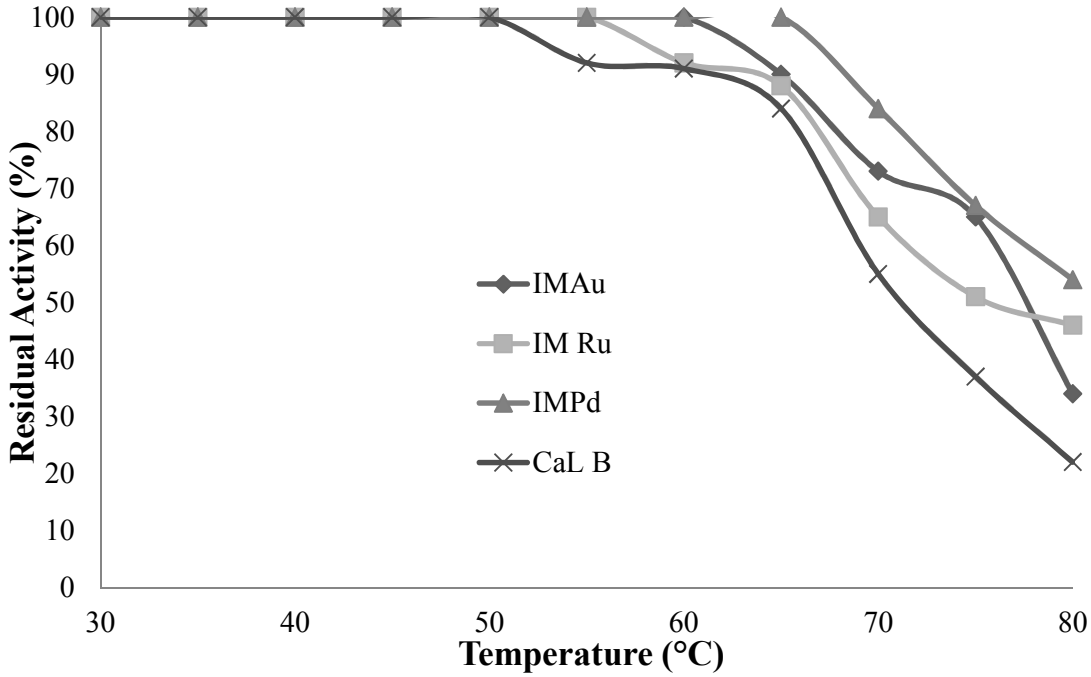


Figure 6**.** Thermal stability comparison for IM-metal materials and CalB.

Table 1. Immobilization efficiency and amount of protein quantified in the bio-nanohybrid materials.

Preparation	Immobilization efficiency (%)	Amount of protein (mg/g of support)
IM CaLB	79	5.5
IM Au	82	5.8
IM Pd	75	4.9
IM Ru	68	3.2

Table 2. Esterification activity of biosilicified nanohybrids containing Au, Ru and Pd nanoparticles (IM Au, IM Ru and IM Pd) as compared to the free enzyme (CaLB) and biosilicified CaLB (IM CaLB).

Organic Solvents	Esterification Activity (mmol . min^{-1}. mg)*				
	IM Au	IM Pd	IM Ru	IM CaLB	CaLB
n-hexane	219.3	201.3	187.3	193.5	134.5
n-heptane	198.9	172.2	133.8	175.6	124.4
Iso-octane	144.7	122.2	116.8	120.5	98.5
MTBE	109.4	100.2	95.1	98.7	88.2

*Measured by Lowry-Tinsley method and confirmed by CG-MS