

Co-immobilization of Laccase and TEMPO in the Compartments of Mesoporous Silica for a Green and One-pot Cascade Synthesis of Coumarins via Knoevenagel Condensation

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In memory of Prof. Abbas Shafiee

Abstract: Co-immobilization of bio- and chemo-catalysts produces sustainable, recyclable hybrid systems that open new horizons for green cascade approaches in organic synthesis. Here, a coimmobilization of laccase and TEMPO in mesoporous silica was used for one-pot synthesis of 30 coumarin-3-carboxylate derivatives in aqueous medium under mild conditions via condensation of in situ oxidized 2-hydroxybenzyl alcohols and malonate derivatives. A maximal yield was obtained after 24-h incubation at pH 6.0 and 45 °C. An efficient organic synthesis was catalyzed by the hybrid catalyst in 10% organic solvent. More than 95 % of the initial enzyme activity was preserved after 10 cycles, and no significant catalyst deactivation occurred after 10 runs. This new system efficiently catalyzed in situ aerobic oxidation of salicyl alcohols, followed by Knoevenagel condensation, confirming the possibility of producing efficient hybrid catalysts by co-immobilization of catalytic species in mesoporous materials.

Improvement of heterogeneous catalysts is stimulated by some limitations that restrict the use of homogeneous catalytic systems, such as inefficient product separation, complicated catalyst recovery, and problematic recycling methods.^[1] The use of multifunctional heterogeneous catalysts based on homogeneous catalytic systems has recently emerged as a promising field for green and sustainable cascade synthesis methods.^[2] These catalysts are crucial for reactions that require multiple active components, as heterogeneously catalyzed one-pot organic syntheses combine individual steps, thereby enhancing the efficiency of chemical processes by reducing cost and time expenditures through catalyst reusability.^[3]

The heterogenization of homogeneous bio- and chemocatalysts uses immobilization methods based on mesoporous supports, which have desirable properties (large pore size and surface areas) that make them suitable for high loading of

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catalytic species.^[4] Co-immobilization also improves catalytic entities of both bio- and chemo-catalysts (such as transition metals and non-metal species) by providing suitable environments that allow these species to reside in close proximity to each other and to work cooperatively.^[5] Green and sustainable methodologies for cascade reactions require the use of bio-catalysts with interesting properties, such as biodegradability or functionality under mild reaction conditions and in environmentally friendly reaction media.^[6] The use of enzymes and green synthetic methods that generate less waste are also more attractive than traditional organic synthesis approaches.^[7]

Blue enzymes are one of the most important bio-catalysts applied in organic synthesis. Their typical substrates are phenols and amines, and their major reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates.^[8] The use of combined laccase-mediator systems further expands the range of substrates that undergo biocatalytic aerobic oxidation, leading to amination, cyclization, and Michel addition reactions.^[9] Several reports have described the application of immobilized laccase in the synthesis of organic compounds, but some laccase-catalyzed reactions inevitably require the use of costly mediator reagents, such as 2,2,6,6tetramethyl-1-piperidinyloxy (TEMPO), making recycling of both enzyme and mediator highly desirable, especially for large-scale reactions.^[10] Co-immobilization of a mediator and a laccase in porous materials should therefore be beneficial, as each cavity of the support will contain both active species.^[5]

This paper describes an approach for the construction of an efficient bifunctional hybrid catalyst by co-immobilization of laccase and TEMPO into the same cavities in mesoporous silica. This hybrid catalyst was tested for aerobic oxidation of benzyl alcohols. The catalytic ability of the catalyst in a multicomponent total synthesis was also confirmed using the green and one-pot synthesis of coumarins via Knoevenagel condensation under mild conditions as a model reaction. These findings further advance green chemistry principles for the development of a new sustainable approach.

Mesoporous silica SBA-15 was prepared according to reported procedures (Supporting Information) and then with (3-aminopropyl) triethoxysilane functionalized and glutaraldehyde spacers, followed by reductive amination to construct the corresponding co-immobilized TEMPO and enzyme. The hybrid catalyst was characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), Brunauer-Emmett-Teller (BET) surface analysis, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and thermal gravimetric analysis (TGA) (Figure 1). FTIR analysis showed the absorption bands in the 2850-2980 cm⁻¹ related to asymmetric and symmetric vibrations of alkyl groups in the catalyst and the broad signals overlapping in the 1070-1220 cm⁻ corresponding to Si-O-Si symmetric stretch. Surface analysis

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of SBA-15 and the hybrid catalyst revealed a surface area decrease after co-immobilization (Supporting Information). High-resolution atomic force microscopy (AFM) indicated surface changes in the SBA-15, suggesting the presence of catalytic species on the silica support surface (Figure 2). Barrett-Joyner-Halenda (BJH) plots also indicated a decrease in the pore size after immobilization, confirming occupation of the pores by laccase and TEMPO.

the prepared catalyst catalyzed the reaction with a yield of corresponding salicylaldehydes of up to 90 to 100 % conversion.





Figure 1. a) SEM, b) TEM images of SBA-15, c) TGA curve of consecutive steps, d) Nitrogen adsorption/desorption isotherms, e) corresponding pore size distribution profiles of SBA-15 and hybrid catalyst, and f) XRD pattern of SBA-15. The inset in (f) shows the low angle range of XRD pattern of SBA-15.



Figure 2. Surface topography of a) SBA-15, and b) the hybrid catalyst obtained by atomic force microscopy.

The hybrid catalyst was initially examined for the oxidation of a broad range of benzyl alcohols, in the absence of any transition metal or co-catalyst (Table 1). Under these conditions,

 Table 1. Aerobic oxidation of salicyl alcohols using hybrid catalyst.

Entry	R1	R2	R3	R4	R5	Time	e (h)	Yield	(%) ^[a]
						O ₂	Air	O ₂	Air
1	Н	Н	Н	Н	Н	12	15	94	91
2	н	Н	н	CI	н	12	14	96	95
3	н	Н	Н	NO_2	н	10	14	98	95
4	н	OMe	Н	н	н	13	17	93	91
5	н	н	OMe	Н	Н	13	17	91	89
6	н	н	CH ₂ Ph	Н	н	14	20	91	89
7	н	Me	Me	Н	Н	12	16	93	90
8	н	н	Н	OH	Н	12	15	96	95
9	Н	OH	Н	н	Н	12	15	97	95
10	Н	OH	н	н	OH	13	17	96	94
11	OH	OMe	Н	н	Н	13	20	93	90
12	OH	Н	OMe	Н	Н	13	20	93	91
13	OH	Н	н	н	Н	12	15	95	94
14	OH	H	н	н	Br	11	13	97	93
15	OH	H	н	н	NO_2	11	14	96	93
16	CI	Н	н	н	Н	10	15	95	95
17	CI	H	CI	н	Н	10	13	96	95
18	н	Н	CI	н	Н	10	13	97	95
19	н	н	Br	н	Н	10	14	97	94
20	н	Br	Br	Н	н	10	13	96	92

[a] Yield was obtained based on calibration curve using HPLC.

The two most important issues requiring resolution for heterogeneous catalysts are expanding the lifetime of the catalyst and reducing the possibility of leaching of the active components (enzyme and TEMPO) from the solid into solution, as this would lead to gradual deactivation of the catalyst. In the present study, a filtration experiment, in which the reaction mixture was filtered after 5 h (60 % conversion), confirmed the heterogeneous nature of the catalyst and verified that no catalytic activity was present in the filtrate at 10 h after filtration. Use of the recovered catalyst in a second run further established that no catalyst deactivation had occurred during the aerobic oxidation reaction.

The turnover number (TON), which was normalized by the total amount of TEMPO catalyst, was determined using various substrate/catalyst ratios. At a 100:2 ratio, the TON of the heterogeneous hybrid catalyst was 30, or about 3 times higher than that of the homogeneous laccase and TEMPO after a 12 h reaction (Figure 3a). Increasing the ratio (1000:2) confirmed the inadequate efficiency of the homogeneous laccase and TEMPO (Figure 3b). A catalytic system containing immobilized laccase and free TEMPO showed a slight decrease in the TON toward that of the homogeneous catalysts.

The hybrid catalyst (2 mol %) was also a highly catalytic heterogeneous system for the aerobic oxidation of a range of salicyl alcohols. Air can be conveniently used instead of pure oxygen for this reaction without affecting the efficiency, but the use of air requires slightly longer reaction times (Table 1). The oxidation of benzyl alcohols possessing electron donating groups in the aromatic ring can also be much slower that can be explained by the presence of a negative charge in the transition state for the oxidation involving oxoammonium salts. For this COMMUNICATION

reason, several studies have reported the use of TEMPO in combination with transition metals, such as Ru, Ce, and Cu, as alternative catalysts for the oxidation of alcohols using molecular oxygen.^[11] Brunel et al.^[12] developed a heterogeneous system for catalytic oxidations of alcohols in aqueous media by immobilizing TEMPO in mesoporous silica (MCM-41) and using hypochlorite as the primary oxidant. However, hypochlorite is an oxidizing substance that leads to formation of undesirable halogenated by-products, thus necessitating further purification steps. Recently, Hu et al.^[13] presented a novel metal-free TEMPO-catalyzed aerobic oxidation of alcohols. This method was an interesting achievement in the field of aerobic oxidation of alcohols, but it required expensive Teflon-lined apparatus, high loadings of TEMPO (homogeneous, up to 10 mol %) that was not recoverable, high operating pressures (up to 9 bar), relatively high temperatures (up to 80 °C), and the use of an environmentally undesirable chlorinated solvent (CH₂Cl₂). Thus, the hybrid catalyst presented here is still highly desirable for development of cleaner, milder, cheaper, and recyclable TEMPO catalytic systems for the aerobic oxidation of alcohols.



Figure 3. Comparison of the turnover number (TON) of the time-dependent reaction catalyzed by the heterogeneous (hybrid catalyst) versus a homogeneous (laccase and TEMPO) catalytic system with substrate/catalyst ratios of a) 100:5, b) 100:2, and c) 1000:2.

Karimi et al.^[14] showed that SBA-15 can be used to support the nitroxyl radical TEMPO to form a reusable catalyst for the aerobic oxidation of alcohols in the presence of 10 mol % NaNO2 and 8 mol % nBu4NBr. Sodium nitrite is recognized as a toxic compound that can induce a number of physiological disturbances, due to the production of nitrosamines and nitrosamides by its reaction with amines and amides, respectively.^[15] The hybrid catalyst formed by co-immobilization of laccase and TEMPO in the mesoporous silica has the benefit of using an enzyme instead of transition metals or inorganic oxidants. The hybrid catalyst also showed excellent tolerance for a broad range of substituted benzyl alcohols (Table 1). The ability to synthesize natural and synthetic coumarins with several interesting biological activities suggests promising therapeutic applications for this catalyst.

The potential application of the hybrid catalyst in organic synthesis was further tested by using the prepared catalyst in a multicomponent reaction for the one-pot synthesis of coumarins via Knoevenagel condensation (Table 2). Salicylaldehydes were produced in situ from corresponding salicyl alcohols, together with a condensation reaction with various malonates to yield coumarin-3-carboxylates. The oxidation of salicyl alcohol was complete after 12 h (Figure 4a), and explorations of the effects of pH and temperature on the yield of coumarin-3-carboxylates showed that pH 6.0 at 45 °C were optimal reaction conditions (Figure 4c-d). Examination of the effect of temperature on the

reaction yield indicated that increasing the reaction temperature up to 45 °C improved the yield of the product by more than 90%, but further increases in temperature decreased the yield due to enzyme deactivation. Karimi et al.^[14] used acetic acid in the oxidation reaction catalyzed by immobilized TEMPO, whereas the present condensation was carried out in aqueous medium. The laccase activity was decreased only 5% after 10 runs and no significant deactivation of the hybrid catalyst was observed after 5 runs (Figure 4b). This reusability also demonstrates the high stability of this new heterogeneous system.



Table 2. One-pot synthesis of coumarin-3-carboxylates in aqueous medium using hybrid catalyst under mild conditions.

Entry	R1	R2	Time (h)	Yield (%) ^[a]
1	Н	CH ₃	24	93
2	Н	CH ₂ CH ₃	24	94
3	Н	CH ₂ CH ₂ CH ₃	24	92
4	H	$CH_2(CH_3)_2$	24	88
5	Н	C(CH ₃) ₃	24	85
6	Н	CH₂Ph	24	88
7	3-OCH ₃	CH ₃	26	89
8	3-OCH ₃	CH_2CH_3	26	88
9	3-OCH ₃	CH ₂ CH ₂ CH ₃	26	86
10	3-OCH ₃	$CH_2(CH_3)_2$	26	86
11	3-OCH ₃	C(CH ₃) ₃	26	84
12	3-OCH ₃	CH₂Ph	26	85
13	4-OCH ₃	CH ₃	27	88
14	4-OCH ₃	CH_2CH_3	27	88
15	4-OCH ₃	CH ₂ CH ₂ CH ₃	27	86
16	4-OCH ₃	$CH_2(CH_3)_2$	27	86
17	4-OCH ₃	C(CH ₃) ₃	27	85
18	4-OCH ₃	CH₂Ph	27	85
19	5-Br	CH ₃	23	93
20	5-Br	CH_2CH_3	23	93
21	5-Br	CH ₂ CH ₂ CH ₃	23	94
22	5-Br	$CH_2(CH_3)_2$	23	91
23	5-Br	C(CH ₃) ₃	23	92
24	5-Br	CH₂Ph	23	92
25	5-NO ₂	CH ₃	22	95
26	5-NO2	CH_2CH_3	22	95
27	5-NO ₂	CH ₂ CH ₂ CH ₃	22	93
28	5-NO ₂	$CH_2(CH_3)_2$	22	92
29	5-NO ₂	C(CH ₃) ₃	22	92
30	5-NO ₂	CH₂Ph	22	89

[a] Yield was obtained based on calibration curve using HPLC.

The best solvent for the reaction was determined by studying the laccase-catalyzed oxidation of 3 in aqueous media and in diverse organic solvents. Citrate buffer (pH 4.5, 0.1 M) was found to be the best reaction medium (Supporting Information). No reaction took place in pure organic solvents, such as THF, DMF, and MeCN, due to deactivation of the enzyme. Interestingly, product was formed, with a yield of 65%, in 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim]PF₆), a prevalent ionic liquid (IL), as a green organic medium. A mixture of citrate buffer and organic solvents supported laccase-catalyzed oxidation with moderate yields, in the range of 21 to 44 %.

Three-dimensional ordered mesoporous carbon with large surface area and thermal and mechanical stability could provide a favorable microenvironment for the immobilization of enzymes. Although many ordered mesoporous carbon materials synthesized by silica templates have been used as enzyme supports, the activity of the immobilized enzyme may be COMMUNICATION

decreased because the pore size of these materials cannot be adjusted for enzyme dimensions. Carboxylated facilelv mesoporous carbon with pore size 10 nm has been used for the immobilization of lysozyme by covalent cross-linking to purify the drinking water contaminated with pathogenic bacteria.^[16] Tang et al.^[17] applied mesoporous silica with a pore size 9.7 nm in the immobilization of zero-valent iron particles for effective degradation of *p*-nitrophenol. Piontek et al.^[18] studied the crystal structure of a laccase from the fungus T. versicolor and it was found that unit cell dimensions were a= 83.6 Å, b= 85.0 Å, and c= 91.5 Å at 1.90 Å resolution with a corresponding V_m of 2.3 Å³/Da, assuming one molecule per asymmetric unit. Bertrand et al.^[19] reported the laccase crystals belonging to the monoclinic space group P21, with unit cell dimensions a= 87.72 Å, b= 110.52 Å, and c= 123.20 Å. In our study, the synthesized SBA-15 with pore diameter 13.48 nm provides conformational stability. Pore diameters less than 10 nm cause a decrease in protein loading as the physical restrictions may change three dimensional structure of enzymes.



Figure 4. Time course study of the oxidation of salicyl alcohol using a heterogeneous (hybrid catalyst) and homogeneous (laccase and TEMPO) catalytic system. b) Relative laccase activity of the hybrid catalyst (dark gray) and yield of the aerobic oxidation reaction by the heterogeneous catalyst (light gray). Effect of pH and temperature on the yield of the oxidation reaction using c) laccase and TEMPO d) the hybrid catalyst.

In summary, a highly efficient multifunctional hybrid catalyst was prepared based on heterogeneous materials containing fundamentally different catalytic species. In this hybrid catalyst, the laccase and mediator cooperate in an unprecedented method. The hybrid catalyst was well characterized, and the co-immobilization of TEMPO and enzyme in the same cavities of the SBA-15 was confirmed. Furthermore, the proximity of the two catalytic species conferred an enhanced the efficiency of the biotransformation. The concept of co-immobilizing catalysts with different functions into mesoporous materials shows great promise for creating other hybrid catalysts with unprecedented reactivity. This will be important for the

future improvement of catalytic entities that can operate by cooperative tandem catalysis for cascade chemical syntheses of the future. In addition, enzyme-catalyzed methodologies can be advanced as a straightforward approach to replace simple biotransformations for the targeted synthesis of valuable compounds.

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Conflict of interest

The authors declare no conflict of interest.

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