APL MATERIALS **2**, 113304 (2014)

Location of laccase in ordered mesoporous materials

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(Received 1 August 2014; accepted 10 September 2014; published online 14 October 2014)

The functionalization with amine groups was developed on the SBA-15, and its effect in the laccase immobilization was compared with that of a Periodic Mesoporous Aminosilica. A method to encapsulate the laccase *in situ* has now been developed. In this work, spherical aberration (C_s) corrected scanning transmission electron microscopy combined with high angle annular dark field detector and electron energy loss spectroscopy were applied to identify the exact location of the enzyme in the matrix formed by the ordered mesoporous solids. [http://dx.doi.org/10.1063/1.4897281]

Mesoporous silica materials have received increasing attention due to their optimum to encapsulate enzymes within the pores providing a more stable environment in comparison to that at a soluble enzyme.[1–3] The advantage of using porous supports for immobilization of enzymes is evident, the large internal surface area can provide a safe haven for the enzyme.[4,5] The most important factor in this use is that the pore diameter must be sufficiently large to accommodate the enzyme.[6,7] Recent progress has been achieved in the preparation of silica devices materials which can be used in enzyme nanoarchitectures with soft functions.[8–10]

This work focusses on one particular type of enzyme, laccase from *Myceliophthora thermophila* (Figure 1). Laccases are attractive biocatalysts for manufacturing pharmaceutical intermediates, specialty chemicals, and for bioremediation.[11–20] They exhibit excellent catalytic activity even with water-insoluble substrates such as lignin. One of the most important aspects of using these enzymes is the sensitivity to phenolic compounds that are considered very toxic.[21–23] In these reactions, the oxygen is reduced directly to water without the intermediate formation of toxic hydrogen peroxide. In this direction, the immobilization of laccase in ordered mesoporous materials (OMM) provides the optimal control of the stabilization of the enzyme towards different media.

So far our group have successfully immobilized laccase in ordered mesoporous materials such as SBA-15[24] and periodic mesoporous aminosilica (PMA)[25] via post-synthesis immobilization. Enzyme encapsulation is an attractive method among the different immobilization strategies to improve the reusability and stability of enzymes; however, current encapsulation methods have limitations including enzyme leakage. Silica based sol-gel encapsulation is the most used technique for enzyme encapsulation without the need of surfactants. OMM, which are prepared using surfactants, can provide a high specific surface area, controllable pore diameter, ordered porous network, and large pore volume. Many of the classical OMM are formed using acidic pH, high temperature processes, and hydrothermal synthesis reaction, which are severe conditions and are not compatible with the presence of enzymes. The goal in this work is to encapsulate laccase into a shell structure with the silica shell serving as a robust and stable encapsulation layer to protect the enzymes where the encapsulated enzymes are physically confined inside the siliceous material. Until recently, it was impossible to corroborate the presence of the enzyme within the pores, only indirect methods, such as comparisons of XRD patterns and/or pore volume before and after adsorption, were applied.[1,3]

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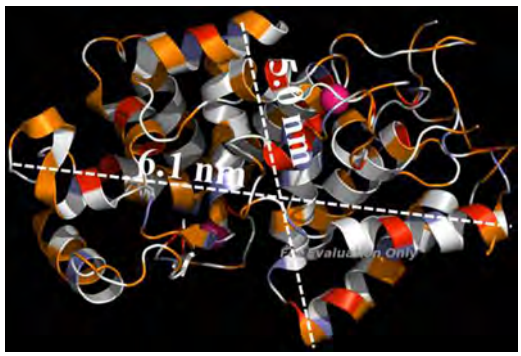

FIG. 1. Average dimensions of laccase measured in PyMOL (~6.1 nm × 5.0 nm × 4.9 nm).

Evidences for the encapsulation of an enzyme in the pores of mesoporous silica were obtained in our group by combining spherical aberration (C_s) corrected scanning transmission electron microscopy (STEM) with a high angle annular dark field detector (HAADF) and electron energy loss spectroscopy (EELS) to show that lipase and laccase were present inside the pores of the host.[26,27] In this work, the presence of individual molecules of laccase within the pores could be directly observed.

The molecular weight estimated for the laccase is around 80 kDa, obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[28,29] and the average molecular size was found to be approximately 6.1 nm high, 5.0 nm wide, and 4.9 nm deep.[29] According to the modelling studies and the textural properties of the supports it is expected that the enzyme dimensions enable its adsorption inside the pores of OMMs.

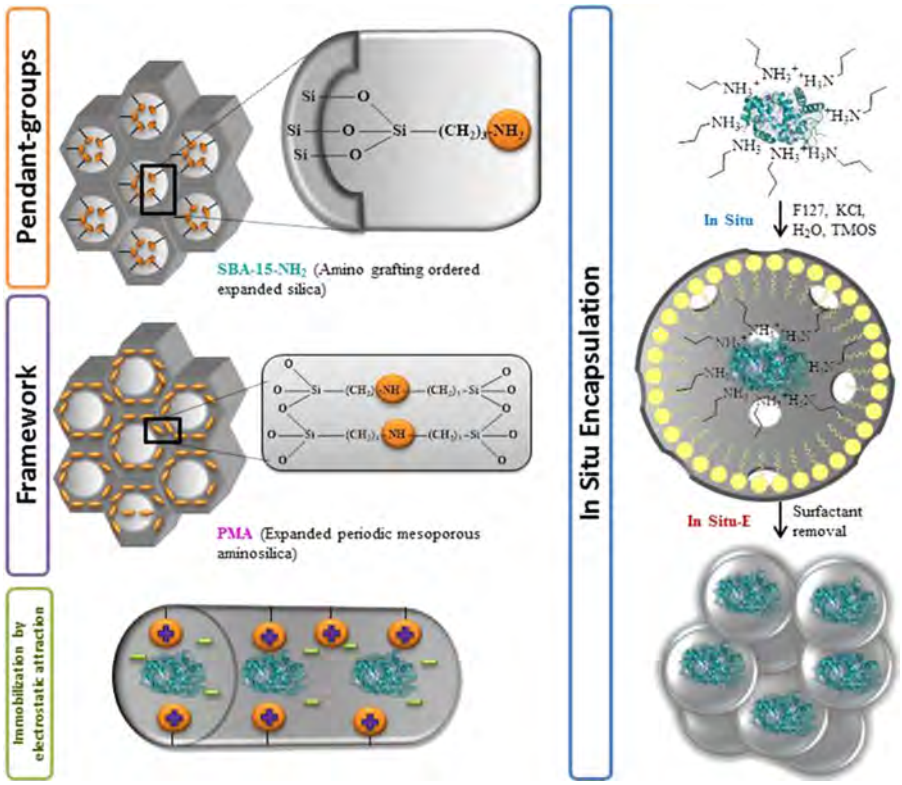

FIG. 2. Left: Incorporation of amine groups in SBA-15 and PMA materials and laccase immobilization post-synthesis. Right: Scheme of the *in situ* laccase encapsulation.

TABLE I. Textural properties, laccase immobilization and catalytic performance.

Material	$D_{P\ BJH}$ (nm)	S_{BET} (m^2/g)	V_P (cm^3/g)	Maximum loading (mg/g)[a]	Biocatalyst activity (U/g)[b]	Catalytic efficiency[c] (U/mg)
SBA-15-NH_2	11.2	339	1.0	170	50.7	0.30
PMA	10.4	264	0.6	88	29	0.33
In Situ	19.5	40	0.2	24.6	8.9	0.36
In Situ-E	20.8	465	1.1	24.6	3.6	0.15

[a]Maximum enzyme loading, expressed in milligrams of enzyme per gram of biocatalysts.
[b]Biocatalyst activity (Units/g biocatalysts) in ABTS test.
[c]Catalytic efficiency = Activity versus loading (Units/mg enzyme).

This protein has an isoelectric point rather low (pI 4). Hence, the useful pH range of immobilization via electrostatic interaction is very narrow (around 3.0-3.5) and close to the isoelectric points of both support and enzyme. Under these circumstances, the introduction of positive charges on the surface of the ordered mesoporous materials at a pH value high enough to ensure deprotonation of carboxylic groups on the surface of the enzyme would be the way to favor the interactions. Therefore, the functionalization with amine groups was developed on the SBA-15 with extended pores aiming to increase the affinity between the enzyme and the support.[24] Figure 2, left side, shows a scheme of the functionalization process and location of the amino groups protruding the walls in SBA-15 and incorporated in the walls in PMA. Following, the materials are put in contact with the enzyme solution for immobilization via *grafting* method or *post-synthesis* approach. The large pores obtained with the use of micelle expanders allow introducing propylamine groups without compromising too much the dimensions of the channels (SBA-15-NH_2). As seen in Table I, the presence of amines (1.07 mmol N/g) leads to a significant increase in enzyme loading, as high as 170 mg/g. Following with our aim of designing an optimum ordered mesoporous material for the immobilization of laccase, PMA was prepared with the aim to design an ordered mesoporous material with increasing affinity but without decreasing the pore size.[25] Figure 3(a) shows the XRD patterns corresponding to *p6mm* symmetry in the case of SBA-15-NH_2, and PMA suggesting that the expanded-micelles method is also working for the PMA materials.[24,25] Figure 3(b) plots the isotherms corresponding to those of functionalized mesoporous materials: SBA-15-NH_2 and PMA with functional groups giving broader hysteresis loop due to a complex mechanism of adsorption and desorption given by a rougher surface. PMA sample shows a second step at higher relative pressure due to the textural porosity, porosity between particles of small size that tend to agglomerate. This trend was corroborated by SEM (data not shown). Nevertheless, the textural properties of the samples allow for immobilization of laccase (Table I). Despite of its slightly smaller pore

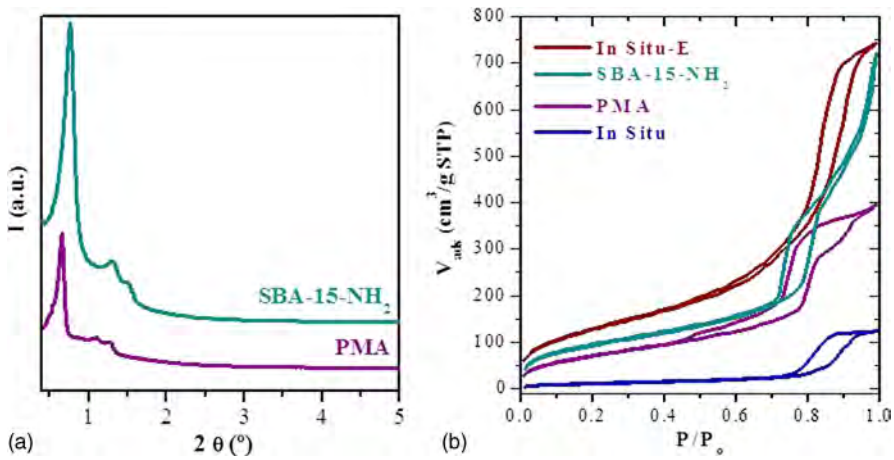

FIG. 3. (a) Low angle XRD patterns of amino-functionalized silica supports; (b) N_2 adsorption-desorption isotherms at −196 °C.

size (10.2 nm), the dimensions are still wide enough to allocate laccase molecules in the proper orientation. The concentration of N in PMA (0.58 mmol/g) is 54% smaller than in SBA-15-NH_2, and the enzyme loading achieved is 52% lower. The higher affinity of laccase for SBA-15-NH_2 can, therefore, be attributed to its higher N content. This leads again to the conclusion that the chemical affinity of the enzyme for the surface of the OMM is crucial to succeed in enzyme immobilization, when the pore size is at least large enough to let enzyme molecules fit inside.

On the other hand, the right side of Figure 2 shows an alternative path to encapsulate enzymes in ordered mesoporous materials,[30] a novel route developed to provide the enzyme with hydrophobic moieties that may help to drive the encapsulation in only one step. At neutral pH, carboxylic groups of laccase (with pI around 4) should be deprotonated, which in the presence of n-butylamine it should interact through positively charged amino groups so that the alkyl chains would be oriented outwards. In the end, the "protected" enzyme would be surrounded by surfactant, and thus, by silica in a *bottle around the ship* approach. This synthesis, denoted as *in situ* (In Situ), was prepared using Pluronic F127 as template, tetramethoxysilane (TMOS) as silica source, and in mild conditions (neutral pH and 35 °C). As a result, a foam like mesoporous material is formed, with large cages of regular size proving the capacity of these butyl chains on laccase surface to drive a correct arrangement of micelles to give rise to siliceous ordered structures.[31]

During the synthesis process, aliquots were taken at given times, and the laccase activity of the suspension and supernatant were determined spectrophotometrically towards the oxidation of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS). The activity of supernatant decreases as the enzyme molecules get encapsulated into the support. When the decrease in the activity reaches a minimum and constant value, it means that no more enzyme molecules can be immobilized. The suspension was then filtered off and washed. The solid sample was first dried under vacuum and then under nitrogen stream, collected, weighted, and finally stored at 4 °C. Surfactant removal (In Situ-E for "extracted") was achieved by mild treatment with 10% ethanol and acidic pH (3.5 in phosphoric acid solution).

The biocatalyst prepared in this manner showed the maximum leaching of the laccase at 4 h and then remained constant proving that the remaining enzyme molecules are successfully encapsulated in the silica mesoporous material. The leaching observed before 4 h is probably due to the enzyme molecules loosely trapped in the material. As shown in Figure 4 and Table I, only 10% is lost in the leaching test. The biocatalyst activity and catalytic efficiency also decrease after the treatment for surfactant removal probably due to the above mentioned loss of active enzyme molecules. For the other two materials (PMA and SBA-15-NH_2), only 4% of the enzyme is lost in the leaching test corroborating that the incorporation of amino groups enhances the affinity of the laccase for the mesoporous supports. Besides, in the case of the PMA, the pore size closer to the size of enzyme results in a confinement effect that favors the versatility of this material as support. In summary,

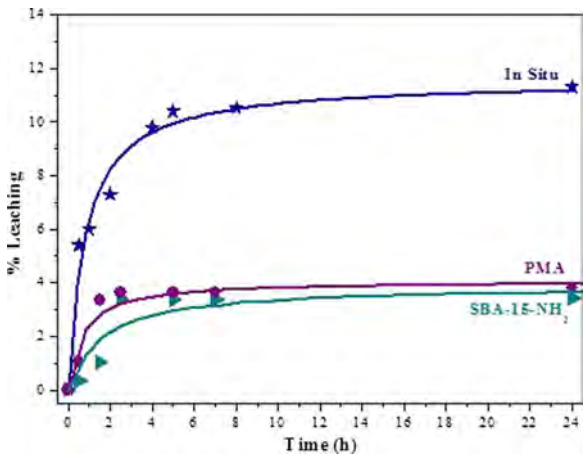

FIG. 4. Leaching of enzyme from channel-like materials (SBA-15-NH_2 and PMA) and *in situ* mesoporous silica, expressed as percent of the initial enzyme loading leached as a function of time.

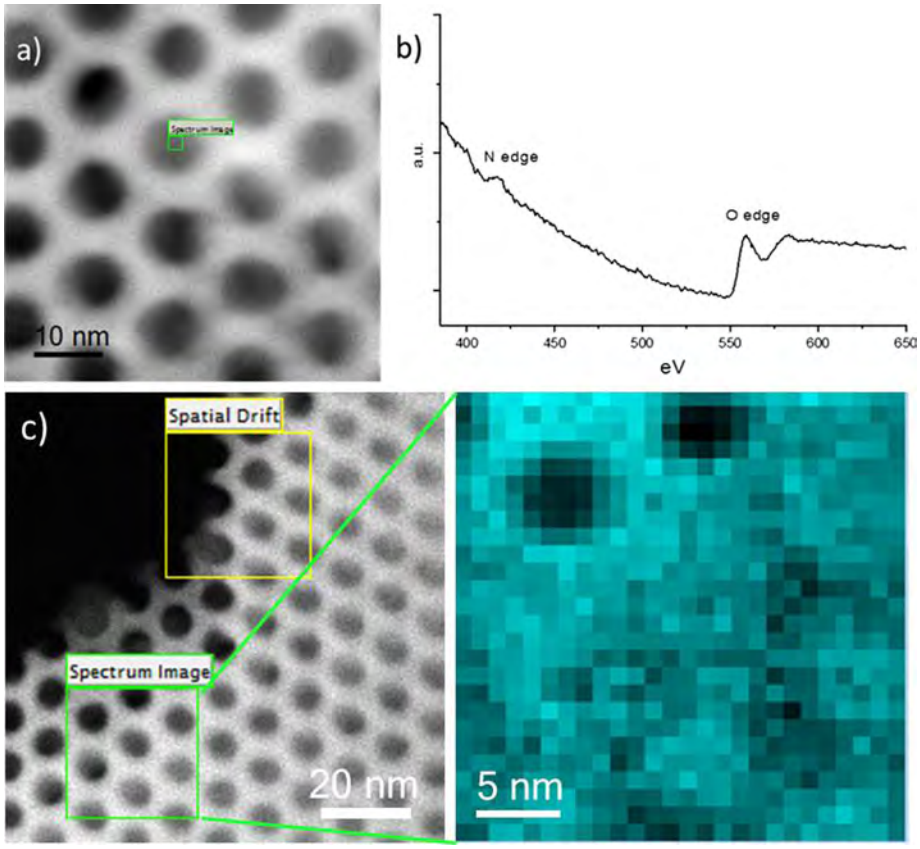

FIG. 5. (a) C_s-corrected STEM-HAADF image of the pore system. The green rectangle represents the area in which the EELS analysis was performed. (b) EELS profile collected from image a, showing the N-K and O-K edges that confirm the presence of laccase. (c) C_s-corrected STEM-HAADF image showing the hexagonal pore arrangement, the green square denoted as spectrum image corresponds to the area of analysis; the correspondent extracted N signal (blue color) is also displayed. Adapted from Ref. 26.

as observed from the data collected in Table I, despite the lower loading of laccase in the In Situ material, it shows the highest catalytic efficiency regardless of the removal of the surfactant.

In order to corroborate the results discussed above, our aim in this work is to develop a reliable method to prove that the location of enzymes is actually inside the pores. In the past twenty years, the only direct method for the characterization of ordered mesoporous materials has been based on high-resolution transmission electron microscopy[32] combined with electron crystallography[33] which has allowed solving all kinds of structures, giving a three dimensional reconstruction of the porous structure. When the subjects of interest are guest materials, STEM combined with a HAADF detector is more suitable as the contrast is related to the atomic number Z. In the current manuscript, C_s-corrected STEM-HAADF combined with EELS was the sole method for the location and the structural determination of the enzyme and mesoporous solids. A FEI X-FEG Titan 60-300 kV, operated at 80 kV, equipped with a monochromator (not excited for the current experiments), a CEOS C_s-probe corrector (allowing forming an electron probe of 0.12 nm mean size at 80 kV), and a Gatan Energy Filter Tridiem 866 ERS, was used for the experiments. The geometric aberrations of the probe-forming system were controlled to allow a beam convergence of 24.9 mrad half-angle to be selected. The alignment of the microscope at 80 kV was verified through the CETCOR software. A focus/tilt tableau was acquired measuring defocus and two-fold astigmatism as a function of both radial and azimuthal tilt angles. Concerning the EELS measurements, the collection semi-angle was of ~100 mrad, the energy resolution ~1.2 eV, and the data were collected using the spectrum-imaging mode.

Figure 5(a) shows an image of the pore system in the PMA-laccase system. A collection of spectrum has been taken in the same image inside one of the pores (green square) analyzing 8×6

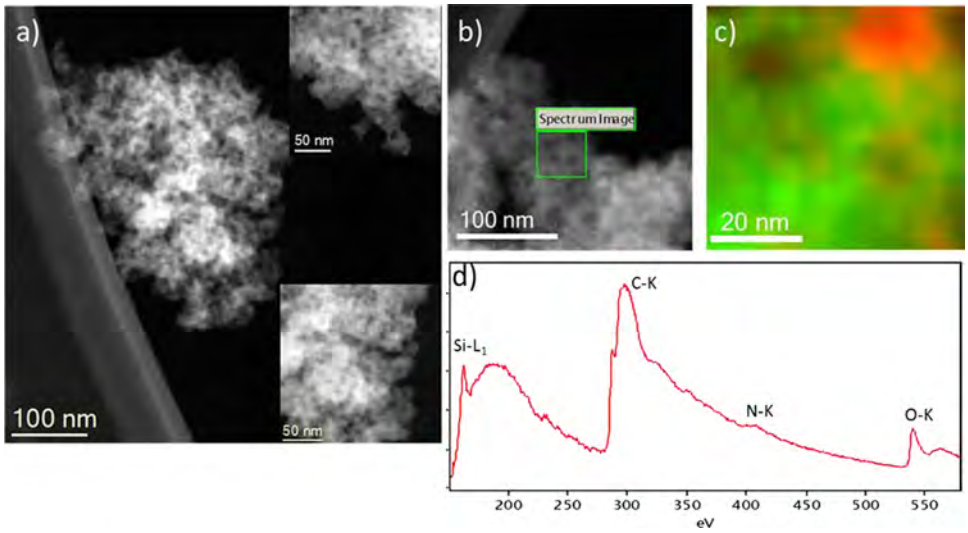

FIG. 6. (a) Cs-corrected STEM-HAADF images of the IS-laccase, a closer observation of the pores is shown in the inset. (b) Area of EELS analysis, green rectangle. (c) EELS compositional map, formed by Si (green) and N (red). (d) EEL spectrum profile of the area analyzed.

pixels with an exposure time of 2.5 s/pixel. The extracted spectrum profile (raw data), Figure 5(b), exhibits the O-K and N-K edges corresponding to the enzyme.[26] The nitrogen map extracted from the spectrum-image collected from the green rectangle in Figure 5(c) is also presented, using a pixel time of 1.5 s in an image formed by 26 × 26 pixels. In light blue, the correspondent N map is shown. As a consequence of the amino functionalization, N was detected in the framework and in certain pores, evidencing that some pores were not filled with laccase or that its content was too low to be detected. A relatively strong electron beam was necessary in order to gain enough counts to identify the species. The total dose was 0.48 e/A^2s which stayed on each pixel during the data collection.

In the case of the material prepared *in situ* (In Situ-E-laccase), the presence of butylamine together with the surfactant yielded to a cage-type of materials although no ordered structure could be identified (Figure 6(a)). Nevertheless, the presence of enzymes inside the cages was proven by the N and C content.[27] Figure 6(b) shows the C_s-corrected HAADF image of certain pores, taken at 80 kV, where the spectrum image was acquired. The compositional map formed by Si-L_1 signal in green and N-K signal in red corroborates the presence of the enzyme in some of these cavities (Figure 6(c)). The EEL spectrum is depicted in Figure 6(d), after background subtraction, proving the presence of all elements coming from the mesoporous silica and the enzyme.

In summary, current studies in our laboratory prove the feasibility of designing functionalized ordered mesoporous materials for enzyme immobilization. A rational design of the synthesis conditions, surface properties, pore size, and connectivity of ordered mesoporous material is, nowadays, straightforward. Furthermore, C_s corrected STEM simultaneously combined with EELS analysis allows imaging the functionalities of the mesopores of silica structures with ultra-high resolution and to unambiguously determine the presence of enzyme molecules inside the mesopores of the hybrid biocatalysts.

The authors would like to acknowledge the Spanish Government (MAT 2012-31127) for funding and V.G. for a FPU Ph.D. fellowship (AP2010-2145). A.M. acknowledges the European Union Seventh Frame-work Programme under Grant Agreement No. 312483—ESTEEM2 (Integrated Infrastructure Initiative—I3).

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